Early Rise in Serum VEGF and PDGF Levels Predisposes Patients With a Normal \textit{MBL2} Genotype to Restenosis After Eversion Endarterectomy

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\textbf{Background and Purpose}—Recently we found that the incidence of restenosis after carotid endarterectomy was significantly higher in patients homozygous for the normal genotype of mannose-binding lectin (\textit{MBL2}) than in with patients with \textit{MBL2} variant genotypes. Several growth factors are also known to contribute to restenosis. Therefore, we investigated whether early postoperative changes in serum vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) concentrations and \textit{MBL2} genotypes interact in the development of restenosis.

\textbf{Methods}—Eighty-two patients who underwent carotid eversion endarterectomy and were followed up by carotid duplex scan sonography for 14 months were studied. Growth factors were measured preoperatively and 4 days after surgery.

\textbf{Results}—Pronounced significant increases in both VEGF and PDGF predicted restenosis but only in patients who were homozygous for the normal \textit{MBL2} genotype. In this group, the adjusted odds ratios of restenosis at 14 months in patients with high versus low early VEGF and PDGF increases were 27.73 (2.42 to 317.26) and 9.23 (1.45 to 58.70), respectively.

\textbf{Conclusions}—These findings indicate that the development of restenosis depends on both complement activation regulated by the \textit{MBL2} gene and pathologic processes leading to enhanced production of VEGF and PDGF during the very early postoperative period. (\textit{Stroke.} 2007;38:2247-2253.)

\textbf{Key Words:} atherosclerosis \textbullet endarterectomy \textbullet growth factors \textbullet mannose-binding lectin \textbullet restenosis

The risk of clinically significant restenosis after carotid eversion endarterectomy during a median 1-year follow up period is 2.5\% (32/1290) according to a meta-analysis by Cao et al.\textsuperscript{1} When carotid restenosis progresses, reinvention may become necessary.\textsuperscript{2,3} According to studies in animal models and patients, early (within 24 months) restenosis is due to myointimal proliferation.\textsuperscript{3,4} Growth factors could have a direct pathophysiologic role in this process. Platelet-derived growth factor (PDGF) was found to be the main factor responsible for proliferation of vascular smooth muscle cells. No data on the relation between PDGF levels and restenosis after carotid endarterectomy are, however, available. Another growth factor, vascular endothelial growth factor (VEGF), is able to promote the growth of vascular endothelial cells derived from arteries and veins and an angiogenic response in different in vivo models.\textsuperscript{5} Epidermal growth factor (EGF) is a very potent proliferation stimulator of various epithelial cell types and, to a lesser extent, of smooth muscle cells.\textsuperscript{6}

Recently in a prospective study performed in patients with severe carotid atherosclerosis, we found that the degree of early restenosis was significantly higher in patients homozygous for the normal (A) genotype of mannose-binding lectin (\textit{MBL2}) compared with patients with \textit{MBL2} variant (R52C [D], rs5030737; G54D [B], rs1800450; and G57E [C], rs1800451) genotypes.\textsuperscript{7} The common designation of variant alleles is \textit{O}. Each variant reduces the amount of functional MBL subunits in heterozygous individuals by 5- to 10-fold, and in homozygotes, only low amounts of dysfunctional MBL are present. On binding to a ligand, MBL may activate the lectin pathway of complement.\textsuperscript{8}

No data on the relation between the incidence of restenosis and either the preoperative levels or early postoperative changes in the circulating levels of different growth factors have been reported. Therefore, we investigated whether there might be a significant correlation between preoperative PDGF, EGF, and VEGF concentrations and their changes in
described earlier. Carotid duplex scan (CDS) sonography was undertaken at 5.7 (4.6 to 8.0) weeks (rounded to 6 weeks), 6.8 (6.2 to 7.9) months (rounded to 7 months), and 13.8 (12.3 to 19.0) months (rounded to 14 months). Two patients died during the observation period, 1 before the 7-month and 1 before the 14-month visit. The CDSs were performed by an experienced radiologist. The common carotid, internal carotid, and external carotid arteries on both sides were examined in a standardized fashion. We recorded the peak systolic and end-diastolic velocities in the common carotid, internal carotid, and external carotid arteries. The spectral measurements were taken with a Doppler angle of 55° to 65°. The diagnostic criteria for internal carotid artery stenosis and restenosis were based on peak systolic and end-diastolic velocities as well as internal carotid artery to common carotid artery ratios. The velocity spectra of the internal carotid arteries were further categorized as mild (<50%), moderate (50% to 69%), and severe (≥70%).

the early postoperative period on one hand and the development of marked (≥50%) restenosis over the follow-up period of 14 months’ duration on the other. The influence of the MBL2 genotype on the association between early postoperative changes in the levels of growth factors tested and the degree of restenosis was also investigated.

