Alterations in the expression of protease-activated receptor 1 and tumor necrosis factor-α in the basilar artery of rats following a subarachnoid hemorrhage

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Abstract. The present study aimed to investigate the expression of protease-activated receptor 1 (PAR1) and tumor necrosis factor (TNF)-α in a rat model of subarachnoid hemorrhage (SAH)-induced cerebral vasospasm (CVS). The rat models were established by twice injecting blood into the cisterna magna, after which the following experimental groups were established: The normal group, the SAH3d group, the SAH5d group and the SAH7d group. The rats were perfused and the basilar artery was removed for histological examination. The cross-sectional area of the basilar artery lumen was measured using computer software; and the protein expression of PAR1 and TNF-α was detected by immunohistochemistry. The cross-sectional area of the basilar artery of the rats in the SAH model groups was significantly decreased in a time-dependent manner, as compared with the normal group. The protein expression of PAR1 and TNF-α in the SAH3d, SAH5d and SAH7d groups was significantly increased over time ($P<0.05$), as compared with the normal group. CVS was detected in the basilar artery, and was associated with wall thickening and significant narrowing of the lumen, thus suggesting that the present model may be used for investigating cerebrovascular disease following SAH. The immunohistochemical analyses demonstrated that the protein expression of PAR1 and TNF-α was significantly increased in the basilar artery of the SAH model rats, and were positively correlated with the degree of CVS.

Introduction

Subarachnoid hemorrhage (SAH)-induced cerebral vasospasm (CVS) is the predominant cause of the high mortality and disability rate associated with SAH (1). Numerous factors, such as bleeding (location and volume), age, smoking, hypertension and operation time, have previously been implicated in the pathogenesis of CVS. In addition, increased levels of thrombin in catabolic processes following SAH can cause strong and lasting CVS in the cerebrovascular system of the SAH animal model (2). Thrombin and its receptor protease-activated receptor 1 (PAR1) have previously been reported to have an important role in vascular smooth muscle cell proliferation, contraction and CVS pathogenesis (3).

The most common predisposing factor for vasospasm onset following intracranial tumor resection is SAH (4). Blood accumulation in the basal cisterns is thought to be an important factor in initiating the vasospasm (5). Brain metabolism and blood flow are regulated by the trigeminal system, which seems to be a significant factor involved in cerebral vasospasm. Vasodilation and upregulation in the cerebral blood flow are induced by stimulation of the trigeminal nerve endings. It is considered that vasoactive substances, direct mechanical trauma, tumor location and tumor compression are the probable causes of vasospasm.

Tumor necrosis factor (TNF)-α has been demonstrated to affect the incidence of CVS following SAH, and is closely associated with a poor prognosis (6-8). However, the role of TNF-α in promoting the upregulation of PAR1 in cerebral vessels following SAH has rarely been reported in current literature. In order to investigate the underlying mechanism of SAH-induced CVS, the present study detected alterations in the histological characteristics of the basilar artery in a rat model of SAH-induced CVS. In addition, the expression of PAR1 and TNF-α was analyzed by immunohistochemistry.

The present study aimed to investigate the alterations in the expression of PAR1 and TNF-α in the basilar artery of rats following a subarachnoid hemorrhage.

Materials and methods

Experimental rats and grouping. A total of 24 healthy male Sprague Dawley rats (n=4/cage; weight, 280-320 g) were purchased from the Faculty of Test Animals at Central South University (Changsha, China). The rats were divided into four groups using a random number table: The normal group, the SAH3d group, the SAH5d group and the SAH7d group. Each group had *ad libitum* access to food and water, and the

