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

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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 n metalloproteinases n polymorphism n stroke n thrombolysis

In ischemic stroke treatment, the beneficial effect obtained by thrombolysis-induced recanalization may be decreased by the risk of hemorrhagic transformation (HT).1 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,2 has emerged as a predictor of tissue plasminogen activator (tPA)–induced hemorrhages after stroke.3 Moreover, MMP inhibition has demonstrated to reduce the presence and extent of hemorrhagic complications in tPA-treated animals using an embolic ischemia model.4,5

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.6 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.7 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. Some 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, transhoracic echocardiography, and Holter monitoring. From this information and the neuroimaging data, previously defined etiologic subgroups were determined.8 Clinical examination was performed on admission and 12, 24, and 48 hours from symptom onset. Stroke severity was assessed by use of the National Institutes of Health Stroke Scale (NIHSS). We defined neurological improvement as a decrease in the NIHSS score by ≥4 points and neurological deterioration as either death or an increase by ≥4 points at 48 hours.9 Intravenous heparin was not administered during the study period, nor were antiplatelet agents given during the first 24 hours. This study was approved by the Ethics Committee of the hospital, and all patients or relatives gave informed consent.

Computed Tomography
On admission, all patients underwent CT within the first 3 hours of stroke onset, which was repeated after 48 hours (or earlier when rapid neurological deterioration occurred) to evaluate the presence of HT.

CT scans were reviewed by a neuroradiologist with extensive experience in acute stroke who was blinded to clinical details and MMP results. Presence and type of HT were defined according to previously published criteria.10,11 Hemorrhagic infarction (HI) was defined as a petechial infarction without space-occupying effect, and parenchymal hematoma (PH) was defined as hemorrhage with mass effect. For statistical analysis, both subtypes of HI and PH were considered (HI-1, small petechiae along the margins of the infarct; HI-2, more confluent petechiae within the infarcted area; PH-1, when hematoma involved ≥30% of the infarcted area with some slight space-occupying effect; and PH-2, when hematoma involved >30% of the infarcted area with substantial mass effect or clot remote to the infarcted area). Symptomatic intracranial hemorrhage was defined as blood at any site in the brain on the CT scan and documentation of neurological deterioration.

The presence of hyperdense MCA sign, early focal hypodensity, or swelling as a result of developing infarction on baseline CT was assessed according to European Cooperative Acute Stroke Study criteria.10,12 No patient with a hypodensity involving >33% of the MCA territory received tPA in this study.

MMP-9 Immunoassay
Peripheral blood samples were drawn from each patient at study entry (before tPA administration). EDTA tubes were used to collect the blood. Plasma was immediately separated by centrifugation at 3000 rpm for 15 minutes and stored at −80°C. MMP-9 levels were determined in duplicate by commercially available enzyme-linked immunosorbent assay (ELISA; Biotrak Amersham Pharmacia), and the mean value of both determinations was used. All ELISAs were performed according to manufacturer’s instructions. Our laboratory reference range for MMP-9 measured in healthy control subjects was 41–27.8 ng/mL (n = 62; 58% men; mean age, 43 years; normal range <97 ng/mL). The mean intra-assay coefficients of variation was <10%.

Determination of C-1562T Genotypes
DNA samples were extracted from whole blood with the QIAMP DNA Blood Midi Kit (QIAGEN). 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’ AATGGCCTGGCATAAgT TAGGG 3’) and Meta R-1 (5’ ACTCTTTCTTCC TAGG C 3’).

PCR was carried out in a 50-μL mixture containing 40 to 200 ng genomic DNA, 5 μL (2 μmol/L) of each primer, 0.5 μL Taq DNA polymerase 5 U/μL, 4 μL dNTPs 2.5 mmol/L, 5 μL 10× PCR buffer (TaKaRa Taq), and water to 50 μL. After an initial denaturation at 95°C for 5 minutes, the samples were subjected to 30 cycles of amplification, consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 60.5°C, and extension for 1 minute at 72°C, followed by a final extension at 72°C for 5 minutes in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400). Amplicons were digested with SphI restriction enzyme (New England Biolabs) that cuts the T allele, and the digests were then subjected to gel agarose 1% electrophoresis and visualized by ethidium bromide staining. Genotypes were scored according to the patterns of DNA bands.

Results were confirmed by cycle sequencing on a Perkin Elmer GeneAmp 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 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 95°C for 5 minutes, the samples were subjected to 30 cycles of amplification, consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 60.5°C, and extension for 1 minute at 72°C, followed by a final extension at 72°C for 5 minutes in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400). Amplicons were digested with SphI restriction enzyme (New England Biolabs) that cuts the T allele, and the digests were then subjected to gel agarose 1% electrophoresis and visualized by ethidium bromide staining. Genotypes were scored according to the patterns of DNA bands.

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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.
Our data confirm that plasmatic 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 Asiatic 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 atherogenesis-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. Plasmatic 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 increase 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 plasm-in-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 α2-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 possible 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.

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References

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