Reduced Pericardial Levels of Endostatin Correlate With Collateral Development in Patients With Ischemic Heart Disease

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OBJECTIVES We investigated whether pericardial levels of a pro-angiogenic factor (vascular endothelial growth factor, VEGF) or an anti-angiogenic factor (endostatin) related to the presence of coronary collateral circulation in patients with significant coronary artery disease (CAD).

BACKGROUND Coronary collateralization favorably alters the prognosis of patients with occlusive CAD. The specific factors that mediate and maintain collateral formation in coronary vessel occlusion are yet to be identified.

METHODS Coronary angiograms from 39 patients undergoing coronary artery bypass surgery were evaluated for the absence of collaterals (n = 20) or the presence of Rentrop classification grade 3 collaterals (n = 19). Pericardial fluid samples were obtained at the time of surgery and were assayed for the VEGF and endostatin by enzyme-linked immunosorbent assay comparing the two groups of patients.

RESULTS Vascular endothelial growth factor levels were not significantly different between the groups (28.86 ± 4.67 pg/ml vs. 24.39 ± 3.08 pg/ml, p = 0.43). However, pericardial fluid endostatin levels were nearly 40% lower in patients with grade 3 collateralization compared with those lacking angiographic evidence of collaterals (15.17 ± 1.87 ng/ml vs. 24.25 ± 2.08 ng/ml, p < 0.0025).

CONCLUSIONS Pericardial fluid levels of endostatin, but not VEGF, are associated with the presence or absence of collaterals in patients with CAD. These data suggest that the angiogenesis inhibitor endostatin levels may locally modulate coronary collateral formation.

The presence of coronary collateralization improves the prognosis of patients with obstructive coronary artery disease (CAD). Collateral limits infarct size and improves ventricular function and overall perfusion in ischemic myocardium (1–3). The development of coronary collaterals appears to be initiated by an occlusive event resulting in opening of preexisting anastomotic channels through increase in shear forces and pressure or by formation of novel capillary sprouts (angiogenesis) from chronic ischemia (4). Subsequent maintenance and maturation of these vessels (arteriogenesis) is believed to be mediated by a balance of pro-angiogenic and anti-angiogenic factors that favor neo-vascularization (4).

Assessment of these substances is difficult as they are produced locally in ischemic cardiac tissue and are lower in systemic circulation because of dilution upon washout (3). Coronary sinus and arterial samples can demonstrate a small “step-up” in concentration of factors produced in the myocardium (5), but are subject to the same washout influences and may not necessarily demonstrate a gradient (6,7).

Pericardial fluid analysis provides a modality that may reflect the local angiogenic milieu more accurately (8). For example, angiogenic substances such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF) are more concentrated in pericardial fluid than in serum and are elevated in patients with ischemic heart disease (9,10). In addition, pericardial fluid has been shown to accelerate vascular smooth muscle cell growth and induce endothelial cell apoptosis, suggesting that substances in pericardial fluid are active and may exert physiologic effects locally (11,12).

Our goals in the present study were to measure VEGF and endostatin levels in the pericardial fluid of patients with significant obstructive CAD and to investigate their relation to the presence of coronary collaterals.

Vascular endothelial growth factor is an angiogenic growth factor that is implicated in both normal angiogenesis (development, wound repair) and abnormal angiogenesis (e.g., tumor growth and retinopathy) (13). It is expressed early in patients with acute myocardial ischemia and implicated in neovascularization (14,15). Recent research has shown that VEGF can enhance therapeutic angiogenesis in experimental models (16).

Endostatin is a potent inhibitor of angiogenesis that was first isolated from in vitro murine hemangiendothelioma cell line cultures. It is an endogenous 20kD protein that is the noncollagenous carboxy-terminal fragment of collagen XVIII produced by proteolytic cleavage (17). Elastase, cathepsin L, and various other enzymes have been shown to produce endostatin from collagen XVIII (18).