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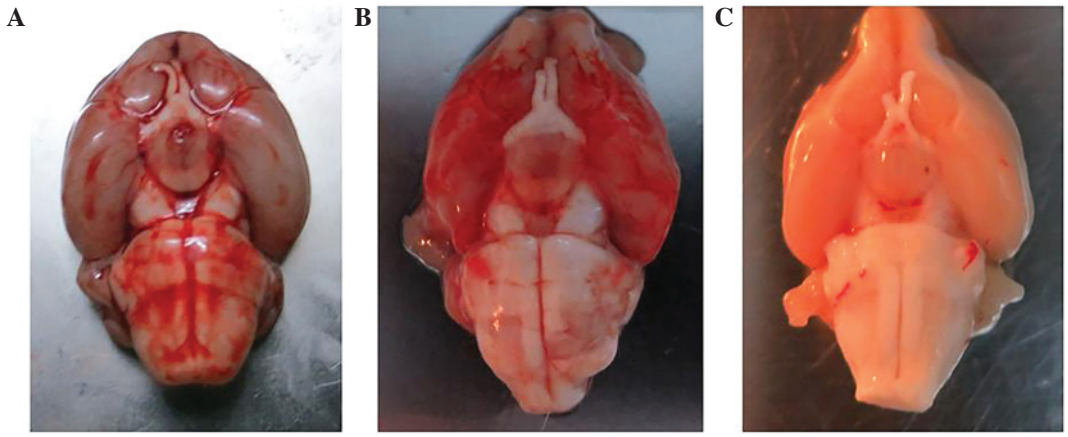

Figure 1. Underside view of the brain from the (A) SAH3d, (B) SAH5d and (C) SAH7d groups. SAH, subarachnoid hemorrhage.

rats were maintained at a temperature of 25˚C and humidity of 66%.

Reagents and instruments. The PAR1 Monoclonal Antibody kit and the TNF-α Immunohistochemical Detection kit were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The stereotaxic instrument was obtained from RWD Life Science Co., Ltd. (Shenzhen, China), and the epidural catheter was obtained from Xinghua Medical Equipment Co., Ltd. (Shanghai, China). The microtome was purchased from Leica Microsystems (Wetzlar, Germany). The DM3000 microscope was purchased from Leica Microsystems, and the IX71 inverted microscope was obtained from Olympus Corp. (Tokyo, Japan).

Preparation of the rat models of SAH. The femoral artery blood collection tube was prepared as follows: One end of a one-time epidural catheter was pulled in order to generate a thin tube (~5 cm). The outer diameter of the thin end was ~0.5 mm, and the thin end was trimmed to a 45˚ slope. The rat model of SAH was established by administering double injections of blood into the cisterna magna. Briefly, the rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (350 mg/kg body weight; Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), and were placed onto the operating table in the prone position. Skin preparation and disinfection was performed in the middle of the back of the neck, and the neck muscle tissue was separated following incision, in order to expose the atlanto-occipital fascia. Subsequently, in the supine position, skin preparation and sterilization was performed on the right lower extremity; followed by skin incision and exposure of the femoral artery. Following femoral artery anterior incision, the thin end of the femoral artery blood collection tube was inserted into the femoral artery centripetally, using a 1 ml syringe to draw 0.3 ml arterial blood. The rats were quickly placed onto the stereotaxic instrument, and the cisterna magna was punctured.

Cerebrospinal fluid (0.05 ml) was drawn prior to slow injection of 0.25 ml anticoagulated autologous arterial blood into the subarachnoid space within 1 min (anticoagulant, heparin sodium; Qilu Pharmaceutical Co., Ltd., Shandong, China). Following the injection, the puncture point was pressed with a gelatin sponge for ~30 sec, and the incision was sutured. The rats were placed into the head-down (30˚) prone position for ~30 min, in order to facilitate the distribution of blood within the basilar artery. After 48 h, blood was taken from the left femoral artery in the same manner. The SAH3d, SAH5d and SAH7d groups were maintained and observed, according to the grouping demand. The normal group did not undergo treatment.

Preparation of tissue samples. The rats in the SAH3d, SAH5d and SAH7d groups were perfused with 4% paraformaldehyde on day 3, 5 and 7, respectively, following model establishment. The rats were sacrificed by cervical dislocation, and the brains were immediately removed and fixed in 4% neutral paraformaldehyde at 4˚C for 24 h. The basilar artery was isolated, together with the brainstem (Fig. 1).

Hematoxylin and eosin (HE) staining of the basilar artery and evaluation of spasm degree. The basilar artery was stained using HE (Shanghai Jianglai Biotechnology Co., Ltd.), and the lumen cross-sectional area was measured as an indication of spasticity. Tissue slices (0.2 mm) were obtained from blood vessels of the following four breakpoints: 0.2 mm below the superior cerebellar artery; upper breakpoint of anterior inferior cerebellar artery; lower breakpoint of anterior inferior cerebellar artery; and the intersection of basilar artery and vertebral artery (9). The histological characteristics of the basilar artery were observed under a microscope by a single pathologist blinded to the treatment group, and Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the average cross-sectional area of the basilar artery lumen from each slice (10).

