Interindividual Heterogeneity in the Hypoxic Regulation of VEGF
Significance for the Development of the Coronary Artery Collateral Circulation

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Background—The coronary artery collateral circulation may be beneficial in protecting against myocardial ischemia and necrosis. However, there is a tremendous interindividual variability in the degree of new collateral formation in patients with coronary artery disease. The basis for this interindividuality heterogeneity is not understood. In this study we test the hypothesis that failure to generate collateral vessels is associated with a failure to appropriately induce with hypoxia or ischemia the angiogenic factor, vascular endothelial growth factor (VEGF).

Methods and Results—We correlated the VEGF response to hypoxia in the monocytes harvested from patients with coronary artery disease with the presence of collaterals visualized during routine angiography. We found that there was a highly significant difference in the hypoxic induction of VEGF in patients with no collaterals compared with patients with some collaterals (mean fold induction 1.9 ± 0.2 versus 3.2 ± 0.3, P < 0.0001). After subjecting the data to ANCOVA, using as covariates a number of factors that might influence the amount of collateral formation (ie, age, sex, diabetes, smoking, hypercholesterolemia), patients with no collaterals still have a significantly lower hypoxic induction of VEGF than patients with collaterals.

Conclusions—This study provides evidence in support of the hypothesis that the ability to respond to progressive coronary artery stenosis is strongly associated with the ability to induce VEGF in response to hypoxia. The observed interindividual heterogeneity in this response may be due to environmental, epigenetic, or genetic causes. This interindividual heterogeneity may also help to explain the variable angiogenic responses seen in other conditions such as diabetic retinopathy and solid tumors.

Key Words: collateral circulation • angiogenesis • ischemia • hypoxia • growth substances
namically insignificant atherosclerotic plaque. However, this certainly cannot explain the marked heterogeneity in compensatory angiogenesis observed in many subjects with chronic stable coronary artery and peripheral vascular disease and in hibernating myocardium.

In the present study we test the hypothesis that failure to generate collateral vessels in many patients with chronic vascular insufficiency is associated with a failure to appropriately increase VEGF production with hypoxia or ischemia. For this purpose we have correlated the VEGF response to hypoxia in monocytes harvested from patients with coronary artery stenosis with the presence of coronary artery collaterals in the same patients. The extent of the coronary collateral circulation was determined by use of accepted criteria during routine angiography. The results revealed a highly significant correlation, with increased hypoxic induction of VEGF in those patients with collaterals in comparison to those without.

**Methods**

**Patient Recruitment**

This study was approved by the Human Research Ethics Committee of the Rambam Medical Center. Informed consent was obtained from all patients. Patients were recruited consecutively from those undergoing diagnostic coronary artery catheterization at the Rambam hospital over a 3 month period. The indications for catheterization in all patients were presence of stable or unstable angina pectoris or suspected significant myocardial ischemia. Only patients with at least ≥1 coronary stenosis of ≥70% by visual analysis of the angiogram were included in this study. Exclusion criteria were age <18 and the presence of anemia.

**Patient Data Collection**

For each patient, a data sheet was completed with the patient’s name, identification number, age, sex, previous revascularizations, history of hypertension, diabetes, cigarette smoking, family history of premature coronary artery disease, or hypercholesterolemia. On a separate sheet the patient’s coronary anatomy (number of diseased vessels and collateral score (0, 1+, 2+) was recorded by an experienced angiographer. The collateral scoring system used was modified from the TIMI system by grading from 0 to 2 rather than 1 to 3 but maintaining a 3 point scale. The ranking from 0 to 2 was based on the presence of collateral vessels and opacification of the recipient vessel. A grade of 0+ was given for no visible collaterals; 1+ for visible collaterals but no filling of the recipient epicardial vessels; and 2+ for filling (partial or complete) of the recipient epicardial vessel by collaterals. A representative frame from a patient with 2+ collaterals is shown in Figure 1. Coronary anatomy and collaterals were reviewed again by a cardiologist blinded to the initial reading with a >85% concordance rate between the 2 reviewers in the collateral score. In instances of discrepancy between the 2 reviewers, a third reviewer blinded to the readings of the first 2 reviewers was used and served as arbitrator. The coronary anatomy and collateral score was not revealed to those involved in the VEGF assay until after all patient samples had been analyzed for VEGF.

