Involvement of Mitogen-Activated Protein Kinase Signaling in Growth and Rupture of Human Intracranial Aneurysms

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Background and Purpose—Mitogen-activated protein kinases (MAPKs) are involved in vascular wall remodeling, but their role in the pathogenesis of intracranial aneurysms (IAs) is poorly known. We investigated the expression and phosphorylation of the 3 major mitogen-activated protein kinases, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase, in unruptured and ruptured human IAs.

Methods—Tissue samples (n=24, 12 unruptured and 12 ruptured IAs) were obtained during microneurosurgical clipping. The localization of the proteins was studied by immunofluorescent staining, and protein levels and phosphorylation state were studied by Western blotting.

Results—The phosphorylation of p54 JNK was increased 1.5-fold in ruptured IAs and the phospho-p54 JNK level and its phosphorylation state directly correlated with IA size. The levels of phosphorylated and total levels of p38 were associated with IA size as well. Extracellular signal-regulated kinase did not associate with IA size or rupture status. Expression of transcription factor c-Jun, a downstream target of JNK, correlated with p54 JNK level and phosphorylation state. Furthermore, the levels of matrix metalloproteinase 9, known to have a role in vessel wall degeneration, correlated with p54 JNK phosphorylation in unruptured IAs and its expression was increased 4.3-fold in ruptured IAs.

Conclusions—Our results suggest that JNK activity and expression are involved in IA growth and possibly rupture and p38 expression in IA growth. Thus, pharmacological therapy affecting the stress-activated mitogen-activated protein kinases, JNK and p38, may enhance the repair of the IA wall in the future. (Stroke. 2008;39:000-000.)

Key Words: extracellular signal-regulated MAP kinases ■ intracranial aneurysm ■ JNK mitogen-activated protein kinases ■ p38 mitogen-activated protein kinases

Intracranial aneurysms (IAs) are the most common cause of subarachnoid hemorrhage with up to 50% case fatality. Risk factors for IA rupture include hypertension, smoking, heavy alcohol consumption, and female gender. Some IAs are familial. However, the mechanisms of how IAs develop and why some of them rupture remain unclear, although histological differences in ruptured and unruptured aneurysms have been observed. Vascular remodeling, increased inflammatory cell infiltration, complement activation, and apoptosis associate with the rupture of IAs.

Mitogen-activated protein kinases (MAPKs) are a family of intracellular signaling proteins consisting of c-Jun N-terminal kinases (JNKs), p38 MAPKs, and extracellular signal-regulated kinases (ERKs). They respond to various extracellular stimuli such as inflammatory cytokines, growth factors, mechanical stretch, and cellular stress. MAPKs have a critical role in cell growth, proliferation, differentiation, and apoptosis. They phosphorylate other protein kinases and regulate various transcription factors that control the expression of large variety of genes. To be activated, MAPKs must be phosphorylated by upstream kinases. MAPKs are involved in vascular wall remodeling. In experimental animals, they are rapidly and transiently activated in balloon-injured arteries, hypertrophied cardiac and hypertensive vascular tissue, and have important roles in smooth muscle cell (SMC) proliferation and neointimal formation. Interestingly, JNK was recently shown to be involved in the pathogenesis of abdominal aortic aneurysms where it activates transcription factor c-Jun and regulates the expression of matrix metalloproteinases (MMPs) involved in vessel wall degradation.

The role of MAPKs in the pathogenesis of cerebral artery aneurysms is poorly known. A previous immunohistochemistry study has suggested that the expression of phosphory-
**Table 1. Patient and IA Characteristics**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unruptured IAs</th>
<th>Ruptured IAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
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<td>(n=12)</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>56.8 (43–71)</td>
<td>55.9 (39–82)</td>
</tr>
<tr>
<td>Women*</td>
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Aneurysms resected for study

<table>
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<tr>
<th>Location</th>
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<td>Internal carotid artery</td>
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<tr>
<td>Posterior communicating artery</td>
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<td>1</td>
</tr>
<tr>
<td>Pericallosal artery</td>
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</table>

Largest diameter, mean±SD, mm† 8.2±2.2 12.6±5.9

Largest diameter, median, mm (range) 8.0 (5.8–13) 10.5 (6.5–26)

Time from rupture to sample resection, median (range) (5.5 hours to 48 days)

Note: † Significant at 0.05 level.

