Angiostatin is negatively associated with coronary collateral growth in patients with coronary artery disease

Toshiro Matsunaga,1 William M. Chilian,3 and Keith March2
1Second Department of Internal Medicine, Hirotsuki University School of Medicine, Hirotsuki, Japan; 2Department of Medicine and Center for Vascular Biology, Indiana University Medical School, Indianapolis, Indiana; and 3Department of Physiology, Louisiana State University Health Science Center, New Orleans, Louisiana

Submitted 6 July 2004; accepted in final form 14 December 2004

Angiostatin is negatively associated with coronary collateral growth in patients with coronary artery disease. Am J Physiol Heart Circ Physiol 288: H2042–H2046, 2005; doi:10.1152/ajpheart.00669.2004.—Angiostatin, an inhibitor of tumor angiogenesis, is produced by the actions of matrix metalloproteinases (MMP) on plasminogen. Recently, we reported that angiostatin levels are increased in a model of inadequate coronary collateral growth and angiogenesis in response to ischemia, despite high levels of vascular endothelial growth factor (VEGF). We hypothesized that angiostatin levels are negatively associated with collateral formation in patients. Coronary angiograms from 37 patients undergoing coronary bypass surgery were evaluated for the absence of angiographically visible collaterals (Rentrop scores of 0) or the presence of Rentrop classification grade 3 (well-developed) collaterals. Pericardial fluid was obtained from each patient during the bypass procedure, and the sample was analyzed for angiostatin, plasminogen, and VEGF (Western analysis) and for combined activities of MMP-2 and MMP-9 (zymographic analysis). In patients with no collaterals, angiostatin level was greater compared with that in patients with well-developed collaterals (3.1 ± 0.2 vs. 2.3 ± 0.1 optical density units, P < 0.05). Neither MMP activities nor VEGF levels were different between the two groups of patients. The higher levels of angiostatin in patients with no visible collaterals were reflective of a higher concentration of plasmin/plasminogen (6.2 ± 0.7 vs. 4.2 ± 0.5 optical density units, P < 0.05) compared with those in patients with well-developed collateral vessels. Our results support the concept that the growth inhibitor angiostatin may have a negative impact on coronary collateral growth in patients. Perhaps therapies attempting to provoke coronary collateral growth should incorporate approaches to limit or neutralize the effects of growth inhibitors.

Angiostatin inhibits tumor angiogenesis and metastases (11, 12) and is produced via degradation of plasminogen (1, 12) by matrix metalloproteinases (MMP)-2, -7, and -9 (12, 13). MMP-2 and -9 activities are upregulated in atherosclerotic plaques and failing heart in humans (4, 23), cellular environments under oxidant stress and with reduced nitric oxide (NO) bioavailability. Recently, we reported that angiostatin levels are increased in a model of inadequate coronary collateral growth and angiogenesis in response to ischemia under NO synthesis inhibition, despite high levels of VEGF (8, 9). These results indicate that coronary collateral growth and development may be dependent on a balance between levels of growth inhibitors and growth factors. Therefore, we proposed that the impairment of coronary collateral growth is contributed to by increased production of angiostatin in humans. To test this hypothesis, we measured the expression of angiostatin, VEGF, plasminogen/plasmin, and activities of MMP-2 and -9 in pericardial fluid taken from patients with CAD who underwent coronary bypass surgery.

METHODS

Patient profiles. This study enrolled 37 consecutive patients with CAD who underwent coronary artery bypass grafting (CABG) and met the criteria for stratification into either of two groups: the first consisted of 24 patients with CAD without evidence of coronary collateralization (Rentrop collateral grade 0), and the second group consisted of 13 patients with CAD having the highest degree of coronary collateralization (grade 3). All patients gave their informed consent, and the study protocol was approved by the Indiana University Institutional Review Board. Patients were included if they had significant obstructive coronary disease (>70% stenosis in at least 2 or more vessels requiring CABG). Major exclusion criteria included prior CABG, evidence of neoplastic, hepatic, infectious, and autoimmune disease, and concomitant valvular disease requiring surgery.