Endostatin exhibits potent anti-angiogenic activity by...
inhibiting proliferation and migration of endothelial cells in addition to inducing endothelial cell apoptosis (17,19). The specific receptors and signaling cascades for its mechanism of action are yet to be defined. A variety of malignancies are associated with elevated circulating serum endostatin levels. Endostatin has been shown to inhibit the growth of many types of murine cancers by inhibiting tumor angiogenesis and inducing dormancy. Recently, a phase I clinical trial for treatment of solid tumors has been published demonstrating safety and minor antitumor activity (20).

METHODS

This study was approved by the Indiana University Institutional Review Board. Patients were consecutively enrolled and informed consent was obtained before cardiac surgery.

Patient selection. We reviewed the coronary angiograms of patients serially undergoing coronary artery bypass grafting (CABG) and assessed for coronary collateralization using the classification scale described by Rentrop et al. (21). This scale ranges from grade 0 (representing absence of contrast filling an epicardial vessel) to grade 3 (complete filling of the epicardial vessel by a contralateral artery). Patients were included if they had significant obstructive coronary disease (>70% stenosis) in at least two vessels requiring CABG and could be stratified into either one of the following two groups: the first group consisted of patients without evidence of coronary collateralization (grade 0, n = 20); the second consisted of patients demonstrating the highest degree of coronary collateralization (grade 3, n = 19).

Major exclusion criteria consisted of prior CABG, evidence of ongoing malignancy or neoplasm, infection, and concomitant valvular disease requiring surgery.

Factor measurements. Vascular endothelial growth factor and endostatin levels were determined by enzyme-linked immunosorbent assay according to the manufacturer’s instructions. Briefly, the pericardial fluid samples were collected during cardiac surgery at the time of pericardiotomy. The fluid was centrifuged to separate debris and then frozen at −70°C for subsequent analysis. Factors were measured by enzyme-linked immunosorbent assay (VEGF: R & D Systems, Minneapolis, Minnesota, sensitivity 1 pg/ml; endostatin: Cytimmune, College Park, Maryland, sensitivity 1.953 ng/ml). The absorbance was measured by optical densitometry at 450 nm.

Data collection and statistical analysis. Clinical data were collected by chart review. Categorical demographic data were compared by Fisher exact test. Continuous variables are presented as the mean ± SEM, and compared by Student t test and multivariate stepwise logistic regression. All probability values are two-tailed.

RESULTS

The patient demographics in the first group with absence of collateral development (grade 0) and with the greatest degree of collateral development (grade 3) are presented in Table 1. As expected, there was a high prevalence of coronary risk factors in both groups including hypertension, smoking, and hyperlipidemia. No significant differences in patient characteristics were evident between groups, although a trend toward more men and younger patients was noted in the collateral group. A two-way analysis of variance (ANOVA) using both gender and collateral grade as grouping factors revealed no evidence of general gender-related effect on pericardial fluid endostatin levels (F = 0.3, p = 0.578).

The clinical presentation of patients before surgery and fluid collection are presented in Table 2. This demonstrated a heterogeneous group comprised of patients with either stable angina or recent acute coronary syndromes (within 24 to 48 h). Both syndromes were similarly represented in both groups.

The extent of CAD in the patient groups is characterized in Table 3. The ischemic burden was similar in both patient groups. The number of diseased vessels with 70% or greater diameter stenosis, grafts required for revascularization, ejection fraction, and coronary vessels involved were not different between groups with the exception of more right CAD in the collateral group (p = 0.05).

Table 1. Patient Demographics

<table>
<thead>
<tr>
<th>Grade 0</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>65.4 ± 2.1</td>
</tr>
<tr>
<td>Males (n)</td>
<td>12/20</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>13/20</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>18/20</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>14/20</td>
</tr>
<tr>
<td>Hyperlipidemia (n)</td>
<td>15/20</td>
</tr>
<tr>
<td>Peripheral arterial occlusive disease (n)</td>
<td>10/20</td>
</tr>
<tr>
<td>End-stage renal disease</td>
<td>4/20</td>
</tr>
</tbody>
</table>

ns = not significant.