Immunohistochemical detection of PAR1 and TNF-α. Immunohistochemical detection of PAR1 and TNF-α protein expression in the basilar artery was conducted, according to the manufacturer's protocol. Brown coloration was indicative of positive expression. A total of five horizons per slice were randomly selected using a light microscope, in order to measure the average optical density (OD) of positive reactions, and statistical analyses were conducted using the mean OD of each slice.

Statistical analysis. All data were analyzed using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are

presented as the mean ± standard deviation. Statistical inference was evaluated using one-way analysis of variance (ANOVA) and inter-group comparison was assessed using the least significant difference (LSD) method. Heterogeneity of variance was investigated using the Brown-Forsythe method and Kruskal-Wallis nonparametric test. Pair wise comparisons between groups were evaluated using Tamhane's T2 method. Correlation analysis between two groups was conducted using Pearson correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

General condition and neurological score of the SAH rat models. After successfully modeling for 24 h, the rats exhibited listlessness, a loss of appetite and poor self-cleaning behavior. Neurological scores were given, according to the four point method of Endo *et al* (11): 1 point corresponded to no neurological deficit; 2 points corresponded to mild neurological deficit (lethargy, activity reduction); 3 points was indicative of a moderate nerve deficit (limb weakness, lameness); and 4 points corresponded to a severe neurological deficit (circling movement or difficulty in walking). The neurological scores were as follows: In the SAH3d group, two rats were given 2 points (33.3%) and four rats were given 3 points (66.7%); in the SAH5d group, three rats were given 1 point (50%) and three rats were given 2 points (50%); and in the SAH7d group, four rats were given 1 point (66.7%) and two rats were given 2 points (33.3%). The rats in the normal group were all given 1 point. Following SAH modeling, the nerve dysfunction of the rats presented remission, which may have been associated with abundant collateral circulation in the rats, as described in a previous study (9).

Histological examination. Rats were perfused using 4% paraformaldehyde in order to isolate tissue samples. The SAH model rats exhibited a diffuse SAH, predominantly located around the basilar artery and the cisterna ambiens (Fig. 2). Basilar artery HE staining demonstrated that there was a large number of erythrocytes and inflammatory cells surrounding the basilar artery, and the inflammatory cells were predominantly neutrophils. In addition, the majority of erythrocytes had a complete morphology and only a few had disintegrated. The basilar artery lumen was at a maximum size in the normal group, and appeared rounded and oval-shaped, with a thin wall and smooth intima without wrinkles. Conversely, the basilar artery lumen of the SAH rats was narrow, with a thickened wall and wrinkled intima. The SAH7d group rats exhibited the most severe histological characteristics, followed by the SAH3d group, whereas basilar artery spasms in the SAH5d group had eased (Fig. 3).

Analysis of the cross-sectional area of the basilar artery between the groups. ANOVA demonstrated that the cross-sectional area of the basilar artery among the four groups was significantly different ($P<0.05$). LSD comparisons between two groups demonstrated that the cross-sectional area of the basilar artery was significantly decreased in the SAH3d, SAH5d and SAH7d groups, as compared with the normal group ($7.3438\pm0.0612\times10^{-2}$ mm^2; $P<0.01$). In addition, the cross-sectional area of the basilar artery lumen of the SAH3d rats (3.6003 ± 0.1034x10-2 mm^2) was significantly smaller, as compared with the SAH5d group rats ($3.9195\pm0.0481\times10^{-2}$ mm^2; $P<0.05$). Furthermore, the cross-sectional area of the basilar artery lumen of the SAH3d rats was significantly larger, as compared with the SAH7d group rats ($2.2945\pm0.0995\times10^{-2}$ mm^2; $P<0.05$), and the cross-sectional area of the basilar artery lumen of the SAH5d group rats was significantly larger, as compared with the SAH7d group rats (data not shown).