Sampling pericardial fluid during surgery. Immediately after a small incision of the pericardium was made, undiluted pericardial fluid was obtained. The samples were collected in sterile tubes and immediately placed on ice. The average amount of pericardial fluid obtained from each patient was 3.2 ± 0.3 ml. After clarification of the cellular components by centrifugation at 3,000 g for 10 min at 4°C, supernatants were rapidly frozen and stored at −80°C until use.

Western analyses. Pericardial fluid (15 μl) was collected and diluted 1:1 in SDS sample buffer. Each sample (30 μl) was separated in a 10% SDS-PAGE gel. We used mouse monoclonal angiostatin...
primary antibody (Upstate Biotechnology) and VEGF primary antibody, and the secondary antibody (IgG) was labeled with horseradish peroxidase (HRP). After incubation with HRP substrate (Pierce SuperSignal), membranes were exposed on film, and signals were visualized, digitized, and analyzed using NIH Image software, in units of density × band area, termed optical density units.

**SDS-PAGE zymography.** We performed quantitative gelatin zymography as previously described (9). Pericardial fluid samples (3 μl) were separated by dilution into zymography sample buffer. The samples were loaded into the wells of a 7.5% gelatin gel and electrophoresed. The gel was then removed and incubated for 1 h at room temperature in 100 ml of renaturing buffer (2.5% Triton X-100) on a rotary shaker. The buffer was decanted and replaced with 100 ml of development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 1 μM ZnCl₂, and 0.02% Brij 35). The gel was incubated at 37°C for 18 h. Each gel was stained with 100 ml of 0.5% Coomassie blue G-250 in 30% methanol and 10% acetic acid for 3 h and then destained with three changes of 30% methanol and 10% acetic acid. Gels were digitized using a scanning digitizing system and analyzed using NIH Image software (product of density and band area). In the preliminary study, we identified the 72-kDa band as MMP-2 and the 92-kDa band as MMP-9 by performing Western blotting.

**Statistical analysis.** All results are expressed as means ± SE. To ascertain whether there was any relationship to risk factors, a comparison of the variables between the two patient groups, with no apparent and well-developed collaterals, respectively, was performed using ANOVA and Pearson’s χ² test, for the analysis of continuous and categorical variables, respectively. A P value <0.05 was regarded as significant.

**RESULTS**

**Patient profiles.** Table 1 summarizes the patient profiles by group. The distribution of age, sex, number of bypassed arteries, history of diabetes, and medication were not different between the two groups. The mean age of the patients was 64 ± 2 yr (mean ± SE). Of these patients, 7 had previous myocardial infarction, 2 presented with acute myocardial infarction, 6 with unstable angina pectoris, and 22 with stable effort angina. Total cholesterol, HDL cholesterol, and triglycerides were not different between the two groups. The proportion of patients receiving β-adrenergic and calcium channel antagonists was higher in the groups with collateral development (P < 0.05). Notably, the other medications [lipid-lowering agents and angiotensin-converting enzyme (ACE) inhibitors] were not different between the two groups.

**Table 1. Patient profiles**

<table>
<thead>
<tr>
<th>n</th>
<th>Collateral (−)</th>
<th>Collateral (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rentrop Grade 0</td>
<td>Rentrop Grade 2 or 3</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>Age, yr</td>
<td>63±2</td>
<td>65±3</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>15/9</td>
<td>10/3</td>
</tr>
<tr>
<td>No. of bypass</td>
<td>3.3±0.2</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td>No. with diabetes</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>No. on Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Blocker</td>
<td>7</td>
<td>12*</td>
</tr>
<tr>
<td>Ca²⁺ channel antagonist</td>
<td>4</td>
<td>9*</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Lipid-lowering agent</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

ACE, angiotensin-converting enzyme; n = no. of patients. *P < 0.05, no collateral (−) conductance group vs. well-developed collateral (+) conductance group.