Table 2. Clinical Presentation

<table>
<thead>
<tr>
<th>Grade 0</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous MI (n)</td>
<td>7/20</td>
</tr>
<tr>
<td>Stable angina</td>
<td>9/20</td>
</tr>
<tr>
<td>Acute coronary syndrome*</td>
<td>11/20</td>
</tr>
<tr>
<td>Enzyme positivity†</td>
<td>9/21</td>
</tr>
</tbody>
</table>

*Includes unstable angina and myocardial infarction. †Elevation in troponin I or creatine kinase-MB fractions.

MI = myocardial infarction; ns = not significant.
All patients in the grade 3 collateral group had at least one vessel subtotally or completely occluded, in contrast to the grade 0 group, which evidenced only one such patient (p < 0.0001). This is consistent with previous reports that note the need for transient or permanent coronary vessel occlusion as a stimulus for growth factor modulation and collateral formation (22,23).

Vascular endothelial growth factor and endostatin levels with respect to gender, diabetes, acute coronary syndromes, and ejection fraction are shown in Figure 1. There were no statistical differences in these characteristics for both factors. Enzymatic evidence of myocardial necrosis, age, peripheral arterial occlusive disease, and extent of CAD (2-vessel vs. 3-vessel disease) also demonstrated no differences in the levels of these factors (data not shown).

Factor comparison based upon collateral grade revealed that VEGF was no different between the two groups. However, pericardial fluid endostatin levels were found to be approximately 40% lower in patients with collaterals in comparison to those without (15.17 ± 1.87 vs. 24.25 ± 2.08, p = 0.0025) (Fig. 2).

To identify determinants of collateral growth, multivariate stepwise logistic regression analysis was performed with collateral grade (0 or 3) as the outcome. Several characteristics were evaluated, including various demographic characteristics, gender, acute coronary syndrome/myocardial infarction, and endostatin levels.

In this multivariate analysis, only pericardial fluid endostatin level was found to be predictive. The odds ratio for endostatin level was 0.88 (95% confidence interval 0.81 to 0.97), p < 0.0001.

Subsequent analysis of a subset of patients with at least one subtotally and totally occluded artery who had no myocardial infarction demonstrated a similar difference in pericardial fluid endostatin levels (9.09 ± 2.18 ng/ml grade 3 vs. 22.33 ± 4.21 ng/ml grade 0, p = 0.03).

**DISCUSSION**

This study is the first to demonstrate a correlation between the pericardial levels of an anti-angiogenic factor with coronary

### Table 3. Extent of Coronary Artery Disease

<table>
<thead>
<tr>
<th></th>
<th>Grade 0</th>
<th>Grade 3</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of diseased</td>
<td>Grade 0</td>
<td>Grade 3</td>
<td>p</td>
</tr>
<tr>
<td>vessels &gt;70%</td>
<td>2.5 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>0.13</td>
</tr>
<tr>
<td>Number of grafts reqd</td>
<td>3.2 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>0.83</td>
</tr>
<tr>
<td>Occluded coronary</td>
<td>1/20</td>
<td>19/19</td>
<td>0.001 *</td>
</tr>
<tr>
<td>arteries (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ejection fraction %</td>
<td>45.0 ± 3.5</td>
<td>38.3 ± 4.0</td>
<td>0.22</td>
</tr>
<tr>
<td>Left main disease &gt;50%</td>
<td>5/20</td>
<td>6/19</td>
<td>0.73</td>
</tr>
<tr>
<td>Left anterior desc.</td>
<td>16/20</td>
<td>18/20</td>
<td>0.34</td>
</tr>
<tr>
<td>artery disease &gt;70%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left circumflex desc.</td>
<td>12/20</td>
<td>13/20</td>
<td>0.74</td>
</tr>
<tr>
<td>artery disease &gt;70%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right coronary desc.</td>
<td>15/20</td>
<td>19/19</td>
<td>0.05</td>
</tr>
<tr>
<td>artery disease &gt;70%</td>
<td></td>
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</tbody>
</table>

*Denotes statistical significance.
ns = not significant.