**Blood Collection**

Mononuclear cells were isolated from peripheral blood by a well-established procedure initially described by Boyum, performed with the use of a mixture of polysaccharide and a radiopaque contrast medium. Forty milliliters of blood was collected from the femoral venous catheter placed for the catheterization before angiography was begun. The blood was immediately placed in a 50-mL polypropylene heparinized tube (100 µL of 5000 U/mL heparin) and kept on ice before it was used for monocyte isolation. In all instances, the blood was used within 4 hours of removal from the patients. Twenty milliliters of heparinized blood was gently layered onto 10 mL of Histopaque-1077 (Sigma) in a fresh 50-mL polypropylene centrifuge tube. Tubes were centrifuged at 1800 rpm for 30 minutes at room temperature. Eight milliliters of plasma was removed from each tube and saved for later use. The middle phase (buffy coat) containing the monocytes was isolated and placed in a fresh 15-mL polypropylene centrifuge tube. The isolated mononuclear cells were washed twice with sterile phosphate buffered saline. The cell pellets were resuspended in Dulbecco’s modified Eagle’s medium (Sigma) with 2% fetal bovine serum (Sigma) and antibiotics. The cells were plated in 2 equal aliquots on 2 polystyrene, 10-cm-diameter tissue culture dishes (Corning) and incubated in a 95% room air, 5% CO₂ incubator (Forma) at 37°C for 1 hour to allow for monocyte attachment. The medium from the 2 tissue culture dishes from a single patient was aspirated and replaced with 8 mL of autologous plasma on each dish. One of the tissue culture dishes was placed in a normoxic incubator, 21% O₂, 5% CO₂ (Forma), and the other tissue culture dish from the same patient was placed in a hypoxia incubator, 1% O₂, 5% CO₂, 94% N₂ (Triple Gas Incubator, Jouan). After 20 hours of exposure to either hypoxia or normoxia, RNA was extracted from the cells.

**Figure 1.** Representative frame from a patient with 2+ collaterals visualized by angiography. A, Total occlusion (arrow) of the mid right coronary artery on selective injection of the right coronary artery with contrast media. B, Complete filling by coronary artery collaterals (arrows) of the distal right coronary artery (right posterolateral and posterior descending arteries) after injection of the left coronary system with contrast media.
Characteristics of Patients

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<td>No. of diseased vessels</td>
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RNA Isolation From Monocytes

Total RNA was isolated from the tissue culture dishes containing the monocytes with the TRI Reagent (MRC Inc). Briefly, 1 mL of reagent was added to each dish with vigorous pipetting and transferred to a 1.5 mL Eppendorf tube. Chloroform (200 μL) was added, and the tube was vortexed and centrifuged at 14 000 rpm for 10 minutes. The RNA was precipitated with an equal volume of isopropanol and washed with 80% ethanol. The RNA was air-dried and resuspended in water treated with diethyl pyrocarbonate. The optical density of all of the samples was measured at 260 nm. On average, 20 μg of RNA was obtained from both the normoxic and hypoxic monocytes.

Measurement of the Fold Induction of VEGF mRNA by RNase Protection Assay

The quantity of VEGF mRNA was determined by RNase protection assay by use of a riboprobe to VEGF and to 18S rRNA to allow for sample normalization as previously described. Quantification of signal intensity was performed on a phosphorimager (Fujix). For each patient, a VEGF/18S ratio was calculated for both the hypoxic and normoxic cells. The fold induction of VEGF with hypoxia was calculated by dividing the hypoxic by the normoxic value.

