Safety Profile of Tissue Plasminogen Activator Treatment Among Stroke Patients Carrying a Common Polymorphism (C-1562T) in the Promoter Region of the Matrix Metalloproteinase-9 Gene

Joan Montaner, MD, PhD; Israel Fernández-Cadenas; Carlos A. Molina, MD, PhD; Jasone Monasterio, MD, PhD; Juan F. Arenillas, MD; Marc Ribó, MD; Manolo Quintana; Pilar Chacón, MD, PhD; Antoni L. Andreu, MD, PhD; José Alvarez-Sabín, MD, PhD

Background and Purpose—Matrix metalloproteinase-9 (MMP-9) expression, related to blood-brain barrier disruption, has been implicated in the appearance of hemorrhagic transformation (HT) after tissue plasminogen activator (tPA) treatment in stroke patients. Because an in vitro functional polymorphism of the promoter region of MMP-9 gene (C-1562T) has been described, we hypothesize that patients carrying this mutation might have higher MMP-9 levels and greater susceptibility to developing HT when receiving tPA.

Methods—We studied strokes involving the middle cerebral artery territory of 61 patients who received tPA <3 hours after stroke onset. Blood samples were obtained before tPA administration. Plasmatic MMP-9 determinations were performed (enzyme-linked immunosorbent assay, ng/mL), and C-1562T genotype was determined by polymerase chain reaction. Healthy age-matched control subjects were used to study allele distribution (n = 59). Hemorrhagic events were classified according to CT criteria (petechial hemorrhagic infarctions [HI,1 to 2] and large parenchymal hemorrhages [PH,1 to 2]).

Results—Allele distribution was similar in patients and control subjects (CC/CT/TT: 72.3/27.7/0% versus 79.7/20.3/0%, respectively; P = 0.37). Among patients, mutation carriers (CT/TT alleles) had similar rates of HT and PH than noncarriers (HT: 23.1% versus 38.2%, P = 0.49; PH: 15.4% versus 17.6%, P = 1.0). Although the highest MMP-9 level corresponded to patients who later developed a PH (PH, 191.4 ng/mL; non-PH, 68.05 ng/mL; P = 0.022), no relation between MMP-9 mutation presence and plasmatic levels was found (CC, 127.12 ng/mL; CT/TT, 46.31 ng/mL; P = 0.11).

Conclusions—Although MMP-9 level predicts PH appearance after tPA treatment, no relationship exists with the C-1562T polymorphism, probably because this mutation is not functional in response to cerebral ischemia in vivo. (Stroke. 2003; 34:2851-2855.)

Key Words: hemorrhagic transformation ■ metalloproteinases ■ polymorphism ■ stroke ■ thrombolysis

In ischemic stroke treatment, the beneficial effect obtained by thrombolysis-induced recanalization may be decreased by the risk of hemorrhagic transformation (HT). Recently, a member of the matrix metalloproteinase (MMP) family of zinc-binding proteolytic enzymes, MMP-9, which attacks the major components of the basal lamina around cerebral blood vessels, has emerged as a predictor of tissue plasminogen activator (tPA)–induced hemorrhages after stroke. Moreover, MMP inhibition has demonstrated to reduce the presence and extent of hemorrhagic complications in tPA-treated animals using an embolic ischemia model.

Genetic manipulation in mice has shown a protective effect of MMP-9 gene knockout after cerebral ischemia that is mediated by reduced proteolytic degradation of critical blood-brain barrier components. In humans, a polymorphism has been described in the promoter region of the MMP-9 gene, and in vitro, this C-to-T substitution at the polymorphic site (−1562) results in more promoter activity of the T-allelic promoter. Therefore, we hypothesize that stroke patients carrying the C-1562T mutation might have higher MMP-9 levels and be at higher risk of developing an HT when receiving tPA.

Methods

Study Population

Patients with an acute stroke admitted at the emergency department of a University Hospital were prospectively studied. Our target group consisted of patients who had had an acute ischemic stroke involving
the vascular territory of the middle cerebral artery (MCA) who were admitted within the first 3 hours after symptom onset. These patients underwent urgent carotid ultrasound and transcranial Doppler (TCD) examinations. Sixty patients had a documented MCA occlusion on TCD and received tPA in a standard 0.9-mg/kg dose (10% bolus, 90% continuous infusion over 1 hour) <3 hours after symptom onset. We excluded patients with a known inflammatory or malignant disease. Finally, 61 patients who had had an acute stroke in the MCA territory who received tPA <3 hours after symptom onset were included in the analysis.

