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The study of endogenous hepatocyte growth factor in the pathogenesis of intracranial aneurysms

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Abstract. – OBJECTIVE: Inflammation as a significant influence factor plays an important role in the formation and rupture of intracranial aneurysms. However, based on plenty of related studies, it is believed that the hepatocyte growth factor (HGF) is able to prevent vascular inflammation. This paper, therefore, explored the role and mechanism of endogenous HGF in the pathogenesis of intracranial aneurysms.

MATERIALS AND METHODS: 16 blood samples were collected from the intracranial aneurysms and the lumens of the femoral artery from 16 patients. Comparison and quantitative detection of HGF serum concentrations in an intracranial aneurysm and femoral artery samples according to the immune assay based on Luminex were followed. The tissue of superficial temporal artery (STA) and ruptured or unruptured intracranial aneurysm from the patients (n=10) who were performed surgical clipping of cranial aneurysm was then collected. The intracranial aneurysm was induced by surgery on the 316 mice and grouping administration of c-Met antagonist PF-04217903 or its solvent DMSO for 3 weeks. Then, the brains of experimental mouse were dissected and examined whether there were intracranial aneurysms and subarachnoid hemorrhage (SAH). The procedures followed by treatment of human endothelial cells cultured in vitro, smooth muscle cells and monocytes with HGF and PF-04217903 lipopolysaccharide (LPS). The Real-time fluorescence quantitative PCR, Western blot method, we used the expressions of interleukin normalized inflammatory factor [intercellular adhesion molecule-1 (ICAM-1)], vascular cell adhesion molecule-1 (VCAM-1), E-selectin, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and transforming growth factor-β (TGF-β).

RESULTS: It was found that the concentration of HGF (1076±656) pg/ml from the blood collected from human intracranial aneurysms was significantly higher than that from the femoral artery (196±…) pg/ml ($p<0.01$). Experimental mouse treated with PF-04217903 (c-Met antagonist) and in control group were induced to form intracranial aneurysms. c-Met antagonists did not affect the formation of intracranial aneurysms ($p>0.05$), but significantly increased the occurrence of subarachnoid hemorrhage and reduced the survival rate of mice ($p<0.05$). HGF attenuated the expression of VCAM-1 ($p<0.05$) and E-selectin ($p<0.05$) in human aortic endothelial cells

CONCLUSIONS: The plasma HGF levels were elevated in intracranial aneurysms, and the HGF and c-Met were expressed in STA and intracranial aneurysms. In the treatment of intracranial aneurysms, HGF signaling pathway reduced inflammation in endothelial cells and prevented the rupture of intracranial aneurysm through c-Met.

Key Words:
Intracranial aneurysm, Inflammation, Hepatocyte growth factor, Subarachnoid hemorrhage, c-Met, E-selectin, VCAM-1.

Introduction

According to previous studies[1,2], it was convinced that the inflammation is an important factor in the formation and rupture of intracranial aneurysms. The various components of the inflammatory reaction include cytokines, chemokines, growth factors, reactive oxygen species (ROS), white blood cells, matrix metalloproteinases and vascular smooth muscle cells, which all increase in the occurrence of intracranial aneurysms[3,4]. The treatment, which focuses on inflammation pathways, is more effective under clinical experiments on both human and animal basis[2,5]. Hepatocyte growth factor (HGF) was originally discovered to be a growth factor of the hepatocyte, which showed the activity of mito-

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sis, morphology, anti-fibrosis and anti-apoptotic in a variety of tissues[6,7]. However, the activity of HGF is regulated by proto-oncogene c-Met, which is a type of tyrosine kinase[8]. Up to now, the latest researches and data suggested that HGF is capable of regulating cytokine profiles and protecting various tissues including the arterial wall from inflammatory damages[9]. Moreover, HGF can promote an anti-inflammatory cytokine profile in the abdominal aortic aneurysm. Therefore, the endogenous HGF secretion promoted by drug interventions may be possibly effective in the prevention and treatment of an aneurysm[10]. Nevertheless, there were only few reports to be found concerning the study of HGF in intracranial aneurysms.