**Materials and Methods**

**Human Intracranial Aneurysm Samples**

IA samples (n=24; 12 unruptured and 12 ruptured) were obtained during their microneurosurgical clipping. Cutting a part of the dome followed by the coagulation of the remaining aneurysm was performed to achieve optimal clipping of the aneurysm and to obtain samples (J.H., M.N. Department of Neurosurgery, Helsinki University Central Hospital). Patient and IA characteristics, obtained from the medical records, are described in Table 1. The IA samples were immediately snap-frozen in liquid nitrogen and stored at −70°C. The study was approved by the local ethics committee of the Departments of Neurology, Neurosurgery, Otorhinolaryngology, and Ophthalmology at the Helsinki University Central Hospital.

**Histology and Immunofluorescence**

IA samples were cryosectioned at 4 μm. For histology, sections were fixed immediately in 4% formaldehyde (in PBS) for 15 minutes at room temperature. Sections were blocked in 5% normal goat or horse serum (Vector Laboratories Inc, Burlingame, Calif) diluted in 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, Mo) in PBS (PBS/Triton) for 60 minutes. After blocking, the sections were incubated with the primary antibody against phospho-SAPK/JNK, phospho-p38, and phospho-p44 MAPK (detailed in supplemental Table I, available online at http://stroke.ahajournals.org) diluted in PBS/Triton 30 minutes at room temperature and overnight at +4°C followed by incubation with a fluorochrome-conjugated secondary antibody diluted in PBS/Triton (supplemental Table I). For double staining, an additional incubation with mouse monoclonal Cy3-conjugated anti-α-smooth muscle actin antibody (1:1000 in PBS; Sigma-Aldrich) 30 minutes at room temperature and overnight at +4°C was performed. Sections were mounted in Vectashield containing DAPI (Vector Laboratories Inc). Rat balloon-injured aorta served as a positive control tissue.† For negative controls, the primary antibody was omitted.

**Preparation of Tissue Samples and Western Blot Analysis**

Tissue samples were pulverized in liquid nitrogen with mortar and pestle, homogenized in hot 1% sodium dodecyl sulfate (SDS) in PBS buffer, and sonicated. Samples were kept at +100°C for 10 minutes and immediately frozen on dry ice. For WB, the mixture was centrifuged at 14 000 g for 15 minutes at +4°C. The supernatant protein concentrations were measured by DC-protein assay (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer’s protocol. The samples were mixed (5:1) with sample buffer (0.3 mol/L Tris, pH 6.8, 10% SDS, 5% β-mercaptoethanol, 50% glycerol and bromophenol blue). First, 20 μg of protein was loaded for actin WB. Actin served as an internal loading control to standardize the amount of intracellular protein for each IA sample, resulting in 15 to 42 μg of total protein to be loaded per sample on subsequent gels. Proteins were separated in a 7.5% (MMP-9, c-Jun) or 10% SDS polyacrylamide gel (ERK, p38, JNK, actin) with Tris-glycine–SDS running buffer. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat dry milk in 0.1% Tween-20 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in Tris-buffered saline (milk/Tris-buffered saline) for 1 hour at room temperature. The blots were incubated at +4°C overnight with primary antibodies against JNK, p38, p44/p42 (ERK), c-Jun, MMP-9, and actin and phosphorylated forms of JNK, p38, p44/p42, and c-Jun (supplemental Table I) diluted in 5% bovine serum albumin (Sigma–Aldrich) in Tris-buffered saline or milk/Tris-buffered saline followed by 1-hour incubation at room temperature with secondary antibodies (supplemental Table I) in milk/Tris-buffered saline. Antibody–antigen complexes were visualized by chemiluminescence using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences/GE Healthcare, Piscataway, NJ) according to the manufacturer’s instructions and a Typhoon scanner (Amersham). Western blot scans were quantified using Scion Image software (www.scioncorp.com).

