Circulating Humoral Factors and Endothelial Progenitor Cells in Patients With Differing Coronary Collateral Support

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Background—The mechanisms underlying the variation in collateral formation between patients, even with similar patterns of coronary artery disease, remain unclear. This study investigates whether circulating humoral or cellular factors can provide an insight into this variation.

Methods and Results—Thirty patients with isolated left anterior descending coronary artery disease underwent percutaneous coronary intervention with collateral flow index (CFI) determined using a pressure wire. Patients with inadequate (CFI <0.25) compared with those with adequate (CFI ≥0.25) collateral support had, or tended to have, lower concentrations of coronary sinus growth factors and plasma exerting a weaker effect on endothelial cell migration and angiogenesis in vitro. However, there was an inverse correlation between serum mitogenicity and CFI ($r=-0.61$, $P<0.01$). No significant differences were detected between the 2 groups in plasma levels of total vascular endothelial growth factor, vascular endothelial growth factor$_{165}$, or placentinal growth factor. There was a strong positive correlation between numbers of CD34/CD133-positive circulating hemopietic precursor cells and CFI ($r=0.75$, $P<0.001$). In patients with inadequate, compared with those with adequate, CFI, the numbers of differentiated endothelial progenitor cells (EPCs) appearing in the circulation and in culture were significantly reduced by 75% ($P<0.05$) and 70% ($P<0.05$), respectively.

Conclusions—In this study, inadequate coronary collateral development is associated with reduced numbers of circulating EPCs and impaired chemotactic and proangiogenic but not mitogenic activity. These findings are consistent with current efforts to enhance collateral formation by augmentation of circulating EPCs. (Circulation. 2004;109:2986-2992.)

Key Words: collateral circulation ▪ angiogenesis ▪ coronary disease

There is marked heterogeneity in the degree of coronary collateral development between patients, even when their patterns of coronary artery disease (CAD) are similar.1-3 However, the humoral and cellular mechanisms underlying this variability remain unclear. Clinical trials of angiogenic gene and peptide therapy are based on the paradigm that patients with inadequate collateral support are deficient in mitogenic growth factors.4 Emerging evidence indicates that bone marrow–derived circulating endothelial progenitor cells (EPCs) participate in postnatal neovascularization.5-7 These cells are reduced in both number and migratory activity in patients with risk factors for coronary disease or endothelial dysfunction.8,9 EPCs coexpress CD34, CD133 (hematological stem cell markers), and kinase-insert domain-containing receptor (KDR) (vascular endothelial growth factor [VEGF] receptor 2) and develop an endothelial phenotype in culture.10,11 In addition, they incorporate into developing collaterals in animal models of ischemia and thereby enhance neovascularization.12-14 These observations have resulted in the first clinical trial of EPC therapy after myocardial infarction.15

Despite this improved understanding of the circulating factors that may contribute to collateral growth, little is known of their relative contribution to collateral formation in patients. This study, in a carefully characterized population of patients with single-vessel disease of the left anterior descending coronary artery (LAD), was designed to investigate the humoral and cellular arms of the angiogenic response.
Methods

Outline
Patients were divided into the following 2 groups according to their pressure-derived collateral flow index (CFI): ↓ Coll, CFI <0.25; and ↑ Coll, CFI ≥0.25 (see below). Ischemic thresholds on exercise were assessed before percutaneous coronary intervention (PCI). Blood samples collected from the coronary sinus (CS) and aortic root before heparinization were used for determination of plasma growth factor concentration by ELISA and for in vitro biological assays to assess the effects of plasma on angiogenesis and chemotaxis and serum on mitogenicity. Peripheral venous samples taken within 4 weeks of PCI were analyzed by flow cytometry to determine the fraction of circulating EPCs and by cell culture to determine endothelial cell maturation.

Patients
Thirty patients (mean age, 63 ± 4 years; 80% male) with a positive exercise ECG and single-vessel LAD disease awaiting PCI were identified prospectively. The protocol was approved by the St Thomas' Hospital local research ethics committee, and all patients provided written informed consent.