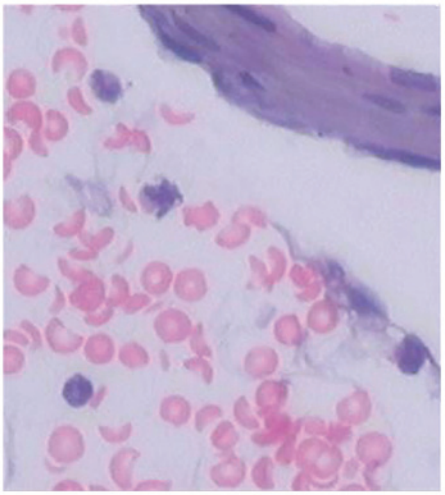

Figure 2. SAH model rats exhibited a diffuse SAH, in which red blood cells and inflammatory cells surrounded the basilar artery (hematoxylin and eosin staining; magnification, x400). SAH, subarachnoid hemorrhage.

PAR1 and TNF-α immunohistochemical analysis. PAR1 and TNF-α protein expression in the basilar artery of the SAH3d, SAH5d and SAH7d group rats increased in a time-dependent manner, as demonstrated by immunohistochemistry. The normal group was negative for the expression of both proteins (Fig. 4).

Differences in the average OD of PAR1 between the four groups. The data were tested using the Brown-Forsythe method and Kruskal-Wallis nonparametric test analysis (variance nonhomogeneity in four groups, $P<0.05$); and the difference among the four groups was statistically significant ($P<0.01$). In addition, Tamhane's T2 comparisons between two groups demonstrated that the mean OD of PAR1 in the basilar artery of the SAH3d (0.1180 ± 0.0042), SAH5d (0.2081 ± 0.0016) and SAH7d (0.2382 ± 0.0103) groups was significantly increased, as compared with the OD of PAR1 in the basilar artery of the normal group (0.0851 ± 0.0035; $P<0.01$). Furthermore, the mean OD of PAR1 in the SAH5d and SAH7d groups was significantly increased, as compared with the SAH3d group rats ($P<0.01$). There was no significant difference in the mean PAR1 OD between the SAH5d and SAH7d groups ($P>0.05$; data not shown).

Differences in the average OD of TNF-α between the four groups. ANOVA demonstrated that the average OD of TNF-α between the four groups was significantly different ($P<0.05$). LSD comparison between two groups demonstrated that the mean OD of TNF-α in the basilar artery of the SAH3d (0.0872 ± 0.0024), SAH5d (0.1170 ± 0.0019) and SAH7d (0.1587 ± 0.0016) groups was significantly increased, as

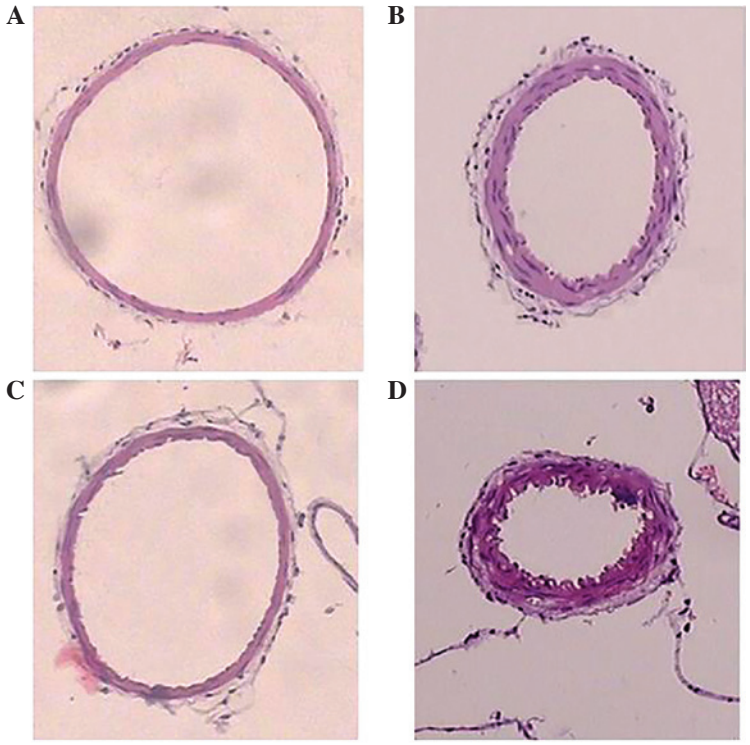

Figure 3. Hematoxylin and eosin staining of the basilar artery from the (A) normal, (B) SAH3d, (C) SAH5d and (D) SAH7d groups (magnification, x100). SAH, subarachnoid hemorrhage.