Age-matched control subjects (n=59) were randomly sampled from a group of healthy volunteers who participated in a nutritional study. None had a history of stroke. All the included subjects were unrelated patients and control subjects of European white ancestry (Mediterranean area).

Clinical Protocol
A detailed history of vascular risk factors was obtained from each patient. To identify a potential mechanism of cerebral infarction, a set of diagnostic tests was performed that included ECG, chest radiography, carotid ultrasonography, complete blood count, and leukocyte differential and blood biochemistry in all patients; when indicated, some patients also underwent special coagulation tests, transthoracic echocardiography, and Holter monitoring. From this information and the neuroimaging data, previously defined etiologic subtypes were assessed according to European Cooperative Acute Stroke Study criteria.10,11 No patient with a hypodensity involving some slight space-occupying effect; and PH-2, when hematoma involved >30% of the infarcted area with substantial mass effect or swelling as a result of developing infarction on baseline CT was excluded.

Determination of C-1562T Genotypes
DNA samples were extracted from whole blood with the QIAMP DNA Blood Midi Kit (QUIAGEN). The region containing the C-1562T polymorphism upstream of the MMP-9 gene was amplified by polymerase chain reaction (PCR) using 2 new primers: Meta F-1 (5’AATGCGTAGGACATAGTAGG 3’) and Meta R-1 (5’ACTCTTCTCTTCCAGCC 3’)

Results were confirmed by cycle sequencing on a Perkin Elmer Gene Amp PCR System 2400 with Big-dye-labeled terminators; sequencing products were purified on Autoseq G-50 columns (Amersham Biosciences) and analyzed on an ABI Prism 310 sequencer (PE Applied Biosystems).

Statistical Analysis
Descriptive and frequency statistical analyses were obtained and comparisons were made by use of the SPSS statistical package, version 9.0. Statistical significance for intergroup differences was assessed by the χ² or Fisher’s exact test for categorical variables such as for deviation of genotype distribution from Hardy-Weinberg equilibrium and to determine whether there was any significant difference in allele or genotype frequencies between cases and controls or patients with or without HT. Mann-Whitney test was used to assess intergroup differences of MMP-9 values because this variable was not normally distributed (Kolmogorov-Smirnov and P-P plot). A value of P<0.05 was considered statistically significant.

Results
We included in the study 61 patients (55.7% women) with an acute stroke in the MCA territory. Mean age was 71±9.3 years (range, 41 to 86 years). A total of 63.6% of patients were hypertensive, 25.4% were dyslipidemic, 28.6% had a history of diabetes mellitus, and 47.4% had a documented atrial fibrillation. NIHSS score of the series on admission was 17 (range, 7 to 22). Baseline TCD detected a proximal MCA occlusion in 61% and a distal occlusion in 39% of the patients.

The allele frequencies in patients and control subjects were consistent with Hardy-Weinberg equilibrium, and there was no significant difference between the patient and control groups in genotype distribution of the studied polymorphism (patients: CC, 72.3%; CT, 27.7%; and TT, 0%; healthy control subjects: CC, 79.7%; CT, 20.3%; TT, 0%; P=0.37).

Among patients with the C-1562T mutation (CT alleles), no differences existed regarding sex or any cardiovascular risk factor compared with patients without the mutation (CC alleles) except for atrial fibrillation (75% for CT versus 38.7% for CC; P=0.033).
HT was present in 21 patients (34.4%; HI, 12 [19.7%]; PH, 9 [14.7%]). Of the 12 patients with an HI, 3 were HI-1 and 9 were HI-2. Among the 9 PH patients, 3 were PH-1 and 6 were PH-2. Of all these bleedings, 7 (11.5%) were symptomatic hemorrhages.

MMP-9 mutation did not influence the presence or absence of HT considered globally (23.1% in CT/TT versus 38.2% in CC; \( P = 0.49 \)) or when HT subtypes were analyzed (the Table and Figure 1).

In more than a half of our study population, the MMP-9 pretreatment determination (100.30 ng/mL; range, 45.15 to 205.0 ng/mL) exceeded the normality interval of our laboratory for healthy control subjects (<97 ng/mL). In contrast to genotype findings, a significantly increased plasmatic MMP-9 expression was found among patients who later developed a PH (191.4 ng/mL for patients with PH versus 68.05 ng/mL for patients without PH; \( P = 0.022; \) Figure 2).

A graded response was found between baseline MMP-9 levels and degree of bleeding on CT (the Table). We observed very low median baseline MMP-9 levels in the HI-1 group (37.4 ng/mL; range, 12.47 to 37.47 ng/mL), abnormally higher levels in the HI-2 (149.32 ng/mL; range, 41.16 to 274.89 ng/mL) and PH-1 (191.4 ng/mL; range, 178.13 to 213.79 ng/mL) groups, and very high levels in the PH-2 group (209.89 ng/mL; range, 127.80 to 361.80 ng/mL).