Subjects
In this prospective study, a total of 82 consecutive patients (55 men, 27 women; mean ± SD age, 66.2 ± 8.9 years) with severe (mean, 83.1 ± 2.9%) stenosis of the carotid artery who were undergoing elective carotid endarterectomy between October 2000 and March 2003 were included and followed up. The patients included in this study represent a subgroup with the same distribution of demographic and clinical parameters (Table 1) and MBL2 genetics as the group from our previous report about MBL2 genetics comprising 123 patients who underwent elective carotid endarterectomy. We compared the full (123 patients) and the present (82 patients) restricted database, and no significant difference in age, sex, or restenosis rate was found. The study protocol was approved by the institutional review committee at Semmelweis University in Budapest, and the subjects gave informed consent.

Surgery and Follow-Up of the Patients
After detailed medical examination, a careful medical history was taken (Table 1). Indication for carotid endarterectomy was in accordance with American Heart Association guidelines. Of the 82 patients, 26 (31.7%) were asymptomatic, 43 (52.4%) had transient ischemic attack, 11 (13.4%) had minor stroke, and only 2 (2.4%) had major stroke. The patients with transient ischemic attack underwent operation within 1 month of the onset of symptoms. The patients with stroke underwent operation 4 to 8 weeks after the last insult; computed tomography scans showed no fresh ischemic signs. The operation and clinical and radiological follow-up were performed as described earlier. Carotid duplex scan (CDS) sonography was undertaken at 5.7 (4.6 to 8.0) weeks (rounded to 6 weeks), 6.8 (6.2 to 7.9) months (rounded to 7 months), and 13.8 (12.3 to 19.0) months (rounded to 14 months). Two patients died during the observation period, 1 before the 7-month and 1 before the 14-month visit. The CDSs were performed by an experienced radiologist. The common carotid, internal carotid, and external carotid arteries on both sides were examined in a standardized fashion. We recorded the peak systolic and end-diastolic velocity in the common carotid, internal carotid, and external carotid arteries. The spectral measurements were taken with a Doppler angle of 55° to 65°. The diagnostic criteria for internal carotid artery stenosis and restenosis were based on peak systolic and end-diastolic velocities as well as internal carotid artery to common carotid artery ratios. The velocity spectra of the internal carotid arteries were further categorized as mild (<50%), moderate (50% to 69%), and severe (≥70%).

Collection of Samples
Blood samples were drawn preoperatively and a median of 4 days (interquartile range, 3 to 5 days) after operation. Serum was separated and stored immediately at −80°C. EDTA-anticoagulated blood samples were obtained preoperatively for DNA preparation.

Laboratory Examinations
Serum concentrations of total cholesterol and triglycerides (Roche/Hitachi), HDL cholesterol, and LDL cholesterol (Human, Wiesbaden, Germany) were measured in a Cobas Mira Plus clinical chemistry analyzer. Serum concentrations of VEGF, EGF, and PDGF-AB were determined by commercial ELISA methods with Quantikine human VEGF, EGF, and PDGF-AB kits (R&D Systems, Minneapolis, Minn).

Genotyping of MBL2
Total genomic DNA was extracted from white blood cells according to the method of Miller et al. Determination of the alleles of the MBL2 gene and the regulatory promoter variants was performed by polymerase chain reaction with sequence-specific priming, as described. Part of the MBL2 genotypes has been published earlier.

Statistical Analyses
Statistical analyses were performed with the GraphPad Prism, version 3.0, for Windows software package (GraphPad Software, San Diego, Calif). Nonparametric tests were used for group comparison and correlation analysis. Comparison of categorical variables was done with Fisher’s exact test. Multiple-regression analysis was done with SPSS 10.0 (SPSS Inc, Chicago, Ill) software. Values are presented as median and (25th to 75th percentile), unless otherwise stated.