**Comparison between absent and well-developed collateral groups.** Figure 1 shows a representative coronary angiography for a patient with marked visible collaterals. Although his right coronary artery is totally occluded, it is well filled by collaterals arising from the left coronary artery. Figure 2 shows an immunoblot of human pericardial fluid, obtained using anti-human angiotatin antibody raised against kringles 1–3, which reveals bands for plasminogen, plasmn, and angiotatin. The level of angiotatin was greater in the group without observable collaterals (3.1 ± 0.2 optical density units) compared with that in the presence of collaterals (2.3 ± 0.1 optical density units) (P < 0.05). The aggregate level of plasminogen/plasmin was also greater in the absence of collaterals (6.2 ± 0.7 optical density units) compared with that in the collateralized group (4.2 ± 0.5 optical density units) (P < 0.05). The level of VEGF was nonsignificantly greater in the absence of collaterals (2.7 ± 0.1 optical density units) compared with that in their presence (2.3 ± 0.2 optical density units) (Fig. 3). However, the level of gelatinase (MMP-2 + MMP-9) activity was not different between the groups (0.97 ± 0.08 and 1.05 ± 0.08 optical density units, respectively) (Fig. 4).

**DISCUSSION**

The present study shows for the first time that an increase in pericardial fluid levels of angiotatin and plasminogen/plasmin correlates with an absence of angiogenically detectable collaterals, whereas pericardial fluid levels of VEGF and MMP activity are not different between these two groups. These findings suggest that the production of angiotatin occurring in the context of an increase in substrate (plasmin/plasminogen) concentration may negatively regulate coronary collateral growth in patients with CAD.

**Coronary collateral growth in CAD.** The growth or recruitment of coronary collateral circulation ameliorates adverse outcomes of CAD such as myocardial infarction, angina, and sudden death (5, 7). Myocardial viability after acute infarction correlates with the extent of collateral blood flow of the affected vascular segment. Therefore, the extent of increase of collateral conductance is pivotal in determining the preservation of myocardium during ischemia. Recent clinical trials...
evaluated the administration of growth factors such as VEGF or bFGF for therapeutic angiogenic effects in CAD (17, 21). However, these clinical trials have not shown significantly positive therapeutic outcomes, suggesting endogenous impediments to coronary collateralization in humans that are not sufficiently complemented by the provision of exogenous growth factors.

A complicating factor in this study is the lack of recruitment of pericardial fluid samples from a control group of normal volunteers. Thus we do not know how the levels of angiostatin we measured in either group compare with those from normal human subjects without CAD. This caveat should be considered in the interpretation of our results, in which we proffer that the conclusions should be confined to the groups of patients with CAD and not extrapolated to normal patients. Another limitation is that our study represents only a relatively small group of patients with varying degrees of collateral development, and as such, our results should be interpreted with caution.

Another caveat to our results pertains to the characteristics and clinical data shown in Table 1. Although the age, gender balance, number of bypasses, and proportion of subjects with diabetes are equivalent in the two groups, and the proportion of patients taking ACE inhibitors or lipid-lowering agents is not different between the groups, the proportion of those on calcium channel antagonists or \( \beta \)-adrenergic antagonists is greater in the group with Rentrop grade 2 or 3 collaterals. We also do not have a complete understanding about the duration of the pathology in the different groups, which also may complicate the interpretations. Despite these caveats, our study shows a difference in the levels of angiostatin in patients with CAD who develop or do not develop coronary collaterals; namely, angiostatin is negatively correlated with collateral development.

We previously reported that NO is important for coronary collateral development in the context of a repetitive coronary occlusion model designed to mimic the clinical circumstances of intermittent ischemia (8). Upon NO synthase (NOS) inhibi-

Fig. 2. A: representative Western blots for angiostatin and plasminogen/plasmin. We used anti-human angiostatin antibody against kringles 1–3 so that we could show the bands of plasminogen, plasmin, and angiostatin. B: quantitative analysis for angiostatin. The level of angiostatin is greater in the no collateral conductance group (open bar) compared with the well-developed collateral conductance group (filled bar). C: quantitative analysis for plasminogen/plasmin. The level of plasminogen/plasmin is greater in the no collateral conductance group (open bar) compared with the well-developed collateral conductance group (filled bar). *\( P < 0.05 \).