Cardiac Catheterization and Sample Collection
Catheters were passed into the distal third of the CS, ensuring collection of venous blood from the LAD territory. Aortic root and CS samples were collected at baseline before heparinization. Blood collected in EDTA for plasma and SST tubes (Becton Dickinson) for serum was placed immediately on ice and within 30 minutes spun at 3000 g for 30 minutes before aliquoting and storage at −80 °C. The severity of the LAD stenosis was measured from orthogonal views using a quantitative coronary angiography package (Quantum).

Measurement of Pressure-Derived CFI
CFI was calculated after at least 120 seconds of balloon inflation from coronary occlusion pressure (Poccl) using the physiological derivation of collateral flow according to Pijs and colleagues. Pharmacological vasodilators were not used.

Assays
Plasma and serum samples were analyzed using ELISAs and in vitro assays of mitogenicity, angiogenesis, and chemotactic potential. Details of these assays can be found in the accompanying online data supplement.

Isolation, Cultivation, and Characterization of Endothelial Progenitor Cells
The peripheral blood mononuclear cell (PBMCN) fraction isolated by density gradient centrifugation from 40 ml peripheral blood underwent both flow cytometric analysis and culture. PBMCNs were plated on chamber slides coated in fibronectin (4 μg/mL) and gelatin (50% vol/vol) at a density of 4 × 10⁶ cells/well. Culture medium was M199 supplemented with endothelial growth medium (EBM-2, Sigma) and 20% FCS. After 72 hours in culture, nonadherent cells were removed by washing with PBS, and remaining cells were maintained in culture for a total of 7 days. They were then fixed in 2% paraformaldehyde and underwent staining for von Willebrand factor (vWF) (rabbit anti-human mAb-Sigma), CD31, VE cadherin, and KDR (mouse Ab, Santa Cruz) and were visualized by confocal microscopy using monoclonal secondary antibodies conjugated to Alexa 488 and 568 (Molecular Probes). Image acquisition and analyses were undertaken by observers blinded to the CFI of the patients. The mean number of EPCs identified per field from 3 random high-power images was recorded from each of 3 triplicate wells per patient or per control. Control PBMCNs were obtained by venesection of 5 volunteers without a history of heart disease.

Results
Patient Characteristics and Clinical Data
A CFI <0.25 is established on the basis of theory and clinical observation as the threshold below which collateral flow is inadequate to prevent rest ischemia during coronary occlusion. This dichotomy limit was validated in our study population, because subjects with ↓ Coll developed 11.6-fold greater peak ST elevation during coronary occlusion than the ↑ Coll group. These well-collateralized patients had a greater mean percentage diameter stenosis (92% versus 84%; P = 0.04). No statistically significant differences existed between the two study groups in terms of age, number of cardiovascular risk factors, and use of statins. Importantly, diabetes and hypercholesterolemia, conditions associated with impaired collateral formation, were equally prevalent (Tables 1 and 2).

Statistical Analysis
Data are expressed as mean ± SEM. Continuous variables tested for normal distribution by the Kolmogorov-Smirnov test were compared by unpaired t test or 1-way ANOVA. In cases of nonnormal distribution, nonparametric tests were used (Mann-Whitney U test). Linear regression analysis was used to assess correlations in the humoral and cellular assays with CFI. Statistical significance was accepted if the null hypothesis could be rejected at P = 0.05. All statistical analyses were performed using SPSS for Windows 7.0.

Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>CFI &lt;0.25 (n=18)</th>
<th>CFI ≥0.25 (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y</td>
<td>60.5 (±4)</td>
<td>66 (±3)</td>
<td>NS</td>
</tr>
<tr>
<td>Male, %</td>
<td>78</td>
<td>83</td>
<td>NS</td>
</tr>
<tr>
<td>Mean CFI</td>
<td>0.09</td>
<td>0.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Risk factors, % each group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td>78</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>33</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>Hypercholesterolemia†</td>
<td>61</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking</td>
<td>56</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td>Medication, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺ antagonist</td>
<td>33</td>
<td>33</td>
<td>NS</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>44</td>
<td>66</td>
<td>NS</td>
</tr>
<tr>
<td>Nitrate</td>
<td>28</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>ACE-I</td>
<td>6</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Statin</td>
<td>56</td>
<td>42</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Hypercholesterolemia defined as cholesterol >5.2 mmol/L. †Hypertension defined as systolic >150mm Hg or diastolic >90mm Hg.
Transmyocardial Growth Factor Concentrations