Results
Relation Between Preoperative Growth Factor Levels and Extent of Carotid Stenosis
We performed CDS sonography 6 weeks after the operations and did not find any residual stenosis. At 6 months after
surgery, marked restenosis defined as \(>50\%\) was observed in 9 patients, and at 14 months, in 12 patients. In these patients, clinically significant (\(>70\%)\) restenosis was observed in the same 4 patients (4/82=4.9\%) at both 6 and 14 months. There was no correlation between preoperative or postoperative levels of any growth factor and the degree of carotid stenosis as measured on either the operated or unoperated side by CDS (data not shown).

**Changes in PDGF, VEGF, and EGF Concentration in the Early Postoperative Period**

Serum PDGF, VEGF, and EGF levels were measured in the samples obtained from the patients before operation and 4 days after surgery. The direction of change in growth factor concentrations varied on a per-patient basis. When we divided the patients by the presence of marked (\(>50\%, \text{vs}<50\%)\) restenosis at 7 months, an increase in VEGF levels was significantly (\(P=0.0044\)) more frequently observed in patients with than in those without subsequent marked early restenosis. A similar tendency (\(P=0.0653\)) was found for PDGF, but no such difference occurred with EGF. There were no differences between symptomatic and asymptomatic patients with respect to early postoperative changes in either VEGF (\(P=0.209\)) or PDGF (\(P=0.248\)). Because these findings suggested an association between early VEGF and PDGF increases and restenosis, we further evaluated these data, as well as with respect to MBL2 genotypes.

**Correlation Between Changes in VEGF and PDGF Serum Concentrations and CDS Values Measured 7 and 14 Months After Surgery Is Restricted to Those With the A/A Genotype**

The extent of the early postoperative increases in VEGF and PDGF levels (ie, \(\Delta\)VEGF and \(\Delta\)PDGF) was significantly correlated with the 7-month CDS values, whereas there was no significant correlation between \(\Delta\)VEGF and \(\Delta\)PDGF and the CDS values measured at the end of the follow-up period (14 months; Figures 1 and 2, A and B). \(\Delta\)VEGF and \(\Delta\)PDGF values were strongly correlated (\(R=0.536, P<0.001\)).

The patients were subdivided into 2 groups according to MBL2 genotype. Group 1 comprised 53 patients who were homozygous for the normal MBL2 allele (genotype A/A), and group 2 comprised 29 patients with 1 or 2 variant alleles (MBL2 genotypes A/O or O/O). When the relations between \(\Delta\)VEGF and \(\Delta\)PDGF values and 7-month restenosis (Figure 1, C and D) or 14-month CDS values (Figure 2, C and D) were analyzed, highly significant Spearman correlation coefficients were found at both time points in group 1 (Figures 1 and 2, C and D). By contrast, no correlation was found in the patients of group 2 (Figures 1 and 2, E and F).

**Clinically Significant Restenosis Is Associated With a Pronounced Increase in VEGF and PDGF Levels in Patients With MBL2 Genotype A/A Only**

The patients were subdivided into 2 subgroups according to high versus low \(\Delta\)VEGF and \(\Delta\)PDGF values (Table 2). For patients with high (\(>90\,\text{pg/mL}\)) versus low (\(\leq 90\,\text{pg/mL}\)) \(\Delta\)VEGF (Table 2) values, odds ratios for developing marked restenosis (CDS >50\%) were calculated by logistic-regression analysis and were adjusted for age, sex, and preoperative body mass index values.

The odds ratios for high versus low early \(\Delta\)VEGF were 16.69 (1.86 to 149.61) and 20.00 (2.25 to 177.63) for the 7- and 14-month CDS values, respectively, in patients homozygous for the MBL2 genotype (A/A). By contrast, marked (\(>50\%\)) restenosis occurred rarely in patients with MBL2 the A/O or O/O genotype, and no significant difference between patients with low versus high \(\Delta\)VEGF was found in this group (Table 2). We found a less notable but still significant risk of marked restenosis in patients who exhibited pronounced (\(>30\,\text{pg/mL}\)) \(\Delta\)PDGF values, with adjusted odds ratios of 7.21 (1.04 to 49.63) and 9.23 (1.45 to 58.70), respectively (data not shown in detail).