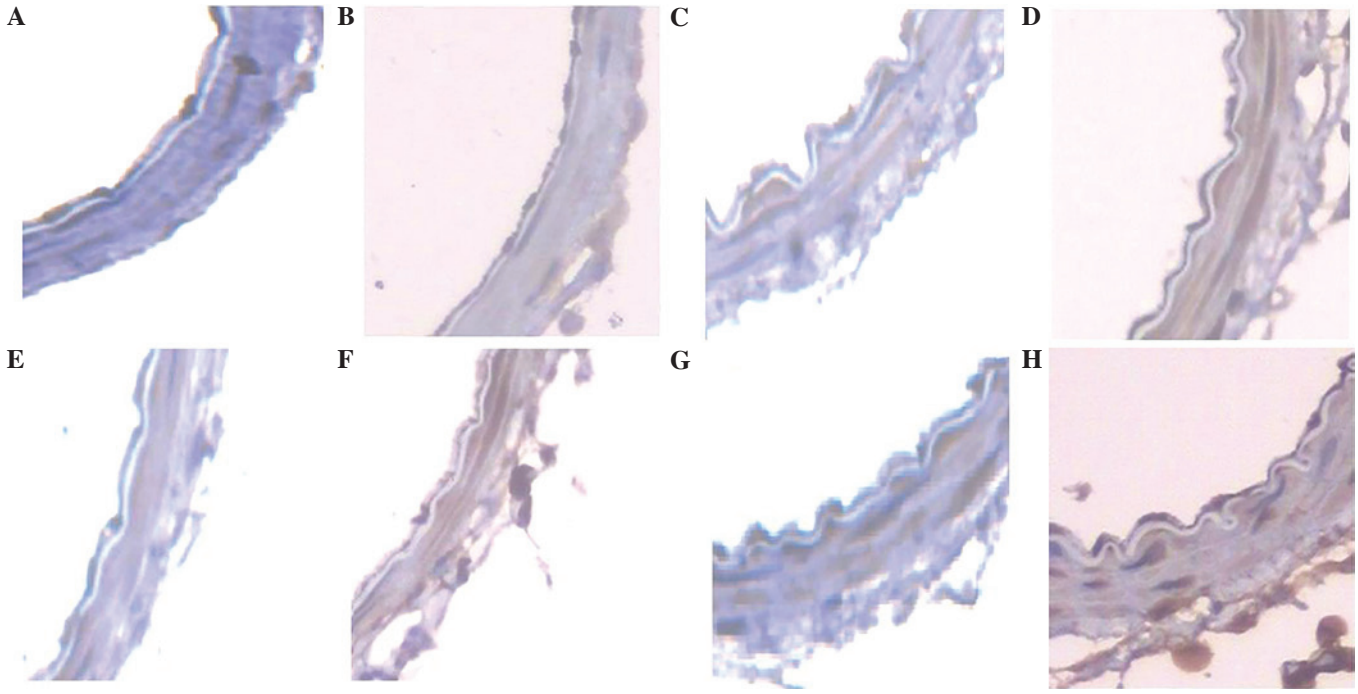

Figure 4. Immunohistochemical detection of (A) PAR1 and (B) TNF-α in the normal group basilar artery and (C) PAR1 and (D) TNF-α in the SAH3d group basilar artery (magnification, x400). Immunohistochemical detection of (E) PAR1 and (F) TNF-α in the SAH5d group basilar artery; and (G) PAR1 and (H) TNF-α in the SAH7d group basilar artery (magnification, x400). PAR1, protease-activated receptor 1; TNF-α, tumor necrosis factor-α; SAH, subarachnoid hemorrhage.

compared with the OD of TNF-α in the basilar artery of the normal group (0.0383±0.0014; P<0.01). In addition, there was a significant difference in the OD of TNF-α between the SAH3d and SAH5d group rats (P<0.01), and there was a significant difference between the SAH3d and SAH7d group rats (P<0.01). Furthermore, there was a significant difference between the SAH5d and SAH7d group rats (P<0.01; data not shown).

Correlation analysis of PAR1 expression, TNF-α expression and the cross-sectional area of the basilar artery lumen.