Quantified optical densities on WBs of all intracellular proteins were standardized to actin levels of the same samples, whereas MMP-9, which is mostly extracellular, was standardized to the total amounts of protein. For all phosphoproteins, two WBs were performed and quantified: one with antibody detecting only the phosphorylated form of the protein and another with antibody detecting both the phosphorylated and unphosphorylated form (total) (Figure 1A). Phosphorylation state was defined as the ratio between phosphorylated and total levels of the proteins of interest. Results are expressed as arbitrary units where the mean of the unruptured group is set at 1.0.

**Statistical Analysis**

Data were analyzed with SPSS for Windows (release 13.0; SPSS Inc). Effects of rupture status, sex, and aneurysm size on protein and phosphorylation levels were compared by t tests, analysis of variance, and covariance after logarithmic transformation of dependent variables, which was necessary to obtain their normal distributions and equality of variances between different groups. Correlation between protein and phosphorylation levels and aneurysm size was estimated using Spearman’s rank correlation coefficients (r). Effects of interactions of rupture status, aneurysm size, sex, and age on protein and phosphorylation levels were studied with 2-way analysis of variance. Probability values less than 0.05 were considered statistically significant.
Results

Mitogen-Activated Protein Kinases in Intracranial Aneurysm Wall

First we studied whether there were activated MAPKs in IAs and in which part of the wall they localized. The phosphorylated forms of all 3 MAPKs were found both in unruptured and ruptured IAs in immunofluorescent staining (Figure 2). Phospho-ERK localized in the cytoplasm of α-smooth muscle actin (αSMA)-positive cells and in some αSMA-negative cells in the outer and luminal side of the wall. Phospho-p38 localized in the cytoplasm of most of and nuclei of some αSMA-positive cells. Few cells were also detected in the luminal and outer sides of the IA wall. Phospho-JNK localized in the nuclei of αSMA-positive cells. Cytoplasm was also weakly positive. In the outer IA wall, some αSMA-negative cells stained positive for phospho-JNK with or without positive nuclei. To conclude, the

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

Figure 1. Examples of WBs obtained in unruptured and ruptured IAs (A). The phosphorylation of p54 JNK was higher in ruptured than in unruptured IAs (B) and correlated with IA size (C). The levels of phospho-p38 correlated with IA size (D).

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

Figure 2. The immunofluorescent staining of a ruptured IA wall showing the localization of phosphorylated MAPKs. (A) ERK, (B) p38, and (C) JNK in green and αSMA in red; DAPI (blue) was used as a nuclear stain. Asterisk indicates the luminal side of the wall.
Table 2. Protein Levels in the IA Wall