The purpose of this study was to evaluate the role of endogenous HGF in the pathogenesis of intracranial aneurysms. Specifically, we sought to determine that: (1) if the concentration of HGF in patients with intracranial aneurysm in the blood is higher than the concentration of HGF in arterial blood; (2) if the HGF and c-Met are expressed in the wall of human intracranial aneurysms; (3) in animal models, if the c-Met antagonist in es the risk of intracranial aneurysm rupt in cultured human endothelial cells in v if the smooth muscle cells and mononuclear in HGF are expressed and regulated by infla matory cells.

Materials an etho

Human Research ogram

The human re h program approved by the Ethics e Qingdao r Medical Center gdac ndong, China). The nature, be fits, and ri f this study have been ex ed to all pati eforehand. All partic s have ead and volu tarily signed the inf d cons orm.

Cell C

man e helial cells (HAECs), Huma rtic sm muscle cells (HASMCs) and Hu Monocy c Cell Line THP-1 were purc merican ATCC cell bank (Manas- VA, USA). The cells were cultured in Dulco's Modified Eagle Medium (DMEM) with level glucose (HyClone, Nampa, ID, USA) wit 0% fetal bovine serum (FBS) (Tianhang Biological Technology Inc. Hangzhou, Zhejiang, China) and 1% penicillin streptomycin (Beijing Suolaibao Technology Inc, Beijing, China), and then placed in the incubator (371 type, Thermo Scientific, Waltham, MA, USA) in constant 37°C and 5% CO_2.

Serum

Human R/c-Met HGF affinit fied polyclonal antibody (AF276) and hum GF affinity purified polyclonal body (A NA) were from Shanghai Ro eida Industri Ltd. (Shanghai, China stase from Da Meilun Biological T logy Ltd. (Dalian, Shenyang, China) ng was f Nanjing Senbeijia ogical olog o., Ltd. (Nanjing, Jia China), c-M onists PF-04217903 Gaochuang emical Technology C Ltd. ghai, China), Dimethyl Sulphoxide (DMSO) om Beijing Huamaike Bio echnology C d. (Beijing, China), P ine phenol blue was from Shanghai Bao- Biological hnology Co., Ltd (Shanghai, a), Papaveri was from Wuhan Haojia Biol Technolo Co., Ltd (Wuhan, Hubei, China), om Shanghai Jingke Chemical Techno o., Ltd. (Shanghai, China), Human combinant HGF was from PeproTech (Rocky USA), LPS was from Weihuan Biologi- nology Co., Ltd (Shanghai, China).

Determination of HGF Concentration in Plasma

All patients enrolled in this study were from the Qingdao Hiser Medical Center (Qingdao, Shandong, China) from the period of November 2015 to December 2015. In this study, all patients were applicants who suffered from cystic (ruptured or unruptured) coil embolization of intracranial aneurysms. Patients treated with corticosteroids, aspirin, or immunosuppressive agents were excluded. A total of 16 patients and 18 aneurysms were finally involved in the study. Through the Seldinger technique, the femoral artery puncture was operated to patients to investigate if an aneurysm was present. Each patient was embedded in a 7-French artery sheath, followed by blood collection from the femoral artery. Then, a guiding catheter was imported into the targeted vessels for each patient to identify the aneurysm. The microcatheter was then introduced into the aneurysm cavity depending on micro guide wires. Blood samples were collected (5 ml) before the import of the guiding wires into the aneurysm, and then the blood samples were stored at -80°C after centrifugation. By using

Human HGF Magnetic Bead Kit (Thermo Fisher Scientific, Waltham, MA, USA), HGF serum concentrations were quantitatively detected in aneurysm and femoral artery samples in Luminex 200 system (Luminex Corp, Austin, TX, USA).

Expression of HGF and c-Met in Intracranial Aneurysms

The aneurysm samples were collected from patients with microsurgical clipping (5 ruptured cases, 5 unruptured cases), then were fixed with formalin and embedded in paraffin after surgical removal. Compared with the superficial temporal artery, the excised aneurysm samples were firstly made to 4 μm slices and then immunohistochemical stained by Human R/c-Met HGF affinity purified polyclonal antibody (AF276) or human HGF affinity purified polyclonal antibody (AF-294-NA). All images were acquired by BX-61 Olympus automatic microscope 20 objective lens (Olympus, Tokyo, Japan).