Growth factor levels measured before heparinization in aortic root and CS are presented in Table 3. A significant transmyocardial gradient for basic fibroblast growth factor (FGF) was identified in both groups, with the highest CS levels measured in the ▼ Coll group. There was a tendency for CS levels of VEGF to exceed those in the aorta in the ▼ Coll, but not the ▲ Coll, group. The lack of statistical significance may be because the study was underpowered to detect such a difference. Placental growth factor did not seem to differ by group or sample site.

[^H]Thymidine Incorporation

The rate of DNA synthesis was 80% greater in human umbilical vein endothelial cells (HUVECs) exposed to serum samples from ▼ Coll versus ▲ Coll patients (Figure 1A). There was a significant inverse correlation between CFI and relative mitogenic activity in HUVECs (Figure 1B) but no transmyocardial gradient in mitogenic activity (data not shown). Anti-VEGF and anti-FGF antibodies diminished mitogenicity to the same extent in CS samples from both groups (data not shown). However, anti-FGF Ab caused a mean 34% inhibition, whereas anti-VEGF antibody caused only 14% inhibition.

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**TABLE 2. Angiographic and Exercise Data**

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Collateral Flow Index &lt;0.25 (n=18)</th>
<th>Collateral Flow Index ≥0.25 (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Diameter stenosis</td>
<td>84±10</td>
<td>92±6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Proximal LAD*</td>
<td>75%</td>
<td>80%</td>
<td>NS</td>
</tr>
<tr>
<td>Translesional pressure gradient, mm Hg</td>
<td>41±28</td>
<td>46±26</td>
<td>NS</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>102±12</td>
<td>103±17</td>
<td>NS</td>
</tr>
<tr>
<td>Mean distal coronary pressure, mm Hg</td>
<td>58±25</td>
<td>57±20</td>
<td>NS</td>
</tr>
<tr>
<td>Exercise ECG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise test duration, s</td>
<td>483±159</td>
<td>431±125</td>
<td>NS</td>
</tr>
<tr>
<td>Time to 0.1-mV ST depression, s</td>
<td>337±171</td>
<td>340±167</td>
<td>NS</td>
</tr>
<tr>
<td>Ischemia recovery time, s†</td>
<td>298</td>
<td>141</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PCI</td>
<td>0.93±0.3</td>
<td>0.08±0.04</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Before first diagonal vessel.
†Time to return from 0.1-mV ST depression to baseline (seconds).

**TABLE 3. Plasma Growth Factor Concentrations Measured by ELISA**

<table>
<thead>
<tr>
<th>Collateral Flow Index &lt;0.25 (n=18)</th>
<th>Collateral Flow Index ≥0.25 (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF, pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic root</td>
<td>19±2</td>
<td>22±2</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>18±2</td>
<td>32±6*</td>
</tr>
<tr>
<td>Total VEGF, pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic root total</td>
<td>22±1</td>
<td>25±2</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>24±3</td>
<td>34±6</td>
</tr>
<tr>
<td>Basic FGF, pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic root</td>
<td>7±1</td>
<td>16±5</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>16±3†</td>
<td>32±7‡</td>
</tr>
<tr>
<td>Placental growth factor, pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic root</td>
<td>16±2</td>
<td>19±2</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>16±1</td>
<td>20±4</td>
</tr>
</tbody>
</table>

*P=0.27 vs Aortic root in same CFI group.
†P<0.01 vs aortic root in same CFI group.
‡P<0.005 vs aortic root in same CFI group.