MMP-9 levels were not related to baseline NIHSS or infarct volume, and infarctions were not significantly larger in those patients who developed a PH (data not shown). MMP-9 levels were compared between patients with different allele distributions to analyze C-1562T polymorphism functionality in vivo, but no difference in plasmatic MMP-9 levels appeared regarding the presence of the mutation. In fact, MMP-9 level was slightly lower among the polymorphism carriers (CC: 127.12 ng/mL; range, 48.97 to 266.74 ng/mL; CT/TT: 46.31 ng/mL; range, 18.76 to 193.81 ng/mL; \( P = 0.11; \) Figure 3).

**Discussion**

A genetically predetermined risk of bleeding after thrombolysis might exist in some stroke patients. The present study sought to ascertain whether a common polymorphism in the promoter region of MMP-9 gene influences the safety profile of tPA-treated stroke patients.

<table>
<thead>
<tr>
<th>Genotype, n (%)</th>
<th>MMP-9 Phenotype, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>C/T+T/T</td>
</tr>
<tr>
<td>Non-HT</td>
<td></td>
</tr>
<tr>
<td>21 (61.8)</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>HI-1</td>
<td></td>
</tr>
<tr>
<td>2 (5.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HI-2</td>
<td></td>
</tr>
<tr>
<td>5 (14.7)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>PH-1</td>
<td></td>
</tr>
<tr>
<td>1 (2.9)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>PH-2</td>
<td></td>
</tr>
<tr>
<td>5 (14.7)</td>
<td>1 (7.7)</td>
</tr>
</tbody>
</table>

Phenotype study corresponding to plasmatic protein level is given for each subgroup of patients (medians are shown).

![Figure 1](http://stroke.ahajournals.org/)

**Figure 1.** Number of patients who develop PH according to the C-1562T MMP-9 polymorphism allele distribution. Similar percentages of bleedings were found in CC (17.6%) and CT (15.4%) groups (\( P = 1.0 \)).

![Figure 2](http://stroke.ahajournals.org/)

**Figure 2.** PH was associated with plasmatic MMP-9 level measured before tPA administration (\( P = 0.022 \)). Dashed line indicates laboratory reference interval (normal MMP-9 <97 ng/mL).

![Figure 3](http://stroke.ahajournals.org/)

**Figure 3.** Plasmatic MMP-9 level distribution according to presence of the C-1562T polymorphism (\( P = 0.11 \)) in those patients in whom both genotype and phenotype studies could be performed (\( n = 38 \)).
Our data confirm that plasmatric MMP-9, increased before administration of thrombolytic therapy, accurately predicts intracerebral hemorrhage, but MMP-9 gene promoter mutation (C-1562T) is not responsible for this high MMP-9 levels or for the bleeding complication itself.

MMP-9 Gene Promoter Polymorphism in Ischemic Stroke

This is the first study to explore the role of MMP-9 gene mutations in ischemic stroke. Although a large number of stroke patients were heterozygotes for the C-1562T polymorphism, this rate was not different from the healthy population used as controls in our study. To the best of our knowledge, this is the first time that the C-1562T mutation was studied in a Mediterranean population. The prevalence of the mutation in our control subjects (20.3%) was more comparable to that in North American (22.9%), Swedish (30%), and English (30.1%) populations than to Asian data (6%).

MMP-9 contributes to plaque instability, and this mutation has previously been related to the severity of coronary atherosclerosis in a cohort of patients with coronary heart disease. However, most of our patients had a cardioembolic stroke; perhaps in a different stroke population with more atherosclerosis-related subtypes, a different allele distribution might be expected.

MMP-9 Gene Promoter (C-1562T) Polymorphism Is Not Functional In Vivo

To address the question of whether the MMP-9 genotype is a genetic determinant of bleeding complications after thrombolysis, we analyzed the promoter C-1562T polymorphism in the MMP-9 gene described as a functional single nucleotide polymorphism in an in vitro study. Surprisingly, no relationship between genotype and phenotype could be found in our population. In fact, the protein level of MMP-9 was even slightly higher in patients with the theoretically low-function alleles (CC). Our findings might explain why other authors failed to relate this single nucleotide polymorphism to other neurological diseases that are clearly related to MMPs expression such as multiple sclerosis and intracranial aneurysms.

In a Finish study of mildly hypercholesterolemic young men, the T allele carriers (12%) had MMP-9 serum concentrations (41.1 µg/L) similar to those with the CC genotype (88%; 36.9 µg/L; P=NS). Moreover, a recent study also failed to demonstrate differences in the activity of −1562 T and C alleles in primary cultures of human amnion cells.

