Identification of a Serum Gelatinase Associated With the Occurrence of Cerebral Aneurysms as Pro-Matrix Metalloproteinase-2

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Background and Purpose—Subarachnoid hemorrhage from intracranial aneurysm rupture produces a severe form of stroke. Extracellular matrix remodeling is associated with cerebral aneurysms and may play a role in the formation or rupture of these lesions. We previously reported a 3-fold increase in a 72-kDa serum gelatinase in a subgroup of aneurysm patients. The purpose of the present study was to further characterize and identify this gelatinase.

Methods—Serum samples were collected from surgical patients with intracranial aneurysms. The following series of experiments was designed to further characterize and identify the predominant serum gelatinase found in the subgroup of patients with increased gelatinase activity. Gelatin zymography was performed on native serum samples and compared with serum that had been pretreated with a known metalloproteinase activator (4-aminophenylmercuric acetate [APMA]). Gelatin zymography was repeated in the presence of a matrix metalloproteinase (MMP) inhibitor (EDTA) and a serine proteinase inhibitor (phenylmethylsulfonyl fluoride [PMSF]). Final identification was made by Western blotting with the use of monoclonal antibodies to MMP-2 and MMP-9.

Results—A consistent gelatinolytic band (72 kDa) was identified in all serum samples (n = 60). Pretreatment of the serum by APMA (n = 60) lowered the molecular weight of the band to 66 kDa. The band was inhibited by EDTA (n = 10) but not PMSF (n = 10), thus characterizing the circulating 72-kDa gelatinase as an inactive pro-MMP. Western blotting (n = 20) identified the 72-kDa band as MMP-2.

Conclusions—These findings confirm that the increased gelatinolytic activity observed in vitro in a subset of cerebral aneurysm patients is due to pro-MMP-2. (Stroke. 1998;29:1580-1583.)

Key Words: cerebral aneurysm ■ gelatinases ■ metalloproteinases

Cerebral aneurysms usually remain asymptomatic until rupture occurs. Despite modern therapy, aneurysmal subarachnoid hemorrhage remains one of the most severe forms of cerebrovascular disease, with a mortality approaching 50%.1–6 Understanding the biology of cerebral aneurysms may lead to new therapeutic strategies for these extremely ill patients.

Previous work by this laboratory has found a 3-fold increase in a 72-kDa serum gelatinase in a subgroup of aneurysm patients. In that study, aneurysm patients (52% ruptured, 48% unruptured) were compared with surgical patients known to be free of cerebral aneurysms. One half of the aneurysm patients had a 3-fold increase in serum gelatinase activity as measured by quantitative gelatin zymography compared with the controls. This increased activity was irrespective of their hemorrhage status.7 Vascular extracellular matrix (ECM) remodeling is known to be orchestrated and regulated by a complex network of proteases and protease inhibitors.8-10 Molecular markers of matrix remodeling have been found in both cerebral and aortic aneurysm patients.7,11

The purpose of the present study was to further characterize and identify this circulating gelatinase.

The families of matrix metalloproteinases (MMPs) and serine proteinases both participate in ECM remodeling, and each contains proteases capable of digesting gelatin.12 The MMPs are a homologous group of zinc- and calcium-dependent matrix proteases that are secreted as zymogens.13 Two members of this family, MMP-2 and MMP-9, are known respectively as gelatinase A (72 kDa) and gelatinase B (92 kDa) because of their strong affinity to that substrate in vitro.14 Of the serine proteases known to degrade gelatin, plasmin (75 kDa) has the closest molecular weight to the gelatinase in question.

Characterization of this gelatinase as an MMP or a serine protease was accomplished by studying its behavior in the presence of known protease inhibitors. Ethylenediaminetetraacetic acid (EDTA), a divalent cation chelator, is known to inhibit activation of MMPs, while phenylmethylsulfonyl fluoride (PMSF) is a known serine protease inhibitor. 4-Aminophenylmercuric acetate (APMA), a known MMP
an activator, was used to determine whether this gelatinase was an inactive zymogen or active protease. Characterization of the gelatinase in this way allowed for presumptive identification as an MMP. Final identification was confirmed by Western blotting with the use of monoclonal antibodies to MMP-2 and MMP-9.

Materials and Methods

Patients

Patients undergoing surgical treatment of cerebral aneurysm as documented by cerebral angiography were included in the study. Informed consent was obtained in accordance to the protocol approved by the Institutional Review Board before collection of any samples. The sex, age, and presence or absence of subarachnoid hemorrhage was recorded for each patient at the time of sample collection.

Sample Collection

Blood was collected immediately before surgery by venipuncture in an evacuated tube. After we allowed for clot formation, the blood was centrifuged at 2000 rpm for 10 minutes, aliquoted, and stored at −70°C.

Gelatin Zymography

Gelatinase substrate gel electrophoresis was performed with the use of precast gels (10% polyacrylamide containing 0.1% gelatin, Novex). Samples were prepared by dilution (1:200) into a loading buffer consisting of 0.4 mol/L Tris, pH 6.8, 5% SDS, 20% glycerol, 0.03% bromophenol blue, and 20 μL of serum loaded per lane. After electrophoresis at 125 V, the gels were incubated in renaturing solution (2.5% Triton-X-100) for 30 minutes at room temperature and then for 72 hours at 37°C in a developing buffer containing 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 4 mmol/L CaCl₂, and 0.02% Brij-35. When appropriate, the developing buffer contained 20 mmol/L EDTA, a known inhibitor of MMPs, or 1 mmol/L PMSF, a known inhibitor of serine proteases. The gels were then stained with Coomassie blue, and regions without staining were indicative of gelatin lysis.