**Discussion**

We found that early marked restenosis, defined as \(>50\%\) in the carotid arteries after eversion endarterectomy, occurs with a high probability in patients who are homozygous for the normal MBL2 A allele and exhibit a marked elevation (in the upper tertile of the whole group) in serum VEGF and/or PDGF levels on day 4 after surgery. In contrast, restenosis occurred much less frequently in patients with the variant MBL2 O allele or in those who had a low early VEGF and/or PDGF increase. According to our unpublished study, the high VEGF and PDGF levels observed 4 days after surgery remained elevated in most patients at least until the 6-week follow-up visit. The odds ratios for restenosis, adjusted for possible confounding variables at 7 and 14 months, were \(>16\) and \(>20\), respectively, for a high versus low early increase in serum VEGF concentration, and similar, albeit lower, odds ratios were found for the early PDGF increase. By contrast, we did not find a significant correlation between early changes in EGF values and restenosis.

These novel findings extend our previous results obtained in the same patient population,
7 in whom we reported a dramatically decreased risk of developing restenosis after carotid eversion endarterectomy in patients with variant MBL2 O alleles. A possible conceptual mechanism of the events that lead to restenosis is suggested in Figure 3. The carotid eversion endarterectomy procedure denudes the intima and atherosclerotic plaque from the vessel wall, thus exposing a new surface composed of mainly smooth muscle cells, extracellular matrix proteins, and local inflammatory cells. In addition, during the procedure, cross-clamping of the carotid artery is performed for an average of 20 minutes, which leads to ischemia/reperfusion injury of the carotid artery. These pathologic events are followed by the release of PDGF and VEGF. Both growth factors are essential for smooth muscle proliferation, migration, and matrix formation and for triggering neointimal hyperplasia, giving rise to a vicious circle of events that result in restenosis. In addition, activation of the lectin route of complement initiated by MBL binding to intracellular components from endothelial cells has been shown to occur during ischemia/reperfusion injury.\textsuperscript{13,14} As a result of subsequent complement activation, highly potent activation products (C3a, C5a, and C5b-9) are generated, which may also release PDGF and activate smooth muscle cells and macrophages.\textsuperscript{13} Our findings indicate that
both growth factor release and complement activation are necessary for the development of restenosis (Figure 3).

Several reports support the mechanism suggested in Figure 3. Under acute hypoxic conditions, PDGF and VEGF mRNA and protein are upregulated, which may mediate hypoxia-initiated angiogenesis. Results of studies in several experimental models and clinical observations definitely indicate that PDGF, through interaction with its receptors, has a major role in those early pathologic events that lead to the myointimal proliferation responsible for narrowing of carotid arteries. Moreover, inhibition of PDGF receptors by antibodies, receptor antisense, or different drugs administered before experimental arterial injury were found to markedly decrease the subsequent neointimal proliferation. Inhibition of PDGF receptors by triazolopyrimidine (Trapidil), a mild competitive inhibitor of PDGF receptors, has

Figure 1. Correlation of the early (4 days, before surgery) changes in serum VEGF (left) and PDGF (right) levels and the extent of restenosis measured by CDS 7 months after surgery in all patients (A, B), in patients with only the normal (A/A) alleles of MBL2 (C, D), and in patients with either only the variant (O/O) or both variant and normal (A/O) alleles of MBL2 (E, F). Nonparametric Spearman rank correlation coefficients and their probability values are indicated.
successfully been used in patients for preventing restenosis after percutaneous transluminal coronary angioplasty.\textsuperscript{22} Similar, albeit somewhat controversial, findings were obtained for the relation between VEGF and restenosis. VEGF as a strong endothelial cell mitogen can promote reendothelization\textsuperscript{23} and consequently may play a protective role against restenosis. On the other hand, increased VEGF synthesis may trigger proliferation of endothelial cells and neovascularization of smooth muscle tissue.\textsuperscript{24}

As for the role of complement activation, high serum levels of MBL were associated with high serum levels of interleukin-1\beta, tumor necrosis factor-\alpha, and interleukin-8 after cross-clamping of the aorta in a human model of ischemia/reperfusion.\textsuperscript{25} In a rat model of ischemia/reperfusion injury, infarct size was reduced by anti-MBL antibodies.\textsuperscript{26} In other experiments, MBL knockout mice were partially protected against kidney and heart ischemia/reperfusion injury.\textsuperscript{27} In addition, the complement activation product C5b-9 was found to