In agreement with our results, and paradoxically, a previous pharmacogenomics study in coronary patients randomized to glycoprotein IIb/IIIa antagonists demonstrated that, among the placebo group, carriers of the C-1562T mutation had a protective effect for bleeding complications.

MMP-9 Level Effects in tPA-Induced Hemorrhages

In our study, a positive graded response exists between MMP-9 production and the degree and extension of brain bleedings. Until recently, only clinical or radiological parameters such as age, severity of neurological deficit, or extent of initial hypodensity on baseline CT scan have been associated with the occurrence of a PH. Plasmatric MMP-9 findings may thus be considered very useful because, in addition to its diagnostic utility, MMP-9 is potentially modifiable in contrast to other HT risk factors.

MMP-9 in PH patients may be responsible for the disruption of some components of the blood-brain barrier, and tPA may contribute to the overall rate and extension of this complication by several mechanisms. First, tPA facilitates activation of MMPs; second, tPA-induced sudden clot lysis with abrupt reperfusion may promote bleedings in the areas where MMP-9 has already disrupted the blood-brain barrier. Because plasmin is involved in the cascade that processes pro-MMP-9 to the active form, tPA administration may activate and promote the destructive potential of this enzyme, although very recent reports have identified plasmin-independent activation pathways.

Other Nongenetic Mechanisms Responsible of the Overexpression of MMP-9

The negative result of the C-1562T polymorphism is not incompatible with the important role of MMP-9 in tPA bleeding complications because other factors can also lead to overexpression of this protein.

A number of regulatory mechanisms can influence the impact of an MMP on extracellular matrix degradation. These mechanisms include regulation of transcription, activation of latent MMPs, and inhibition of MMP activity by tissue inhibitors of metalloproteinases. Several cis elements in the MMP-9 gene promoter have been shown to be important in the regulation of its transcription. These include 2 AP-1 sites (bound by transcription factors c-Fos and d-Jun), a PEA3 motif (recognized by transcription factor Ets), and a consensus sequence for binding of nuclear factor-κB. Through them, the promoter of the gene responds to different regulators such as interleukin-1, platelet-derived growth factor, tumor necrosis factor-α, and epidermal growth factor. After MMP-9 is activated, tissue inhibitors of metalloproteinases and other inhibitors such as α1-macroglobulin and α1-antitrypsin may regulate its level. Unfortunately, neither MMP-9 active forms nor tissue inhibitors of metalloproteinases have been properly studied in human stroke. The duration of the vascular occlusion may be another clue because we have demonstrated that delayed tPA-induced recanalization is related to PH and that a time-occlusion dependence exists for MMP release.

Study Limitations

In this report, we have a small absolute number of patients with PH; therefore, results should be interpreted with caution. Although the overall rate of PH is higher than that reported in the National Institute of Neurological Disorders and Stroke trial, this difference may be explained by the greater severity of our patients, most of whom experienced cardioembolic strokes. In the control group, we could not measure MMP-9 levels because only DNA (not plasma) was available; thus, we can confirm the lack of functionality of the C-1562T polymorphism only among stroke patients.

MMP-9 contributes to plaque instability, and this mutation has previously been related to the severity of coronary atherosclerosis in a cohort of patients with coronary heart disease. However, most of our patients had a cardioembolic stroke; perhaps in a different stroke population with more atherosclerosis-related subtypes, a different allele distribution might be expected.

MMP-9 Gene Promoter (C-1562T) Polymorphism Is Not Functional In Vivo

To address the question of whether the MMP-9 genotype is a genetic determinant of bleeding complications after thrombolysis, we analyzed the promoter C-1562T polymorphism in the MMP-9 gene described as a functional single nucleotide polymorphism in an in vitro study. Surprisingly, no relationship between genotype and phenotype could be found in our population. In fact, the protein level of MMP-9 was even slightly higher in patients with the theoretically low-function alleles (CC). Our findings might explain why other authors failed to relate this single nucleotide polymorphism to other neurological diseases that are clearly related to MMPs expression such as multiple sclerosis and intracranial aneurysms.

In a Finish study of mildly hypercholesterolemic young men, the T allele carriers (12%) had MMP-9 serum concentrations (41.1 µg/L) similar to those with the CC genotype (88%; 36.9 µg/L; P=NS). Moreover, a recent study also failed to demonstrate differences in the activity of −1562 T and C alleles in primary cultures of human amnion cells.