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Unruptured IAs (mean ± SD)</th>
<th>Ruptured IAs (mean ± SD)</th>
<th>P</th>
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<tbody>
<tr>
<td>Phospho-p54 JNK</td>
<td>1.00 ± 0.30</td>
<td>1.48 ± 1.01</td>
<td>NS</td>
</tr>
<tr>
<td>Total p54 JNK</td>
<td>1.00 ± 0.21</td>
<td>0.96 ± 0.40</td>
<td>NS</td>
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<tr>
<td>Phospho-p54/p54 JNK ratio</td>
<td>1.00 ± 0.23</td>
<td>1.52 ± 0.61</td>
<td>0.028</td>
</tr>
<tr>
<td>Phospho-p46 JNK</td>
<td>1.00 ± 0.24</td>
<td>1.44 ± 1.01</td>
<td>NS</td>
</tr>
<tr>
<td>Total p46 JNK</td>
<td>1.00 ± 0.23</td>
<td>1.66 ± 1.12</td>
<td>0.081</td>
</tr>
<tr>
<td>Phospho-p46/p46 JNK ratio</td>
<td>1.00 ± 0.15</td>
<td>0.90 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td>Phospho-p38</td>
<td>1.00 ± 0.28</td>
<td>0.97 ± 0.46</td>
<td>NS</td>
</tr>
<tr>
<td>Total p38</td>
<td>1.00 ± 0.28</td>
<td>1.09 ± 0.40</td>
<td>NS</td>
</tr>
<tr>
<td>Phospho-p38/p38 ratio</td>
<td>1.00 ± 0.14</td>
<td>0.90 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>Phospho-ERK</td>
<td>1.00 ± 0.24</td>
<td>1.04 ± 0.70</td>
<td>NS</td>
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<tr>
<td>Total ERK</td>
<td>1.00 ± 0.12</td>
<td>1.16 ± 0.56</td>
<td>NS</td>
</tr>
<tr>
<td>Phospho-ERK/total ERK ratio</td>
<td>1.00 ± 0.19</td>
<td>0.85 ± 0.27</td>
<td>NS</td>
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<tr>
<td>Phospho-c-Jun</td>
<td>1.00 ± 0.46</td>
<td>1.69 ± 1.06</td>
<td>0.066</td>
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<tr>
<td>Total c-Jun</td>
<td>1.00 ± 0.50</td>
<td>1.82 ± 1.28</td>
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<tr>
<td>Phospho-c-Jun/total c-Jun ratio</td>
<td>1.00 ± 0.30</td>
<td>0.95 ± 0.22</td>
<td>NS</td>
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<tr>
<td>MMP-9</td>
<td>1.00 ± 0.43</td>
<td>4.34 ± 3.29</td>
<td>&lt;0.001</td>
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Student’s independent samples t test.
NS indicates not significant.

Table 3. Correlation Between JNK and c-Jun

<table>
<thead>
<tr>
<th>Variables</th>
<th>Phospho-c-Jun</th>
<th>Total c-Jun</th>
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<tbody>
<tr>
<td>Phospho-p54 JNK</td>
<td>0.65*</td>
<td>0.58*</td>
</tr>
<tr>
<td>Total p54 JNK</td>
<td>0.426†</td>
<td>0.458†</td>
</tr>
<tr>
<td>Phospho-p54/p54 ratio JNK</td>
<td>0.533*</td>
<td>0.420†</td>
</tr>
<tr>
<td>Phospho-p46 JNK</td>
<td>0.576*</td>
<td>0.536†</td>
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<tr>
<td>Total p46 JNK</td>
<td>0.375†</td>
<td>0.463†</td>
</tr>
<tr>
<td>Phospho-p46/p46 ratio JNK</td>
<td>0.213</td>
<td>0.038</td>
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*P<0.01, †P<0.05, ‡P=0.071, Spearman’s rho correlation analysis.

The median size of all 24 IAs was 8.0 mm. The phosphorylation state of p54 JNK was increased 1.5-fold in large IAs when compared with small IAs (1.51 ± 0.62 for large IAs [≥8.0 mm in diameter, n = 12], 1.01 ± 0.23 for small ones [≤8.0 mm, n = 12], P = 0.037). The levels or phosphorylation state of other MAPKs did not differ according to aneurysm size.

By use of covariance analysis, it was revealed that aneurysm size, but not rupture status, was significantly associated with the level of phospho-p54 JNK (P = 0.027 and P = 0.894, respectively). The phosphorylation of p54 JNK was also associated with IA size but not with rupture status (P = 0.023 and P = 0.296, respectively). The levels of total p38 associated with IA size but not with rupture status (P = 0.003 and 0.210, respectively). On the other hand, the levels of phospho-p38 were associated both with IA size and with rupture status (P = 0.001 and P = 0.027, respectively). However, although the levels of phospho-p38 increased with increasing aneurysm size, they were lower in ruptured IAs. There were no significant interactions between rupture status or aneurysm size and the levels or phosphorylation state of any of the MAPKs.

Mitogen-Activated Protein Kinases versus Intracranial Aneurysm Rupture and Size

Because MAPKs were found in both unruptured and ruptured IAs in the immunofluorescent staining, we studied the expression and phosphorylation of MAPKs more closely by WB (Table 2). The levels or phosphorylation state of any of the MAPKs were not affected by sex or age of the patients (data not shown). First we investigated whether there is any difference between unruptured and ruptured IAs. The total levels of 2 isoforms of JNK, namely p54 and p46, did not associate with the rupture status. However, the phosphorylation state of p54 JNK was significantly (P = 0.028) increased 1.5-fold in ruptured IAs compared with unruptured ones (Figure 1B). Total levels of ERK and p38 or their phosphorylation state did not associate with IA rupture status in this analysis.