Evaluation of the Effect of Endogenous HGF on Intracranial Aneurysm in Mouse Model

In this study, we used 51 adult mice (Beijing Weilitonghuashi Experimental Animal Technology Co., Ltd. Beijing, China), approved Animal Ethics Committee of Zhengzhou University (SCXK(yu)2015-0004). The were randomly grouped in three, with each. Referring to the previous research[4,11], induced intracranial aneurysm in the. The were then anesthetized, scalp slit, the at 2.7 mm back of the former ey, 1 mm at the right side of the dline skull. stereotactic elastase was into the hole depth of 6.3 mm (35 2.5 micro-osmotic pump was subcutaneously implanted and angiotensin II was pumped with a velocity 1000 ng/kg/min at the of blood pressure elevation. The sham operation group was injected with normal saline and ation. were fed with normal drinking during recovery period.

The mark at which the injection of elastase into subcutaneal pool and with the angiotensin II pumped in as day 0, until the end of the experiment (day 21) or the death of mice. Experimental mice had an intragastric administration of c-Met antagonist PF-04217903 (10 mg/kg/d) or its solvent DMSO, which in sham operation control mice only 50 μl DMSO was perfused.

All mice were observed every day, and those that had neurological deficits (forepaw extension disability, dumping at paralyzed side, side dumping, walking disability, consciousness loss) or weight loss (>20%), were sacrificed. All other asymptomatic mice were sacrificed the after the induction of aneurysm. 5 ce were excluded during the experiment: 1 ouse in the sham-operated group died in the plications of gastric lavage; 3 mice in solvent and 1 PF04217903 treated mouse failed ver. The systolic blood pressure of all experimental mice was weekly measured by tail cuff method.

After putting all experimental mice to death, we opened their thoracic and abdominal cavities, checked if there was a major bleeding from aortic aneurysm. We used 10-15 ml containing with (100 μm) heart perfusion to cause systemic vascular relaxation, then pumped in 8% gelatin-ink mixture containing 2 m phenol blue so the visualization of the cerebral blood circulation could be observed. We dissected the brains of mice and examined the presence of intracranial aneurysms and/or subarachnoid hemorrhage (SAH). An aneurysm is defined as times of the size of the diameter of the bo projection of the vessel wall. The survival curve was drawn according to the time or the execution time of the mice.

Effect of HGF on the Expression of Inflammatory Molecules in Cultured Cells In vitro

6 g/ml c-Met antagonists were added in HAECs in the normal medium which containing human recombinant HGF (10 ng/ml) for 1 hour, and 100 ng/ml LPS was added for 3 hours. According to the TaqMan primer reverse transcription of target genes and β-actin, real-time quantitative PCR was introduced, and the genes were expressed by ΔΔCt method for quantitative determination of β-actin standard. The target genes included Intercellular adhesion molecule-1 (ICAM-1), Vascular cell adhesion molecule-1 (VCAM-1), E-selectin, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemotaxis protein-1 (MCP-1), epoxy enzyme-1 (COX-1), COX-2 and transforming growth factor-β (TGF-β). All researches of tissue culture were based on 3-times parallel experiments.

In the same way, human recombination HGF (10 ng/ml) or HGF+ PF-04217903 were added into HASMCs for pre-culture, then LPS was added for 3 hours. The detection of mRNA levels of ICAM-1, VCAM-1, E-selectin, TNF-α, IL-1β, MCP-1, COX-1, COX-2, and TGF-β were as stated previously.

Statistical Analysis

Statistical analysis was performed by Prism 6 and Stata 10. The measurement data were expressed by mean±SD, and the comparison between the two groups was expressed by one-way ANOVA and pairing LSD t-test. Categorical variables were X^2-test and survival rates were estimated using the product-limit method (Kaplan-Meier method) for survival analysis. $p<0.05$ was considered to be statistically significant.