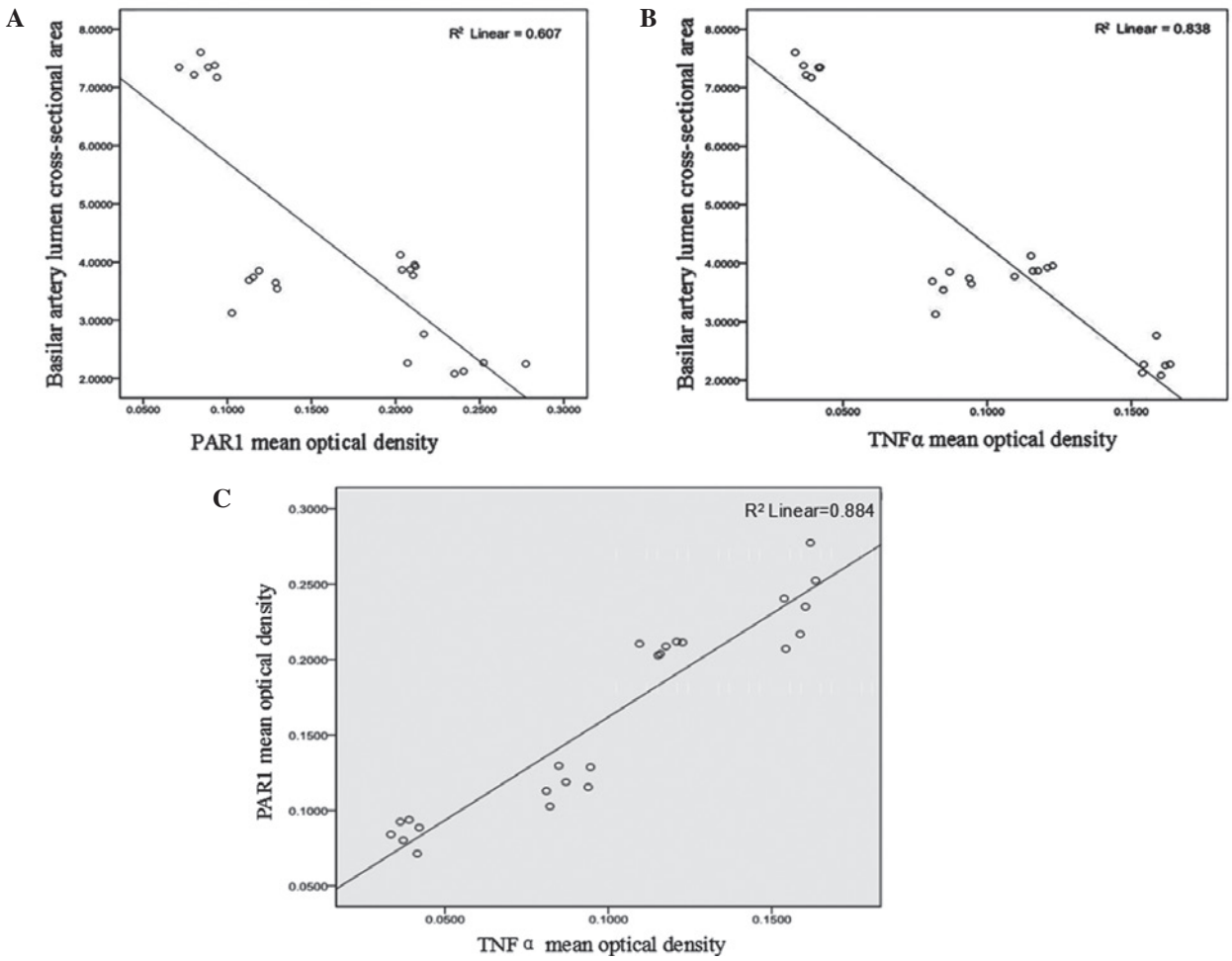

Figure 5. Scatter graphs representing (A) the average optical density of PAR1 against the cross-sectional area of the basilar artery, (B) the average optical density of TNF-α against the cross-sectional area of the basilar artery, and (C) the mean optical density of PAR1 against the average optical density of TNF-α following SAH in the various groups. PAR1, protease-activated receptor 1; TNF-α, tumor necrosis factor-α; SAH, subarachnoid hemorrhage.

Pearson correlation analysis demonstrated that the correlation coefficient (*r*) between the mean OD of PAR1 and the cross-sectional area of the basilar artery following SAH was -0.779 (P<0.01; Fig. 5A); thus suggesting that there was a negative correlation between these factors. In addition, the mean OD of TNF-α and the cross-sectional area of the basilar artery following SAH were negatively correlated (*r*=-0.915; P<0.01; Fig. 5B).