Next we tested whether the expression and phosphorylation of MAPKs correlate with IA size. The level of phospho-p54 JNK and its phosphorylation state directly correlated with IA size (r = 0.490, P = 0.015 and r = 0.501, P = 0.013, respectively; Figure 1C) but the levels or the phosphorylation state of p46 JNK did not. The levels of phosphorylated and total p38 correlated with IA size (r = 0.506, P = 0.012 and r = 0.592, P = 0.002, respectively; Figure 1D). ERK levels or phosphorylation did not correlate with IA size.

As expected,17 ruptured IAs were larger than the unruptured ones (Table 1). Hence, we wanted to study whether the differences in the protein levels and phosphorylation of p54 JNK as well as the differences in the protein levels of p38 are better explained by rupture status than by IA size.
MAP Kinases in Human Intracranial Aneurysms

Discussion

In this study, we investigated 3 key MAPKs in the human IA wall. The immunofluorescent stainings showed that active forms of all 3 key MAPKs are found in both ruptured and unruptured IAs and are mainly localized in the SMCs. MAPKs have different kinds of roles within the intracellular signaling pathways; ERKs respond to mitogenic stimuli, whereas JNKs and p38 are activated mainly by stress signaling.9 To verify the activation status, the phosphorylation state and the levels of each of these 3 MAPKs were studied separately by WB. The results suggest active roles of stress-activated kinases, JNK and p38, in the IA wall.

Possible Activators of Stress-Activated Kinases in Intracranial Aneurysms

JNK and p38 are activated in response to cellular and environmental stresses such as inflammatory cytokines and shear stress.9 IAs tend to develop at the sites of high shear stress20 and thus hemodynamic factors may play a role in activating JNK and p38 in IAs. Inflammation is also associated with the pathogenesis of IAs.5–7 The levels of tumor necrosis factor-α, a cytokine secreted by inflammatory cells, have been previously shown to be increased in ruptured IAs.21 Interestingly, tumor necrosis factor-α is one of the activators of JNK and p38.22,23

The Role of Stress-Activated Kinases in Growth and Rupture of Intracranial Aneurysms

Phosphorylation of p54 JNK was associated more highly with IA size than with the rupture status, which suggests that the activation of p54 JNK is not a response to the rupture but is involved in the pathogenesis of IA and its growth before rupture. The levels of phosphorylated and total p38 directly associated with the aneurysm size. It is known that the increase in IA size associates with elevated rupture risk.13 The pathways that are active in large IAs, like JNK and p38 signaling pathway, may be involved in the inter- and intracellular mechanisms that result in a weaker IA wall being more prone to rupture. However, these may also act as survival and repair pathways that are activated in the weakened IA wall to prevent the IA rupture. It has been observed in vitro and in animal experiments that p38 is activated in cerebral arteries after subarachnoid hemorrhage leading to the development of vasospasm.24 However, in our samples, the levels of phospho-p38 were slightly lower in ruptured IAs. The reason for this discrepancy and the implications of this phenomenon require further investigation. Traditionally, JNK has been seen as a proapoptotic and p38 as a proinflammatory signaling molecule.9,25 Stress-activated kinases may also regulate cell growth and survival. For example, stretch can cause the activation of both JNK and p38 in SMCs followed by the induction of SMC actin expression.26 Clearly, stress-activated MAPKs have different roles depending on the cell type and stimuli and these signaling pathways may also have combinatorial effects on the cell function. Thus, the stress-activated MAPK pathways within IAs should be studied more closely to clarify their roles in the pathogenesis of IAs.