Results

The level of HGF in the Aneurysm Cavity is Higher than that of the Femoral Artery

Among 16 patients with an intracranial aneurysm in this experiment, 13 patients were females, 3 were males, and the average age was 55.23±13.47 years old. The average size of the aneurysms was 10.48±9.13 mm, of which 3 cases (18.75%) were ruptured aneurysms. To compare with the samples collected from femoral artery (196.17±436.25) pg/ml, the samples of a cerebral aneurysm (1076.08±656.33) pg/ml were 5 [illegible] larger ($p<0.001$) in size, and the plasm[illegible] concentration was also significantly inc[illegible]ed ($p<0.05$), as shown in Figure 1.

Fig[illegible]. Comparison of HGF levels in cerebral aneurysm and f[illegible]moral artery samples. Note: the plasma HGF level of the sample from the aneurysm cavity was higher than that from the femoral artery. $^*p<0.05$.

Expression of HGF and c-Met in Intracranial Aneurysms

The samples which collected from the superficial temporal artery (STA), and ru[illegible] unruptured intracranial aneurysms, [illegible] and the receptors c-Met, with positi[illegible]stain. HGF and c-Met were located in endo[illegible] cells and smooth muscle layer (Figure 2).

C-Met Antagonists In[illegible]ased the ris[illegible] Aneurysm Rupture[illegible]Mice [illegible] Reduc[illegible] their Survival Tim[illegible]

After aneurys[illegible] w[illegible]ced, [illegible]pared with sham-ope[illegible]d cont[illegible]oup [illegible]re than 85.00% of th[illegible]e that were [illegible]giotensin II and kil[illegible]ed to have [illegible]acranial aneurysms [illegible]/or s[illegible]hnoid hemorrhage (Figure 3A). However, th[illegible]e, which were given PF0[illegible], had better [illegible]val rate than of the n[illegible]treated with the solvent (25.00% vs. 57.00%, [illegible].05) (Figure [illegible]. Moreover, the systolic blood [illegible]ure was sig[illegible]antly higher ($p<0.05$), which h[illegible]e same r[illegible]nse (Figure 3C) in the mice wit[illegible]or its solvent. The weight loss of mic[illegible]particularly significant in the first two weeks ($p<0.05$), thus there was no significant [illegible]loss observed in sham-operated control [illegible]igure 3D). For the solvent, PF04217903 had no significant effect on blood pressure and weight loss of 85.71% (12/14) of the mice that given solvent and 93.75% (15/16) of the mice that given PF0421790 had an intracranial aneurysm (Figure 3E). The occurrence of subarachnoid hemorrhage in mice treated with PF04217903 was 93.75% (15/16) which was significantly higher than 64.28% (9/14) occurrence in mice treated with the solvent ($p<0.05$) (Figure 3F). In the sham-operated control group, the survival rate was 100%, and there was no intracranial aneurysm or subarachnoid hemorrhage found in mice.

HGF Attenuated the Expression of Inflammatory Molecules in vitro in HAECs

In human arterial smooth muscle cells (HASMCs) cultured with HGF+LPS, the mRNA expression of VCAM-1 and E-selectin was lower than that cultured only with LPS (Figure 4). The inhibitory effect of HGF on VCAM-1 and E-selectin was eliminated by c-Met: the VCAM-1 and E-selectin in HASMCs with HGF+PF-665752+LPS combination remained at the same level as in the HASMCs with only LPS. In HASMCs with HGF+LPS, HGF+PF04217903+LPS and HASMCs