Statistical Analysis

Data are reported as mean±SEM. Analysis between groups for statistically significant differences in enumerative data such as sex, hypertension, hypercholesterolemia, diabetes, cigarette smoking, family history, β-blockers, or prior coronary artery bypass grafting was performed with the use of the χ² test. Analysis between groups for continuous variables such as age and number of diseased vessels was performed with 1-way ANOVA. The fold induction of VEGF mRNA with hypoxia was compared between patients with collateral scores of 0+, 1+, and 2+ by ANCOVA with age, number of diseased vessels, family history of heart disease, diabetes, smoking, hypertension, prior myocardial infarction, and hypercholesterolemia as covariates. Bonferroni post hoc comparisons were performed to compare the adjusted levels of VEGF between the 3 groups.

Results

Patient Enrollment and Study Group Characterization

Over a 3-month period, 81 consecutive patients were approached for inclusion in this study. Six patients declined to participate. Of the remaining patients, 12 were found to have no coronary stenosis at angiography and hence were not included in the study. Twelve patients’ samples were discarded for technical reasons (bacterial contamination of culture in 2 patients and inadequate amount or poor quality of extracted RNA in 10 patients). The profile of the patients with 0+, 1+, or 2+ collaterals with regard to age, sex, hypertension, hypercholesterolemia, cigarette smoking, diabetes, family history, history of prior myocardial infarction, prior coronary artery bypass grafting, β-blocker use, and the number of diseased epicardial coronary vessels is given in the Table. There was no statistically significant difference between the 3 groups in any of these variables.

Measurement of Coronary Artery Collaterals

Coronary artery collaterals were scored by visual analysis by use of conventional criteria by the physician performing the catheterization. All gradings were subsequently reviewed by a single cardiologist blinded to the first reading. There was a >85% concordance between the 2 reviewers. In those cases in which there were disagreements between the 2 reviewers, a third blinded reviewer was asked to review the film and served as arbitrator. Thirty-seven percent of the patients had no collaterals, with 25% of the patients having 1+ and 37% having 2+ collaterals. This distribution of patients with and those without collaterals is in agreement with previous studies in patients with obstructive coronary disease.

Measurement of VEGF mRNA Induction With Hypoxia

A sensitive and quantitative RNase protection analysis was used to precisely quantify the amount of VEGF mRNA in the

<table>
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<table>
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<th>VEGF</th>
<th>18S</th>
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Figure 2. Representative ribonuclease protection assay demonstrating interindividual differences in the hypoxic induction of VEGF mRNA in monocytes from different patients. The ribonuclease assay for the quantitative determination of VEGF mRNA levels in the monocytes was performed as described in Methods. Treatment indicates whether the monocytes were cultured under hypoxic (H) or normoxic conditions (N). The upper band for VEGF represents the protected fragment specific for the 165-amino-acid isoform of VEGF. The second lower VEGF band represents the protected fragment specific for the 189-amino-acid isoform of VEGF. A simultaneous ribonuclease protection assay with 18S rRNA was performed to allow for sample normalization. Quantification of the VEGF mRNA 165-amino-acid isoform and 18S band intensity was performed with a phosphorimager. The fold induction of VEGF by hypoxia was determined by dividing the VEGF/18S ratio under hypoxic conditions by that obtained under normoxic conditions. For representative patients 1 to 4 shown, the hypoxic induction of VEGF mRNA was 3.9-, 2.9-, 1.7-, and 2.4-fold, respectively.
collaterals was highly statistically significant \((P<0.0001)\), as was the difference in the fold induction between patients with no collaterals \((0+)\) and some collaterals \((1+ or 2+)\) \((1.9 \pm 0.2 vs 3.2\pm0.3; P<0.0001)\). In addition, there was a statistically significant difference between the fold induction of VEGF between the \(0+\) and \(1+\) collateral groups \((P<0.04)\). There was no statistical difference between the fold induction of VEGF between the \(1+\) and \(2+\) collateral groups.