In agreement with our results, and paradoxically, a previous pharmacogenomics study in coronary patients randomized to glycoprotein IIb/IIIa antagonists demonstrated that, among the placebo group, carriers of the C-1562T mutation had a protective effect for bleeding complications.

MMP-9 Level Effects in tPA-Induced Hemorrhages

In our study, a positive graded response exists between MMP-9 production and the degree and extension of brain bleedings. Until recently, only clinical or radiological parameters such as age, severity of neurological deficit, or extent of initial hypodensity on baseline CT scan have been associated with the occurrence of a PH. Plasmatric MMP-9 findings may thus be considered very useful because, in addition to its diagnostic utility, MMP-9 is potentially modifiable in contrast to other HT risk factors.

MMP-9 in PH patients may be responsible for the disruption of some components of the blood-brain barrier, and tPA may contribute to the overall rate and extension of this complication by several mechanisms. First, tPA facilitates activation of MMPs; second, tPA-induced sudden clot lysis with abrupt reperfusion may promote bleedings in the areas where MMP-9 has already disrupted the blood-brain barrier. Because plasmin is involved in the cascade that processes pro-MMP-9 to the active form, tPA administration may activate and promote the destructive potential of this enzyme, although very recent reports have identified plasmin-independent activation pathways.

Other Nongenetic Mechanisms Responsible of the Overexpression of MMP-9

The negative result of the C-1562T polymorphism is not incompatible with the important role of MMP-9 in tPA bleeding complications because other factors can also lead to overexpression of this protein.

A number of regulatory mechanisms can influence the impact of an MMP on extracellular matrix degradation. These mechanisms include regulation of transcription, activation of latent MMPs, and inhibition of MMP activity by tissue inhibitors of metalloproteinases. Several cis elements in the MMP-9 gene promoter have been shown to be important in the regulation of its transcription. These include 2 AP-1 sites (bound by transcription factors c-Fos and d-Jun), a PEA3 motif (recognized by transcription factor Ets), and a consensus sequence for binding of nuclear factor-κB. Through them, the promoter of the gene responds to different regulators such as interleukin-1, platelet-derived growth factor, tumor necrosis factor-α, and epidermal growth factor.

After MMP-9 is activated, tissue inhibitors of metalloproteinases and other inhibitors such as α1-macroglobulin and α1-antitrypsin may regulate its level. Unfortunately, neither MMP-9 active forms nor tissue inhibitors of metalloproteinases have been properly studied in human stroke. The duration of the vascular occlusion may be another clue because we have demonstrated that delayed tPA-induced recanalization is related to PH and that a time-occlusion dependence exists for MMP release.

Study Limitations

In this report, we have a small absolute number of patients with PH; therefore, results should be interpreted with caution. Although the overall rate of PH is higher than that reported in the National Institute of Neurological Disorders and Stroke trial, this difference may be explained by the greater severity of our patients, most of whom experienced cardioembolic strokes. In the control group, we could not measure MMP-9 levels because only DNA (not plasma) was available; thus, we can confirm the lack of functionality of the C-1562T polymorphism only among stroke patients.
Because some other polymorphisms that we did not test have been described among the MMP family and may have allele-specific effects on the regulation of MMP gene transcription, future studies should use wider panels of polymorphisms to represent other haplotypes of these genes.

In conclusion, our results confirmed that the plasmatic MMP-9 level is strongly involved in the occurrence of thrombolytic therapy–related PHs, but we failed to demonstrate an influence of MMP-9 gene variations on susceptibility of intracranial bleedings after tPA treatment among stroke patients carrying the common C-1562T polymorphism.

Acknowledgments

Dr Montaner is the recipient of a grant from the Instituto de Salud Carlos III for medical research training. This study was supported by a grant from the Spanish government (FIS 02/0773) and the Spanish Neurological Diseases Network (CIEN). We thank Ana Penalba, Pilar Bermudez, and Dorita Quiroga for technical assistance. This work is part of the GENO-tPA study included in Projecto Luctus to test genetic markers of security and efficacy among stroke patients receiving tPA.

References

Safety Profile of Tissue Plasminogen Activator Treatment Among Stroke Patients Carrying a Common Polymorphism (C-1562T) in the Promoter Region of the Matrix Metalloproteinase-9 Gene

Joan Montaner, Israel Fernández-Cadenas, Carlos A. Molina, Jasone Monasterio, Juan F. Arenillas, Marc Ribó, Manolo Quintana, Pilar Chacón, Antoni L. Andreu and José Alvarez-Sabín

*Stroke*. 2003;34:2851-2855; originally published online November 6, 2003;
doi: 10.1161/01.STR.0000098648.54429.1C

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/34/12/2851

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org/subscriptions/