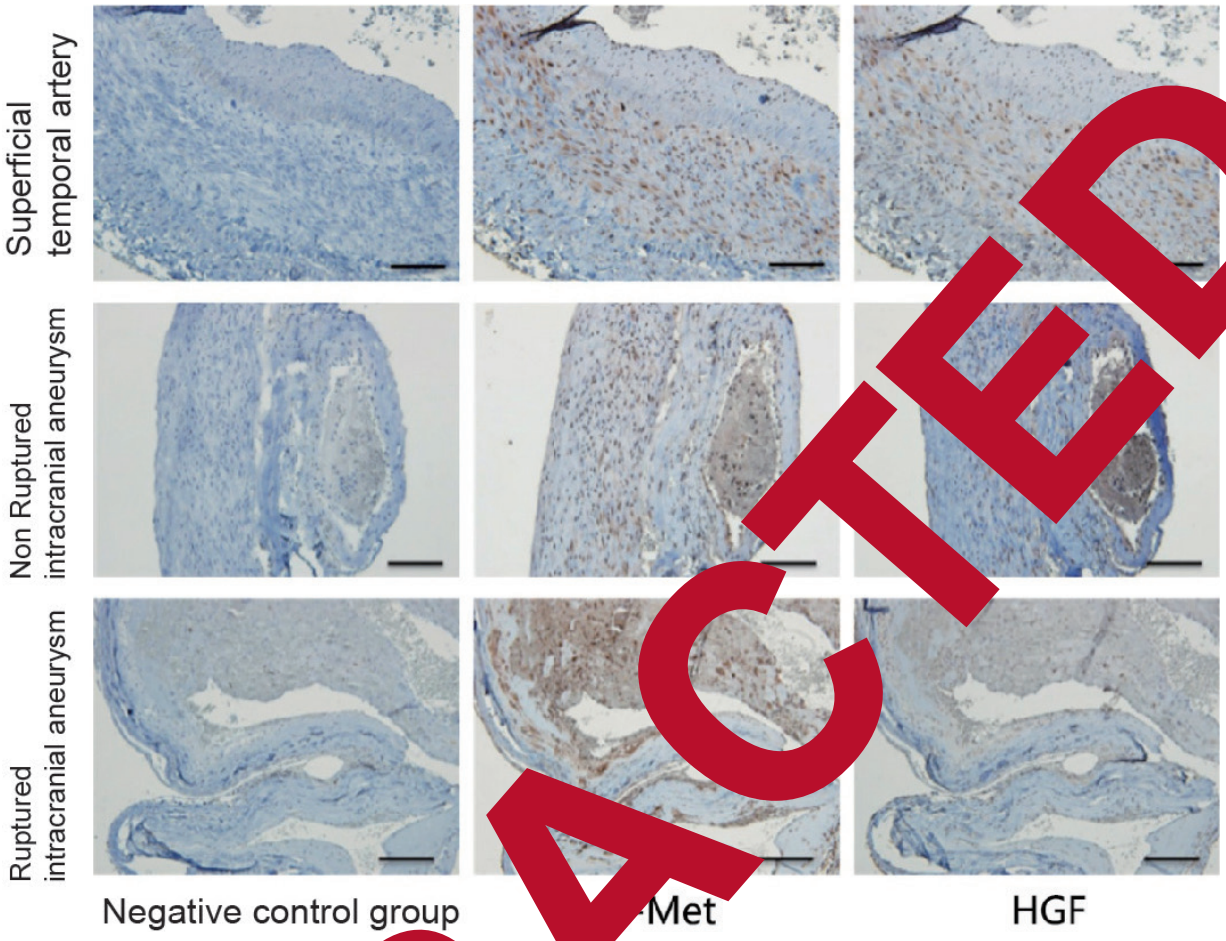

Figure 2. Expression of HGF and c- superficial oral artery and ruptured intracranial aneurysms. Note: HGF and c-Met were detected in the sa of c elium an oth base. HGF and c-Met were excluded from the negative control. The same results were ved in th maining 10 tients, with the ruler =100 μm.

with only LPS as no diffe e in the levels of TN IL-1 CP-1, COX-1, COX-2, TGF-β an CAM-1 ($p>$ In HASMCs with HGF+L HGF+PF0421 PS, HASMCs with L and th THP-1 cells, here was also no diff ce in t evels of TNF-α, IL-1β, MCP-1, CO OX TGF-β CAM-1, E-selectin and ICAM- 05).

Discussion

nflammation is considered as one of the keys the development, growth, and rupture of intra- l aneurysms[12]. The evidence suggests that the o-inflammatory and proliferation pathways are activated in endothelial cells when there is local blood flow load[13]. Subsequently, the mononuclear cells in the arterial wall penetrate, activate and release a variety of pro-inflammatory cells[14]. The final common pathway appears to involve in the release of matrix metalloproteinases and the apoptosis of vascular cell components, and then leads to aneurysm remodeling, development, and rupture[15,16].