Data from the 3 groups with \(0+, 1+,\) and \(2+\) collaterals were subjected to ANCOVA using the variables outlined above (ie, age, sex, prior myocardial infarction, hypertension, family history, hypercholesterolemia, cigarette smoking, diabetes, and number of diseased vessels) as covariates. This revealed an overall significant difference \((F \text{ ratio } 7.7, P<0.002)\). Bonferroni post hoc comparison between the groups \(0+\) and \(1+\) and between the groups \(0+\) and \(2+\) collaterals revealed a statistically significant difference in VEGF induction with hypoxia \((1.5\pm0.4 vs 2.6\pm0.4, P<0.02,\text{ and } 1.5\pm0.4 vs 3.2\pm0.4, P<0.0004, \text{ respectively})\). No significant difference was found between the groups with \(1+\) and \(2+\) collaterals \((2.6\pm0.4 vs 3.2\pm0.4, P<0.3)\). Finally, combining groups \(+1\) and \(+2\) into a single group and repeating the ANCOVA for groups \(+0\) (no collaterals) and the combined group \(+1\) and \(+2\) (some collaterals) revealed a statistically significant difference between these 2 groups in the induction of VEGF with hypoxia \((1.5\pm0.4 vs 2.9\pm0.3, P<0.003)\).

**Discussion**

In this study, we have provided evidence in support of the hypothesis that the ability to respond to progressive coronary artery stenosis by growing coronary artery collaterals is strongly associated with the ability to induce VEGF in response to hypoxia. We have shown that the presence of collaterals is correlated with a significantly higher level of induction of VEGF mRNA in monocytes derived from the same patients. Moreover, the response is graded such that the mean fold induction of VEGF is greater in patients with \(2+\) as compared with \(1+\) collaterals. Perhaps more significantly there is a highly statistically significant difference in the fold induction between the patients when divided into 2 groups: no collaterals \((0+\) collaterals) versus some collaterals \((\text{patients with } 1+\text{ or } 2+\text{ collaterals})\). This is important because although the presence of \(1+\) or \(2+\) collaterals is subject to interpretation of the catheterization films, the presence of none versus some collaterals is clear-cut. The significant difference in the induction of VEGF was maintained even after adjustment of the data for a number of variables (age, sex, prior revascularization, hypertension, hypercholesterolemia, cigarette smoking, and diabetes) that may have an affect on collateral density.\(^{15-22}\) None of these variables have previously been shown to have an effect on the hypoxic regulation of VEGF in monocytes. The failure to detect a role for any of these variables in terms of collateral formation in this study does not prove that they do not influence collateral formation because the power of this study may have been too small to observe such effects.

**Statistical Analysis of Data From Patients With Coronary Artery Disease**

The unadjusted fold inductions of VEGF mRNA for coronary artery disease patients with \(0+\), \(1+\), and \(2+\) collaterals were \(1.9\pm0.2, 2.8\pm0.4,\) and \(3.4\pm0.3\), respectively. The difference in the fold induction between patients with \(0+\) versus \(2+\) samples. All values were normalized to 18S mRNA as previously described.\(^9\) A representative RNase protection assay demonstrating a range of differences in the hypoxic induction of VEGF is shown in Figure 2. There was no significant difference in the mean basal (normoxic) level of VEGF mRNA among the 3 collateral groups. For patients with \(0+\) collaterals the mean normoxic VEGF/18S ratio was \(0.19\pm0.005\), for patients with \(1+\) collaterals the mean normoxic VEGF/18S ratio was \(0.023\pm0.005\), and for patients with \(2+\) collaterals the mean normoxic VEGF/18S ratio was \(0.023\pm0.006\). A fold induction score was determined for each patient, comparing the ratio of VEGF/18S under hypoxia and normoxia (Figure 3).