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Figure 1. Rate of [^H]thymidine incorporation normalized to control pooled human serum (relative mitogenicity). A, Relative mitogenicity is displayed by collateral grade, as follows: ▲ Coll (CFI =0.25) and ▼ Coll (CFI <0.25). B, The same information plotted against CFI as a continuous variable.
In Vitro Angiogenesis Assay
Four patients were excluded from the in vitro angiogenesis assay because of loss of viability of the HUVECs or cell clumping in the Matrigel wells. CS plasma 10% from 1 Coll patients stimulated significantly greater in vitro tube formation than plasma from 2 Coll patients. In addition, the resulting primitive tubular connections were more stable and had increased branching (Figure 2A).

Transwell Migration Assay
Six patients were excluded as a result of bacterial contamination. The chemotactic activity of patients’ plasma mirrored the in vitro angiogenesis assay activity. The weakest migration response to CS plasma occurred in the ↓ Coll CS patients with the lowest tertile of CFI (<0.13) (Figure 2B).

Flow Cytometry
The first 7 patients recruited into this study were not included in EPC characterization because of inadequate optimization of techniques. Circulating EPCs are characterized by coexpression of CD34, CD133, and KDR. There were no significant differences in the total number of CD34-positive cells between age-matched controls without known CAD and the patients with LAD stenoses (0.35%±0.05% versus 0.38%±0.06%). There was a strong positive correlation between CD34/133-positive hemopoietic stem cells and CFI, r=0.75, P<0.0001 (Figures 3A and 3B). We found that 8% of the CD34/CD133-positive cells also expressed KDR in the lymphocyte gate. All KDR-positive cells expressed CD133. Circulating EPC numbers were reduced in the study patients compared with control subjects (CD34/KDR-positive, 0.016% versus 0.04%, respectively; P<0.05). ↑ Coll patients had a 4-fold greater circulating EPC fraction than the ↓ Coll group (Figure 4), approaching that seen in the control subjects. We did not identify any significant correlation between number of risk factors and CD34/KDR+ve or CD34/CD133+ve cell numbers. There were no differences in

Figure 2. A, Effect of plasma from patients with adequate (↑ Coll) and inadequate (↓ Coll) collateral support on rate of in vitro tube formation. Assays are performed on Matrigel, and number of tubes formed are identified per high-power field normalized to control pooled human plasma. Plates illustrate representative wells from each group. B, Transwell migration in a modified Boyden chamber of labeled endothelial cells toward plasma derived from patients in different CFI tertiles. Values are normalized to control plasma. ↓ Coll (CFI <0.25), ↑ Coll (CFI ≥0.25).

Figure 3. Number of CD34/CD133 dual-positive hemopoietic stem cells in patients with adequate (↑ Coll) and inadequate (↓ Coll) collateral support. Cell number according to high and low CFI as a dichotomized variable (A) and related to CFI as a continuous variable (B). ↓ Coll (CFI <0.25), ↑ Coll (CFI ≥0.25).

When the data were analyzed using the dichotomy limit of 0.25 for CFI, the ↓ Coll group had a chemotaxis rate of 48% of control plasma and ↑ Coll group 80% of control plasma (P=0.07).
the numbers of circulating CD34/CD133-ve or CD34/ KDR-ve cells in patients receiving statins versus those not receiving statins (0.23% versus 0.18% or 0.028% versus 0.012%, respectively).

EPC Culture Assay
Cells with an endothelial phenotype were derived from the PBMC fraction and characterized after 7 days of culture. Figure 5A illustrates confocal microscopic images of vWF and KDR expression. A total of 99% of EPCs expressed vWF and KDR, and 30% coexpressed vWF and CD31 (not shown). This culture assay only partially reflects the higher circulating EPC pool identified by flow cytometry in the ↑ Coll patients. The higher number of EPCs cultured from the ↑ Coll patients could have arisen from either higher numbers of cells adhering initially or increased propagation of attached cells from days 3 through 7. As a group, these patients formed fewer endothelial cells in culture than healthy controls (Figures 5B and 5C). However, cultured endothelial cell numbers from ↑ Coll patients approached control levels, with a positive correlation between endothelial cell density and CFI (Figure 5D).

Discussion
This is the first study to investigate the relationship between circulating EPC populations and native coronary collateral development. These data reveal significant differences in the cellular and humoral components thought to contribute to the variability in collateral support in patients with flow-limiting LAD stenoses. In summary, patients with well-developed collaterals have more numerous circulating EPCs coupled with plasma that exerts a more powerful chemotactic and proangiogenic effect on endothelial cells in vitro.