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**Figure 2.** Correlation of the early (4 days, before surgery) changes in serum VEGF (left) and PDGF (right) levels and the extent of restenosis assessed by CDS measured 14 months after surgery in all patients (A, B), in patients with only the normal (A/A) alleles of MBL\textsubscript{2} (C, D), and in patients with either only the variant (O/O) alleles or both variant and normal (A/O) alleles of MBL\textsubscript{2} (E, F). Nonparametric Spearman rank correlation coefficients and their probability values are indicated.
release PDGF from endothelial cells.28 The lack of correlation that we observed between early postoperative rises in EGF levels and restenosis is not unexpected. Although EGF receptors transmit very strong growth signals to many cell types, EGF has only mild effects on vascular smooth muscle cell proliferation.29

In conclusion, our present findings definitely indicate that, at least after eversion-type carotid endarterectomy, restenosis occurs primarily in patients who are homozygous for the normal MBL2 allele (A/A), a genotype associated with high MBL levels, and a marked complement-activating capacity in conjunction with upregulation of PDGF and VEGF production occurs in the early postoperative period. Restenosis can be effectively inhibited by PDGF inhibitors in patients with coronary artery disease after percutaneous transluminal coronary angioplasty.22 This is clearly a hypothesis-generating study, the conclusions of which should be tested by further studies in different cohorts. Such a study is under way in our departments.

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### TABLE 2. Prediction of Clinically Significant (>50%) Restenosis on the Basis of High (in the Highest Tertile) Early (4 Days After Surgery) Increase in Serum VEGF Concentrations in Patients With Normal (A) and/or Variant (O) Alleles of MBL2

<table>
<thead>
<tr>
<th>Restenosis</th>
<th>Patients With A/A MBL2 Genotype</th>
<th>Patients With A/O or O/O MBL2 Genotype</th>
<th>All Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤50% &gt;50%</td>
<td>≤50% &gt;50%</td>
<td>≤50% &gt;50%</td>
</tr>
<tr>
<td>7 Months After Surgery</td>
<td>31 1</td>
<td>21 1</td>
<td>52 2</td>
</tr>
<tr>
<td>∆VEGF low*</td>
<td>13 7</td>
<td>7 0</td>
<td>20 7</td>
</tr>
<tr>
<td>Unadjusted odds ratio (95% CI (P value))</td>
<td>16.69 (1.86–149.61)</td>
<td>0.956 (0.035–26.11)</td>
<td>9.10 (1.74–47.57)</td>
</tr>
<tr>
<td>Adjusted‡ OR (95% CI) (P value)</td>
<td>19.09 (1.77–211.35)</td>
<td>Cannot be calculated</td>
<td>8.06 (1.45–44.75)</td>
</tr>
<tr>
<td>14 Months After Surgery</td>
<td>30 1</td>
<td>19 3</td>
<td>49 4</td>
</tr>
<tr>
<td>∆VEGF low*</td>
<td>12 8</td>
<td>7 0</td>
<td>19 8</td>
</tr>
<tr>
<td>Unadjusted OR (95% CI (P value))</td>
<td>20.00 (2.25–177.63)</td>
<td>0.371 (0.017–8.09)</td>
<td>5.16 (1.39–19.15)</td>
</tr>
<tr>
<td>Adjusted‡ OR (95% CI) (P value)</td>
<td>27.73 (2.42–317.26)</td>
<td>Cannot be calculated</td>
<td>5.50 (1.41–22.45)</td>
</tr>
</tbody>
</table>

* ≤90 pg/mL
† >90 pg/mL
‡ Adjusted for age, sex, and body mass index.
§ Odds ratios could not be calculated. Odds ratios were calculated by multiple-regression analysis.

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**Figure 3.** Putative relation between MBL2 polymorphisms, serum level of growth factors, and myointimal proliferation leading to restenosis after carotid endarterectomy. Operation causes intimal injury and, due to cross-clamping, temporary ischemia/reperfusion, which trigger PDGF and VEGF production in different cell types; the growth factors induce myointimal proliferation. In addition, both injury of the endothelium and hypoxia of the vascular wall result in increased exposure of intracellular components, both leading to increased binding of MBL to endothelial cells, followed by activation of the complement system through the lectin pathway. During activation, several products such as C3a, C5a, and C5b-9 are generated, all of which are known to be potent inflammatory mediators. Complement and growth factors interact in initiating myointimal proliferation, which leads to restenosis.
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Disclosures

None.

References

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