**Reproducibility of VEGF mRNA Induction With Hypoxia in Monocytes**

To use the fold induction of VEGF mRNA of a given patient as an indicator of a true phenotype for that particular patient, the values obtained must be reproducible and consistent. We obtained blood samples on 2 or 3 different days from 5 normal volunteers. These samples were processed in an identical fashion to those from patients from the catheterization laboratory. The fold inductions of VEGF mRNA with hypoxia from different blood samples drawn on different days from the 5 different normal volunteers were volunteer 1, 2.3, 3.1, and 4.4; volunteer 2, 1.6 and 1.7; volunteer 3, 1.0 and 1.5; volunteer 4, 1.6, 2.0, and 2.4; and volunteer 5, 5.5 and 8.1.

**Figure 3. Scattergram of the fold induction of VEGF by hypoxia**

in all patients included in this study separated by collateral score without adjustment for any of the covariates described.  For the group with \(0+\) collaterals, the mean fold hypoxic induction of VEGF mRNA was \(1.9\pm0.2\), for \(1+\) collaterals it was \(2.8\pm0.4\), and for \(2+\) collaterals it was \(3.4\pm0.3\). There was a statistically significant difference in the fold induction of VEGF mRNA between the group with \(0+\) collaterals and \(2+\) collageners \((P<0.0001)\) and between the group with \(0+\) and \(1+\) collaterals \((P<0.04)\).
We have shown that although normoxic levels of VEGF are not significantly different between patients with different degrees of collateral vessel formation, the patients differ markedly in the hypoxic induction of VEGF mRNA. This is significant because regulation at the steady-state mRNA level is known to be of central importance in determining the VEGF response to hypoxia.8,9 The increase in VEGF mRNA in response to hypoxia is due to both an increase in the transcription of the VEGF gene and to an increase in the stability of VEGF mRNA.20,21 We have previously identified the specific trans-acting nucleic acid binding proteins, HIF-1α and HuR,22,23 that mediate this regulation by hypoxia.23,27 It is interesting to note that HIF-1α has recently been shown to be sensitive to posttranslational modifications that inhibit its ability to transactivate target genes.28 Further work will determine whether these or other modifications can explain the striking interindividual differences in the hypoxic induction of VEGF.

It is notable and important for future mechanistic studies that in this study we have used culture conditions in which the patient’s own plasma was used to culture his or her own monocytes. Originally this was performed because the yield of monocytes and RNA was 4 to 5 times higher with the use of autologous plasma rather than a standardized commercial human plasma. It remains to be determined whether the interindividual differences we have seen in this study are due to an unidentified plasma element or due to differences in monocyte activity. The latter differences could be environmental, epigenetic, or genetic in origin. This question may be approached in future studies with monocytes from “low” and “high” VEGF responders with standardized human serum or by plasma complementation experiments. A genetic basis for this phenomenon may be identified by determining the fold induction of VEGF in monocytes of related family members. The role of a genetic component is supported not only by recent studies demonstrating that the induction of VEGF by hypoxia in multiple different human breast tumor cell lines varies widely29 but also by findings in our own laboratory that primary human foreskin fibroblasts from different donors differ markedly in their induction of VEGF with hypoxia (Levy, unpublished observations).

There are a number of immediate conceptual and clinical implications that arise from the results of this study. First, this study provides a potential explanation for the variability in collateral formation in patients with coronary artery disease. Patients identified as low VEGF responders may benefit more from treatment with parenteral recombinant VEGF to enhance collateral growth.30–32 Second, we have developed a simple in vitro assay to identify low and high responders that may be amenable to pharmacological intervention tailored to augment VEGF production in the low responder group. This will require identification of the precise molecular defect responsible for the lower hypoxic induction in this group. Finally, the demonstration of interindividual variability in the hypoxic induction of VEGF has implications beyond the cardiovascular system. VEGF is a key mediator of the pathological angiogenesis seen in tumors33 and in diabetic retinopathy.34 In both instances, hypoxia in the tumor or retina has been proposed to play an important role in this response. For example, it will be of interest to determine if diabetic patients who develop diabetic retinopathy are “higher inducers” of VEGF than those diabetics who do not develop retinopathy.

Acknowledgments

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