Serum samples were prepared as follows for activation with APMA. First 25 μL of 5 mmol/L APMA was added to 100 μL of undiluted serum and incubated in a 37°C water bath for 90 minutes. The reaction was stopped with 10 mmol/L EDTA, and the samples were dialyzed for 18 hours to remove the APMA. The samples were then loaded on gels, and the zymography protocol was followed as above.

Western Blotting

Serum was diluted 1:100 with ultrapure double-distilled water (18-Ω resistance), mixed 1:4 with loading/reducing buffer, denatured at 95°C for 5 minutes, and flash spun at 7000 rpm. A total volume of 30 μL was loaded per lane. The proteins separated electrophoretically on a 10% Tris-HCl polyacrylamide gel for 45 minutes at 200 constant voltage. Commercially obtained protein standards for MMP-2 and MMP-9 (Chemicon) were loaded on every gel. The gel was transferred to a supported nitrocellulose membrane by overnight electrophoresis (190 mA at 4°C). The membrane was blotted according to ECL Western blotting protocol (Amersham RPN2106) with MMP-2 and MMP-9 monoclonal antibodies (Calbiochem) diluted according to manufacturer’s specifications.

Results

Serum samples were collected from 60 aneurysm patients. The mean age of the group was 54 years (range, 25 to 79 years), and 40 patients (67%) were women. Thirty-five were treated for unruptured aneurysms (58%), and 25 patients presented with SAH (42%). Gelatin zymography of the native serum revealed a consistent band of gelatin lysis at 72 kDa in all 60 patients. Ten of the above patients were randomly selected and underwent repeated zymography after treatment.
of the serum with APMA. In these patients, the previously noted band of gelatinase activity at 72 kDa was replaced by a band at 66 kDa (Figure 1).

In 10 patients showing the greatest lysis of gelatin at 72 kDa, EDTA was added to the zymographic developing buffer and in all cases completely inhibited the 72-kDa band of gelatin lysis. PMSF, a known inhibitor of serine proteases, did not inhibit gelatin lysis at the 72-kDa mark when added to the zymographic developing buffer in these patients.

Western blotting in the above 20 patients with a monoclonal antibody against MMP-2 revealed a consistent band at the appropriate molecular weight that corresponded to the commercial MMP-2 standard (Figure 2). The Western blots were repeated in these patients with a monoclonal antibody against MMP-9, and no reactivity was observed except in the protein standard. These data definitively identified the gelatinase in question as pro-MMP-2.

Discussion
Circulating levels of pro-MMP-2 are increased in some patients with cerebral aneurysms. Increased serum-, plasma-, and tissue-bound MMP-2 and other MMPs are associated with the occurrence of vascular diseases such as aortic aneurysms and atherosclerosis. Under normal circumstances, the transcription, secretion, and activation of MMP-2, as well as other MMPs, are tightly regulated. The mechanisms that alter circulatory pro-MMP-2 levels and the nature of their relationship to cerebral aneurysm formation in some patients are unknown.

Although increased levels of MMP-2 are known to be a marker of invasive potential and metastasis in certain neoplasms, the increased circulating levels of pro-MMP-2 in some cerebral aneurysm patients may be an epiphenomenon. MMP-2 is known to be secreted by a variety of different cell types, including fibroblasts, macrophages, leukocytes, smooth muscle cells, and endothelial cells. It is a known participant in normal and pathological ECM remodeling, angiogenesis, and cell migration. It is conceivable that some condition(s) either directly or indirectly related to aneurysm formation may influence circulating pro-MMP-2 levels by acting on sites distant from the cerebral aneurysm. Alternatively, it is possible that pro-MMP-2 may be shed from the region of the cerebral aneurysm representing a circulatory marker of molecular events occurring with the aneurysm wall.

We next considered the possibility that MMP-2 was playing an active role in the matrix degradation that either initiated or propagated the formation of intracranial aneurysms. Although the precise method of pro-MMP-2 activation in vivo has not been characterized, recent publications have identified a unique multistep activation pathway that involves members of both ECM-degrading families. In both pathways, activation occurs on the cell surface, targeting activation of pro-MMP-2 to specific anatomic sites. The transmembrane MMPs (MT1-MMP and MT2-MMP) have been shown to activate pro-MMP-2, in some cases forming a 64-kDa intermediate. Recently, the plasmin activator–plasmin system was implicated in the second stage of MMP-2 activation to the final active 62-kDa form. Other studies show the urokinase-plasmin system playing a larger role in the control of gelatinase activity. This is of interest since we recently localized both plasmin and MT1-MMP in cerebral aneurysm tissue, raising the possibility of localized activation of MMP-2 within the aneurysm.

In conclusion, increased circulating levels of pro-MMP-2 occur in a subset of cerebral aneurysm patients. The relationship of this observation to the mechanisms that result in aneurysm formation and growth remains to be defined.

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