The anti-inflammatory effects of HGF have been studied in recent years, and reported in many other investigations. In human abdominal aortic tissue cultured in vitro, exogenous HGF promotes the secretion of anti-inflammatory cytokines (IL-10) and inhibits the secretion of pro-inflammatory monocyte chemotaxis protein -1 (MCP-1). Likewise, it is found that in the macrophages derived from the bone marrow, HGF reduces the production of pro-inflammatory cytokines IL-6 followed by an increase of

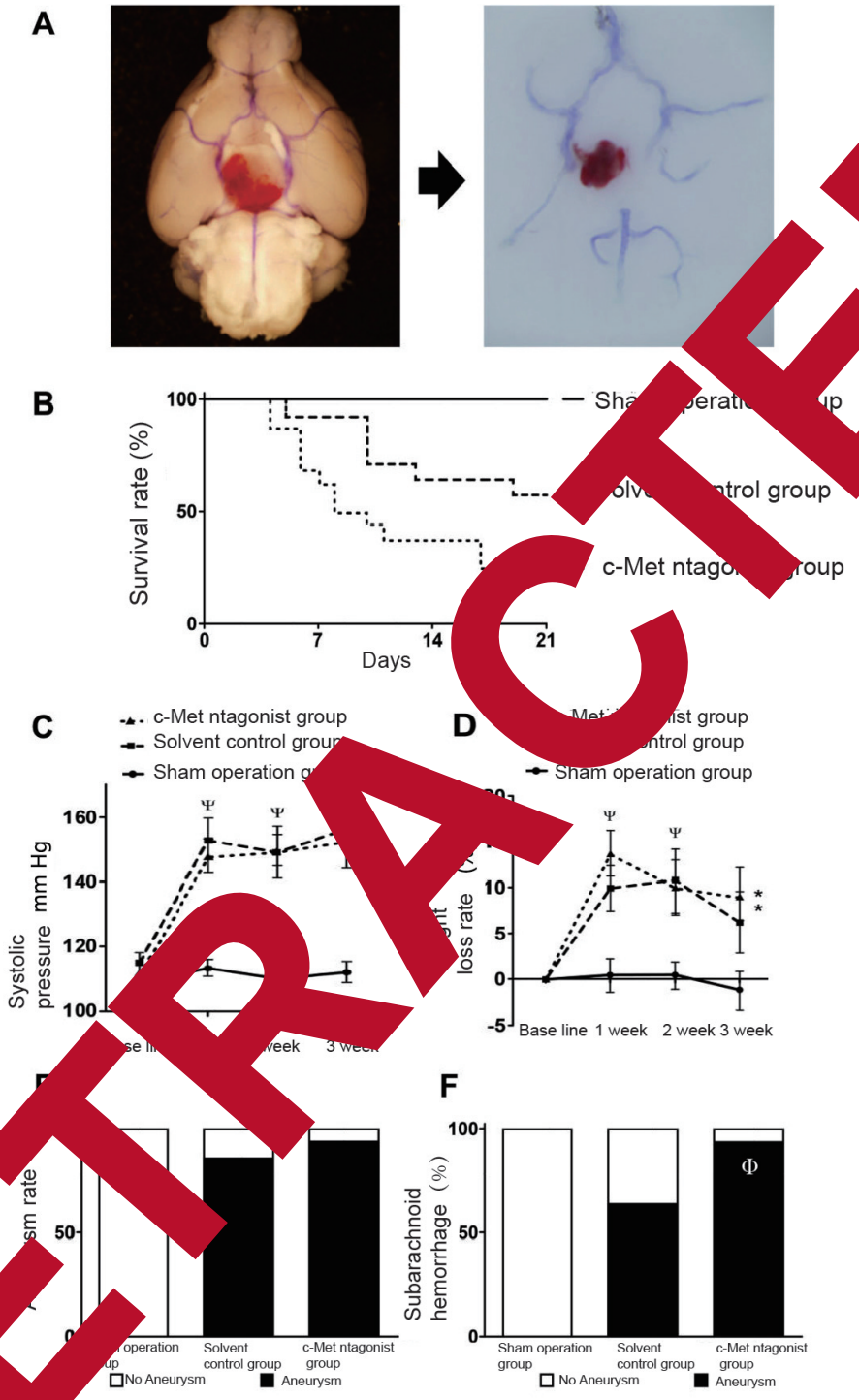

Fig. 3. Effects of c-Met antagonists on the formation and rupture of intracranial aneurysms in a mouse model. Note: ***(A)*** The ... and aneurysm of the left anterior (right) artery can be observed in the left anterior and posterior (right). ... of sham operated control group, PF-04217903 or solvent control group were induced in the aneurysm group. ... The systolic blood pressure of PF-04217903 or solvent control group was induced in sham operation group and control ... ***(D)*** The weight loss of the sham operated control group and the induced aneurysm group were treated with PF-... or solvent control group. ***(E)*** Sham operated control group, induced aneurysm group was given PF-04217903 or solv... control group of mice aneurysm formation. ***(F)*** The sham operation control group, induced aneurysm group received arachnoid PF-04217903 or solvent control group of mice under the bleeding. Compared with the sham operation group $^{*}p<0.05$; compared with the baseline psi $p<0.05$; compared with the control group reagent, $p<0.05$.

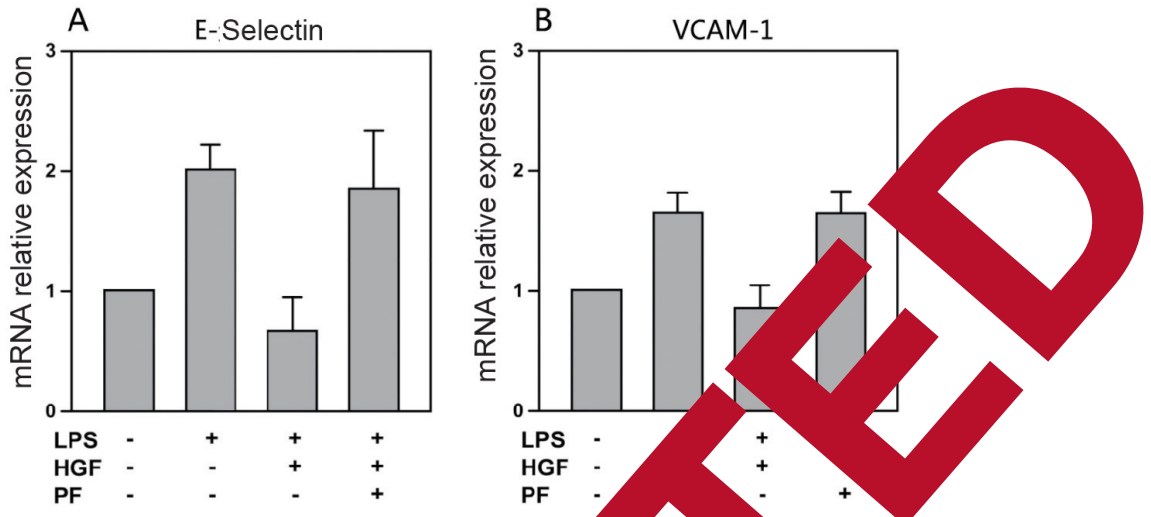