Interrelationship Between Coronary Artery Stenosis Severity, EPCs, and Collaterals
Coronary artery stenosis severity is a long-recognized determinant of collateral development. In the current study, we also found that patients with well-developed collaterals had more severe LAD stenoses. This group of patients also had a greater number of circulating EPCs, and these cells more readily formed mature endothelial cells in culture. These observations can be interpreted in several ways. The most simplistic interpretation is that LAD stenosis severity is the key determinate of CFI and that the changes in EPCs are merely a reflection of the consequences of stenosis severity, such as myocardial ischemia. Within this scenario, the EPCs may either act as innocent bystanders, although this view is not consistent with our current understanding of the biological potential of these cells, or may act as envoys helping to establish the relationship between stenoses and collaterals. A more complex interpretation is that EPCs are a key determinate of collateral formation and of greater potential importance than stenosis severity. We believe this later interpretation is supported by our study and is also consistent with several other observations. For example, it is recognized that the considerable interindividuation variation in collateral formation cannot be explained by variations in coronary artery stenosis severity. This is borne out in our current study, where percentage diameter stenosis only weakly determines CFI (r^2=0.13). Even this determination coefficient may be overly optimistic, because in a much larger database of 450 patients, the value was even lower (r^2=0.01) but nonetheless statistically significant. Lastly, the lack of a close relationship between collaterals and coronary artery stenosis severity has been most convincingly demonstrated by the great variability in CFI that exists between patients without flow-limiting coronary artery stenoses. Taken together, these observations suggest that factors other than stenosis severity mainly determine collateral flow. One such alternative factor may relate to the quantity and quality of circulating EPCs, a view supported by the current study, where the number of CD34/CD133+ve cells was more closely associated with CFI than stenosis severity.
Effects of Humoral Factors on Vascular Cell Behavior

The biological assays examined 3 specific processes relevant to collateral formation: endothelial cell proliferation, chemotaxis, and tube formation in Matrigel. The last of these is a complex assay integrating measures of cellular proliferation, chemotaxis, cell alignment, and apoptosis. The data from the Matrigel and migration experiments indicate that humoral factors in patients with a high CFI drive endothelial cells toward migration and cellular capillary-tube formation.

The inverse relationship between mitogenicity in HUVECs and CFI is counterintuitive but suggests differential effects on endothelial cell behavior, analogous to the differential modulation of migration and proliferation by FGF. This induction of a promigratory, rather than proliferative, endothelial phenotype in the ▼ Coll patients may be an important determinant of in vivo angiogenesis. The finding of more mitogenic CS serum in ▼ Coll patients could be explained by the fact that the myocardium in these patients is ischemic for more prolonged periods at the subendocardial level, illustrated by the longer ischemia recovery times after exercise. Additionally, there may be an intrinsic resistance in the responsiveness of the vascular elements involved in collateral formation to mitogenic stimuli, such that a persistent promitogenic stimulus is required to initiate collateral development. This concept is supported by Waltenberger et al., who showed that the migration response of monocytes is downregulated in diabetic patients, and observations by Metais et al. of an impaired NO-dependent response to VEGF in atrial microvessels of CAD patients.

Study Limitations

Although plasma levels of specific angiogens reflect the general cytokine milieu across the myocardial bed, they cannot necessarily be extrapolated to represent the myocardial interstitium given the effects that extracellular matrix proteins have on growth factor presentation and receptor binding. Indeed, there are an increasing number of reports that describe a complex system of collaboration between growth factors and extracellular matrix components in modulating cellular responses. For example, fibronectin, through its VEGF binding domains, enhances the migration and proliferation of endothelial cells in response to VEGF by promoting a specific physical association between VEGF receptor 2 and integrin α5β1, leading to a sustained activation of mitogen-activated protein kinase.