Extracellular Signal-Regulated Kinase in Intracranial Aneurysms

ERKs are activated in response to growth and mitogenic stimuli and they are not associated with stress signaling like JNKs and p38s.9 In our study, the expression and phosphorylation of ERK did not associate with the size or the rupture status of IAs, which further supports the view that IA wall remodeling is a pathological process in which stress-activated pathways have a role in determining how cells behave.
Downstream Signaling of c-Jun
N-Terminal Kinase

It has recently been shown that JNK has an important role in the pathogenesis of abdominal aortic aneurysms. In that study JNK was shown to program gene expression patterns that enhanced the degradation of the extracellular matrix (eg, increase in MMP-9 expression) and suppressed biosynthetic enzymes, resulting in the destruction of the tissue. JNK inhibition led to the reduced levels of phospho-c-Jun and to enhanced tissue repair. We show here that the levels of phospho-c-Jun and c-Jun correlate with the levels of phospho-JNK in the IA wall, which implies that the JNK pathway is also activated in IAs. c-Jun is one of the AP-1 group proteins and JNK can activate c-Jun by 2 mechanisms: by increasing the expression of c-Jun and by phosphorylating c-Jun. c-Jun itself has diverse biological functions like controlling cell proliferation, differentiation, and apoptosis. In SMCs, c-Jun is involved in SMC proliferation and intimal thickening.

Previously it has been observed that MMP-9 expression and the proteolytic activity of MMPs and serine proteinases are increased in IAs. We showed that the phosphorylation of p54JNK positively correlated with MMP-9 levels in unruptured IAs and that MMP-9 was highly upregulated in ruptured aneurysms. However, in some ruptured aneurysms, there were high MMP-9 levels without increased JNK levels, which indicate that also other mechanisms are affecting MMP-9 expression.

Mitogen-Activated Protein Kinases and the Treatment of Intracranial Aneurysms

Pharmaceutical treatment of IAs is a future goal and, for example, endovascular coils with growth factors have been already tested in animal models. JNK inhibitors have been suggested for the treatment of abdominal aortic aneurysms and p38 inhibitors for the treatment of inflammatory diseases like rheumatoid arthritis and also atherosclerosis. Future studies may clarify the specific roles of MAPKs in the pathogenesis of IAs and show how drugs that modulate MAPK signaling affect the IA wall.

Limitations and Strengths of the Study

Our samples were freshly frozen and thus very reliable for studies of phosphoproteins known to be subject to rapid dephosphorylation after tissue harvest or postmortem. However, because normal fresh human circle of Willis artery samples are extremely difficult to obtain, we did not have any normal arterial controls. It would be interesting to see how signaling pathways in IAs differ from normal intracranial arterial wall. In WB, we used homogenized samples and, of course, there may be local differences in the amounts of signaling proteins within the IA wall. However, quantitative analysis of such ubiquitous proteins is practically impossible using other methods like immunohistochemistry. Based on histological analysis, acellular or highly thrombosed samples were excluded from the study because the aim was to study intracellular signaling proteins within IA wall. Also, because IA samples were heavily denatured to preserve the phosphorylation state of the proteins, we could not study MMP-9 using zymography, which would provide information about the activity of MMP-9.

Summary

All 3 MAPKs, JNK, p38, and ERK, were found both in unruptured and ruptured IAs. The levels of phospho-p54JNK and the phosphorylation state of p54 JNK associated with IA size and possibly with rupture status. The levels of phospho-p38 and the levels of total p38 associated with the aneurysm size. ERK did not associate with the rupture status or the aneurysm size. Furthermore, the phosphorylation state and the phosphorylated and total levels of JNK correlated with the phosphorylated and total levels of c-Jun, and there was a trend for increase in the levels of phospho-c-Jun and total c-Jun in the ruptured IAs as compared with unruptured ones. MMP-9 levels associated with the rupture status and MMP-9 levels correlated with the phosphorylation of p54 JNK in unruptured but not in ruptured IAs.

Acknowledgments

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Disclosures

None.

References


Table. Antibodies and Dilutions

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<th>Dilution</th>
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Elisa Laaksamo, Riikka Tulamo, Marc Baumann, Reza Dashti, Juha Hernesniemi, Seppo Juvela, Mika Niemelä and Aki Laakso

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