Figure 4. Changes of VCAM-1 and E-selectin. Note: the level of VCAM-1 and E- in HGF ... cultured with HAECs was lower in LPS than in HAECs medium. The effect of HGF on inflamm... tors was abo... by the addition of c-Met antagonists. (Single factor analysis of variance; p=0.010 VCAM-1, F ... 5)

anti-inflammatory factor IL-10[26]. In other findings, HGF is found to inhibit MCP-1, IL-6 and IL-1[27], which is related to the pathogenesis of intracranial aneurysms[1,2]. Also, the anti-apo... effect of HGF is also related to the intr... aneurysm. Moreover, it has also been fou... at the pathogenesis of the development and ru... of aneurysm is including apoptosis of endothe... cells and smooth muscle cells[2].

It has been found that H... otective effects in the pathogenesis ... ntracr... l aneurysms and vascular dise... When ti... occurred, HGF as a ... by the side secreti... which ... romotes the regeneration of ... s and wo... epair[17]. In the vascular s... IGF is ass... d with angiogenesis[... d re... s the function of endothelial p... enitor cells ... example, HGF reduces th... ing and oxidati... ess induced by angio... n II in ... endothelial progenitor cells[19]. At ... ellular ... l, the HGF signaling pathway prot... ell... om D... damages through its recepto... t, and ... ulates DNA repair and ... ishes ... to ... HGF also regulates other fac... that a... ciated with vascular biology and ... lammatio... In LPS induced inflammation, H... EGFR degradation by controlling ... translocation of phosphatase (e.g., SHIP2)[12]. ... ny studies[7] suggested that HGF and c-Met are ... d to arterial repair.