**Figure 1.** Vascular endothelial growth factor (VEGF) and endostatin levels in patients grouped with respect to gender, presence or absence of diabetes mellitus (DM), acute coronary syndrome (ACS) (present or absent), and ejection fraction (EF) (<35% or >35%). There are no statistically significant differences shown in either VEGF or endostatin with respect to any of these factors.
collateral development in patients. Our data demonstrate that pericardial fluid endostatin is about 40% lower in patients with robust coronary collateral development than in patients without collaterals. This finding may, in part, explain differential angiogenic responses to ischemia in certain patients.

In addition, all patients in the collateral group exclusively had at least one occluded epicardial vessel. It is well established that vessel occlusion or severe stenosis is a potent stimulus for collateral formation; however, in the context of our study, it can be postulated that such occlusion signals the local downregulation of anti-angiogenic “tone” as a prelude to the formation of collateral vessels.

The levels of endostatin measured in this study are consistent with concentrations known to be inhibitory to endothelial cells in vitro. Furumatsu et al. (24) demonstrated that endostatin specifically inhibited proliferation of human umbilical vein endothelial cells at concentrations of 1 ng/ml, 10 ng/ml, and 100 ng/ml in a dose-dependent manner and also inhibited attachment of endothelial cells to collagen I. More importantly, similar inhibitory effects were also observed in endothelial cells that were stimulated by angiogenic growth factors such as VEGF and basic fibroblast growth factor (bFGF) (25), suggesting that endostatin’s potent effects may be mediated by signaling pathways that are independent of the VEGF and bFGF signaling. Assuming that pericardial fluid concentrations approximate those in the myocardial interstitium, we can postulate that the dose-related inhibitory effects of endostatin at the levels measured are physiologically relevant, and may be more pronounced in the group of patients without collateral development (grade 0) as their pericardial fluid levels were significantly higher. In our analysis, the odds ratio for endostatin levels was 0.88.

It is of interest that VEGF was not significantly different between the two groups and suggests that the process of arteriogenesis and maturation of collateral channels may not necessarily be dependent on persistent VEGF elevation. As discussed earlier, the in vitro inhibitory action of endostatin persists despite VEGF stimulation (25). Vascular endothelial growth factor is clearly important in angiogenesis and is upregulated in response to acute ischemia. However, its role in collateral development is less understood. It has been reported that monocytes derived from patients with collaterals exhibit greater VEGF production; however, this was noted in response to acute hypoxia (26).

These data suggest the hypothesis that the process of neovascularization in patients with ischemic heart disease depends on repression of anti-angiogenic influences, such as endostatin, to facilitate neovascularization. This may help explain the limited clinical success in recent attempts at myocardial angiogenesis (16). Modulation of local anti-angiogenic factors represents an important aspect in future studies of angiogenesis.

**Study limitations.** There are several limitations to the present study. First, there were no control patients without coronary disease for comparison. Serum samples were not available for study, although “washout” and dilution of these factors in systemic circulation limit its utility.

Our study stratifies patients based on angiographically evident collaterals and does not account for inherent limits in detecting vessels smaller than 200 μm, such as recruitable collaterals. These are thought to be pre-existing anastomotic channels that are not angiographically evident until maturation through arteriogenesis. However, these vessels are functionally evident on coronary pressure wire measurements of collateral flow index and other modalities.

The present findings show a correlation of reduced endostatin levels with collateral development but do not prove causation. Experimental models that have been used to demonstrate the inhibitory effects of angiostatin on coronary collateral formation (22) may allow for demonstration of the causal effect of endostatin on coronary collateral formation. Furthermore, future studies may also examine whether inhibition of pericardial or interstitial endostatin with an antibody or similar inhibitor may improve therapeutic angiogenesis.
REFERENCES