Correlation between mean OD of PAR1 and TNF-α. Pearson correlation analysis demonstrated that the average ODs of PAR1 and TNF-α were positively correlated (r=0.940; P<0.01; Fig. 5C).

Discussion

Numerous factors have previously been implicated in the pathophysiology of CVS (12). An intracranial hemorrhage may initiate the production of large amounts of thrombin, which is detected by PAR1 in vascular smooth muscle. In addition, the expression levels of TNF-α have previously been shown to be upregulated during a cerebral hemorrhage (13). The present study aimed to investigate the association between PAR1, TNF-α and CVS in a rat model of SAH.

Hirano (3) reported that upregulation of PAR1 in vascular smooth muscle following SAH may be associated with the proliferation and contraction of vascular smooth muscle cells. Kai *et al* (14) demonstrated that PAR1 was able to regulate thrombin-induced convulsions involved in the pathogenesis of CVS, and increased cerebrovascular reactivity was associated with the upregulation of PAR1. In addition, intrathecal injection of a PAR1 antagonist was able to attenuate the extent of CVS and the upregulation of PAR1 expression levels in the basilar artery of a rabbit double hemorrhage SAH model, in a dose-dependent manner.

Inflammatory factors have an important role in the formation of SAH-induced CVS. TNF-α is a classic inflammatory cytokine, which is able to promote the vascular oxidative stress response. The aggregation of various other inflammatory mediators involved in the pathogenesis of CVS was shown to be dependent on the expression levels of TNF-α in TNF-α transduction system knockout mice (15). In addition, the basilar artery spasm was attenuated by blocking TNF-α transduction in a rabbit model of SAH (6,15-17). Mbebi *et al* (18) demonstrated that TNF-α was able to promote the mRNA and protein expression of PAR1 in myoblast cells *in vitro*. Chieng-Yane *et al* (19) reported that the application of PAR1 antagonists following angioplasty was able to protect against

vascular restenosis by reducing the expression levels and migration of TNF-α and matrix metalloproteinase 7, and by promoting the amplification of vascular smooth muscle cells. Furthermore, Liu and Tang (20) detected TNF-α-induced renal vascular spasm in models of severe pancreatitis and in rat kidney damage experiments.

The present study detected neurological impairments in the rats following SAH modeling, and erythrocytes and inflammatory cells were shown to be highly concentrated in the subarachnoid space, which was associated with basilar artery spasm. The average OD of PAR1 was calculated in order to assess the protein expression of PAR1 in the SAH rats. Notably, protein expression of PAR1 was significantly upregulated in the SAH rats in a time-dependent manner, which was consistent with a previous study (21). In addition, there was a negative correlation (r=-0.779; P<0.01) between the protein expression of PAR1 and the cross-sectional area of the basilar artery lumen, thus suggesting that PAR1 may have an important role in the pathological mechanism underlying CVS.

Positive expression of TNF-α was detected in the basilar artery of the SAH rats, and the expression was significantly increased over time (P<0.01). Furthermore, a correlation analysis demonstrated that the protein expression of TNF-α was negatively correlated (r=-0.915; P<0.01) with the cross-sectional area of the basilar artery lumen, thus suggesting that TNF-α may be associated with SAH-induced CVS. TNF-α was previously shown to promote dose-dependent vasoconstriction in the rat basilar artery (6), and TNF-α in the cerebrospinal fluid is used as a sensitive marker of the severity of CVS (8), thus suggesting that it may have an important role in the development and poor prognosis of SAH-induced CVS (7).

The results of the present study suggested that there was a strong positive correlation (r=0.940; P<0.01) between the positive expression levels of TNF-α and PAR1, while there was a negative correlation between the average OD and the upregulation of TNF-α and PAR1 expression levels. Furthermore, the cross-sectional area of the rat basilar artery, which was an indication of the degree of vascular spasm, was negatively correlated with the upregulation of these factors, thus suggesting that the expression of PAR1 and TNF-α following SAH may influence the severity of CVS. Notably, thrombin may also be associated with the incidence and development of CVS, since it is able to initiate the upregulation of PAR1 and induce inflammatory responses. Future studies should investigate whether inhibition of PAR1 and TNF-α may attenuate SAH-induced CVS.

Acknowledgements

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