... coveries have been found that in the aneurysm, HGF levels may be locally elevated to avoid vascular injury. Spin et al[23] have found that ... ession of H... was found increased in an a... aneurysm... his study[24] indicated that in the ... ... anial aneurysm model, due to the c-M... agonist PF-04217903 significantly increased the rupture of aneurysm and reduced ... ival rate. HGF was up-regulated in the ... n and reduced inflammation and vascular injury.

In this study, we found that the serum concentrations of HGF in the femoral artery were higher than those in the human intracranial aneurysms. Additionally, HGF and its receptor c-Met were expressed on the walls of the ruptured and unruptured human cerebral aneurysms. These findings proved that HGF is associated with human intracranial aneurysms. Therefore, the expression of HGF in the lumen and the wall of the human intracranial aneurysm may result in blood flow loading and local cell damage caused by inflammation. Furthermore, the level of circulating HGF can also be altered in other cardiovascular diseases. Some researches[20,21] have indicated that the HGF level in hypertensive patients is higher than in the normal group. On the other hand, the level of HGF is decreased both in diabetic patients and diabetic animal models[22]. In a word, it is possible that HGF has a protective effect on vascular disease and its level can be regulated by drugs, inflammation, and metabolism.

During the experiment, we have discovered the possible mechanism of HGF in the development of LPS in vitro to prevent the rupture of intracranial aneurysms, and HGF mainly was ef-

fective in endothelial cells (in contrast to smooth muscle cells and monocytes), and diminished the expression of VCAM-1 and E-selectin. The discovery of which HGF selectively reduces the expression of adhesion molecules in endothelial cells is absolutely important. This is because the infiltration of inflammatory cells in the intracranial aneurysm is the major characteristic of the intracranial aneurysm. Aoki et al[25] proved that the formation of the intracranial aneurysm was ceased by inhibiting MCP-1 in mice. Also, VCAM-1 and E-selectin were increased in the experimental aortic aneurysm and rupture of the human brain aneurysm. The overall data of our study indicated that the decrease of the adhesion molecules (VCAM-1 and E-selectin) induced by HGF and the infiltration of inflammatory cells in the intracranial aneurysm tissue might reduce the aneurysm development, growth and related inflammatory reactions of rupture.

The number of patients in this study was relatively small. However, we observed that in some patients, the concentration of HGF in the intracranial aneurysm cavity was significantly higher than the concentration in the blood system. This finding was novel, and it was supportive e... in the view that the HGF can be locally pro...ed in aneurysm tissue. Although the c-Met ant... nists significantly affect rupture and survival ... aneurysms, further experiment... required ... demonstrate whether the for... rupture of aneurysms can be redu... by ad...istering exogenous HGF or c-Me...ptor a...

Conclusions

We proved ... HGF ... c-Met were expressed in the supe...cial tempor...ry and the plasma HGF wa...vated in the int...ial aneurysms. Then ... the mo...e model of an intracranial aneurysm, c-Met ...agonists did not affect the formati... intracranial ...urysms but significantly incre... the sub...hnoid hemorrhage and ...ed ti...vi...ate of experimental mice. Th...otective ...ions of HGF were expressed by ...ering the ...CAM-1 and E- in human aortic e...s. We found that the HGF concen...ion in the intracranial aneurysm was higher ...n that in the peripheral blood, and the HGF ... receptor c-Met were expressed on the wall of ... intracranial aneurysm. According to the experiments, we demonstrated that the inhibition of endogenous HGF signaling pathway in mice could increase the risk of intracranial aneurysm rupture and reduce the survival rate of mice. In vitro, the inhibition of HGF also attenuated the expression of inflammatory molecule... hesion molecules in cultured huma... ...othe... cells. These findings suggested a... ...el role for endogenous HGF in the pathoge... ...of human intracranial aneurysms, and this e... ...an be used in clinical therapy.

Conflict of inter...

The authors declar... conflicts ...rest.

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