Figure 5. Characteristics of peripheral blood mononuclear cells after 7 days of culture. A, Dual labeling for KDR and vWF of peripheral blood mononuclear cells maintained in culture for 7 days. B, Mean number of EPC/high-power field identified in age-matched healthy controls and study patients (CAD). C and D, Numbers of dual-labeled mononuclear cells appearing in culture with the study population against CFI group or CFI as a continuous variable (r=0.54, P=0.01) (D). ▼ Coll (CFI <0.25), ▲ Coll (CFI ≥0.25).
Conclusions
Statin therapy restores EPC numbers\textsuperscript{24,25} and enhances angiogenesis in the ischemic hind-limb model.\textsuperscript{26} Thus, increasing the EPC population seems to play an important role in ischemic protection. The 4-fold increase in the circulating EPC population in patients with \textup{\textup{\upshape Coll}} versus \textup{\textup{\textup{\textup{\downshape Coll}}} supports this contention. Our clinical findings directly support the suggestion that targeted therapeutic strategies to enhance circulating EPC numbers and functional activity either alone or in combination with delivery of proangiogenic cytokines may maximize functional collateral formation.

References
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**ELISA**

Sandwich ELISA was employed to measure the following GFs in plasma samples: total VEGF, FGF, placental growth factor (PlGF) (R&D Systems) and VEGF$_{165}$ (Genentech). These assays were undertaken according to manufacturer’s instructions and repeated in triplicate.

**In Vitro Mitogenicity Assay**

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in M199 and 20% FCS according to the method described by Jaffe et al. To assess the effect of the humoral fraction on proliferation rates in HUVEC, passage 2 cells were plated at a density of 10,000 cells per well in a 96 well plate. Under these conditions, the cells were in their active log proliferation phase for the duration of the experiment and had not reached confluence when harvested. After 24h, HUVEC were growth arrested in 0.5% FCS. They were then exposed to a 10% concentration of patient serum in M199 for 24 h. During the last 6h of culture 1µCi of [$^3$H]thymidine was added to each well. Cells were then isolated for measurement of incorporated [$^3$H]thymidine. Each experiment was performed in 5 wells / condition on 3 different HUVEC populations. Proliferative rate (mitogenicity) was expressed relative to the rate observed in 10% pooled normal human serum, obtained from a commercial source (Sigma, UK), employed as a reference control. Control cultures in 0.5% FCS incorporated 3000-6000 cts/min [$^3$H]thymidine in 6 hours. Blocking antibody experiments were conducted employing 10µg/ml anti-VEGF and anti-FGF monoclonal antibodies (BD). These experiments were performed in triplicate.

**In Vitro Angiogenesis Assay**

This assay employed a growth factor depleted basement membrane matrix to study the effects of patients’ plasma on in vitro angiogenesis by endothelial cells. The induced phenotypic changes
involve cell migration, differentiation and alignment together resulting in the formation of tubular structures in the gel.

The assay was performed as described previously\textsuperscript{5} with the following modifications. Passage 2-4 HUVEC were cultured to confluence in normal growth medium and the monolayer washed with PBS at 37\degree C. This monolayer was then exposed to a 10\% concentration of patient plasma in M199. After 24h, the HUVEC were trypsinised and resuspended in the same medium. 100\mu l of Matrigel (BD) was added to each well of a 96 well plate and incubated at 37\degree C for 1h to promote gelling. Cells were plated at a density of 3\times10^4 cells/well. After 24 hours, a digital image of in vitro tube formation was acquired by light microscopy and the number of intact tubes formed/high power field normalised to control pooled human plasma were measured. Each experiment was performed in triplicate and repeated 3 times. In vitro tube formation was referenced to that observed in cells exposed to 10\% pooled human plasma.

\textit{Migration Assay}

A transwell migration assay was employed to measure the chemotactic activity of plasma samples. Briefly, calcein-AM labelled HUVEC were loaded into the upper well of a modified Boyden chamber at a density of 30,000 cells.\textsuperscript{6} The chamber was placed in a 24 well culture dish containing a 10\% solution of patient plasma in M199. 10\% pooled human plasma in M199 served as a reference control. The amount of cell migration relative to reference control was determined at 3h in a fluorescent plate reader.

\textbf{Reference List}