Fig. 3. The level of VEGF is slightly greater in the no collateral conductance group (open bar) compared with the well-developed collateral conductance group (filled bar), but the difference is not significant.
tion in this canine model, the expected increase in collateral blood flow was not abrogated during repetitive myocardial ischemia despite an increase in VEGF. Many clinical studies have indicated impaired bioavailability of NO in patients with CAD (3, 16). Accordingly, our previous results supported a role of reduced local NO levels in reduced coronary collateral development in patients with risks contributing to endothelial dysfunction.

**Collateral growth and angiostatin.** Angiostatin activity as a potent inhibitor of tumor angiogenesis and the growth of tumor cell metastases (1, 12) is indicated by proteolytic fragments of plasminogen including kringle 1–4 as well as kringle 1–3, with the latter representing the original angiostatin of ~38 kDa reported by O’Reilly et al. (11, 12). The fragment including kringle 1–4, with a molecular weight of ~45–50 kDa, also inhibits angiogenesis (18, 22). It has been reported that MMP-2, -7, and -9 are important enzymes for the generation of angiostatin (13, 15), which are increased in atherosclerotic plaques as well as failing hearts in humans (4, 23). Recently, we reported that angiostatin plays a role in mediating the reduced coronary angiogenesis in the context of inhibition of NO synthesis, despite the presence of high-level expression of VEGF (9). In the present study, pericardial fluid level of angiostatin was found to be increased in patients with absent collateralization compared with that in patients with well-developed collaterals, whereas MMP activity was not different between these two groups. We propose that the higher level of angiostatin is, in turn, due to a locally elevated level of substrate plasmin/plasminogen. One possibility to describe the mechanism of increased level of angiostatin could be myocardial ischemia-mediated increase in vascular leak, partially induced by VEGF (19). According to the increase in permeability, the level of plasminogen in myocardial interstitial fluid increases. Increased plasminogen is then processed by local MMP activity to angiostatin, which restrains both angiogenesis and collateral development. However, one unresolved issue related to this hypothesis is that we did not measure activities of all enzymes capable of generating angiostatin, e.g., macrophage-derived metalloelastase (2), which also could contribute a basis for elevated angiostatin. Nonetheless, our results show elevated angiostatin levels in pericardial fluid of patients with poor coronary collateral development.

It is interesting to note that the extent of increase in the level of angiostatin noted to be correlated with absent collaterals in this study is not as prominent as that seen in the prior study, which showed NOS inhibition in dogs to result in both abrogation of collateral formation and increased angiostatin in myocardial interstitial fluid. This may reflect a difference between the relatively acute imposition of NOS inhibition in the latter study and the chronic endothelial dysfunction that presumably characterized the individuals in the present study. Our recent study demonstrating a similar extent of elevation of intrapericardial endostatin in patients in the absence of collateral response to ischemia complements this finding (14). The extent of both angiostatin and endostatin elevation in these patients with absent visualized collaterals also may emphasize the importance of considering the influence of multiple factors acting in aggregate to determine the overall level of “collateral-forming tone” existing within a region such as the coronary system. It is tempting to hypothesize that the nonischemic adult heart possesses a local bias against new blood vessel formation, which must be overcome by a coordinated downregulation of inhibitors and expression of stimuli to achieve increased collateral vessel supply.

Another point we are compelled to make is that we did not study whether the concentrations of angiostatin in pericardial fluid were high enough to confer a biological effect. Thus we must offer the caveat that our study does not provide a definitive cause-and-effect conclusion; rather, it offers an association of increased levels of angiostatin with decreased coronary collateral development.

In conclusion, locally elevated levels of angiostatin are correlated with reduced coronary collateral development in lumens. These results suggest that therapies attempting to augment coronary collateral growth should incorporate approaches to limit or neutralize the effects of growth inhibitor.

**ACKNOWLEDGMENTS**

This study was supported by National Heart, Lung, and Blood Institute Grants HL-65203 and HL-32788 and by the Cryptic Masons’ Medical Research Foundation.

**REFERENCES**

8. Matsunaga T, Wartlir DC, Moniz M, Tessmner J, Weirucht D, and Chilian WM. Ischemia-induced coronary collateral growth is depen-


