Expression of HGF and VEGF in the cerebral tissue of adult rats with chronic hydrocephalus after subarachnoid hemorrhage

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Received February 14, 2011; Accepted May 27, 2011

DOI: 10.3892/mmr.2011.500

Abstract. The hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) are important cytokines with modulatory actions in the nervous system. The present study aimed to investigate the role and expression of HGF and VEGF in the cerebral tissue of adult rats with chronic hydrocephalus after subarachnoid hemorrhage. Adult female Wistar rats were randomly divided into 4 groups: a control group (n=20) and 3 experimental subgroups (n=60). Subarachnoid hemorrhage was induced by the injection of 0.4 ml of non-heparinized autologous arterial blood into the cisterna magna of experimental animals on day 0 with a second injection 2 days later. The rats were sacrificed within 24 h of magnetic resonance imaging (MRI) examination at 2, 4, or 6 weeks. The excised brains were studied by RT-PCR, immunohistochemical and Western blot analyses as we examined HGF and VEGF mRNA and protein expression. Chronic hydrocephalus was induced in 21 rats after subarachnoid hemorrhage. After 2 weeks, the expression of HGF and VEGF in the cerebral tissue was significantly increased in the experimental group compared to the controls, especially in periventricular white matter. Our results indicate that HGF and VEGF participate in the pathological injury and repair of cerebral tissue in rats with chronic hydrocephalus after subarachnoid hemorrhage.

Introduction

Growth factors, such as the hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) play pivotal roles in a variety of normal and pathological processes, including tissue repair (1,2), embryonic development (3), tumorigenesis and neurodegenerative disorders (4). In the nervous system they are important cytokines with modulatory actions. VEGF is expressed in endothelial cells. It increases peripheral oxygen delivery by promoting angiogenesis, which involves endothelial cell migration, proliferation and differentiation, as well as proteolysis of the extracellular matrix (5). The expression of VEGF increases dramatically after acute hypoxia (6).

Recent attention has focused on the important roles of HGF in the regulation of cell proliferation and motility, embryonic development, morphogenesis, wound healing and angiogenesis. In an earlier study, it was observed that HGF and its receptor c-Met played an important role in the formation and progression of human brain astrocytoma by promoting tumor proliferation and microvascularization. HGF and c-Met expression were closely related to patient prognosis (7). In another study, it was noted that reducing the expression of c-Met with antisense oligodeoxynucleotides potentiated the cytotoxic effect of radiation on human U251 gliomas *in vitro* and *in vivo* (8). Similarly, antisense oligodeoxynucleotide and ribonucleic acid (RNA) interference to lower c-Met expression inhibited U251 glioma growth though apoptosis and increased sensitivity to paclitaxel (9-12). Recently, we observed that stabilization of HGF messenger ribonucleic acid (mRNA) by hypoxia was mediated, at least in part, by hypoxia-inducible factor 1, and HGF may be a factor in radioresistance (13,14).

It is known that VEGF is highly expressed in rat brains following reversible global cerebral ischemia produced by cardiac arrest and resuscitation (15). HGF is also highly expressed in rat brains after ischemia (16). Various researchers, using magnetic resonance (MR) spectroscopy, have found compromised energy metabolism, indicating that cerebral ischemia occurs during experimental hydrocephalus (17). Other researchers have noted VEGF to be highly expressed in the cerebrospinal fluid (CSF) of premature infants with posthemorrhagic hydrocephalus (18). Together, these results led us to question whether HGF is also involved in the pathophysiological processes of chronic hydrocephalus folowing subarachnoid hemorrhage. Therefore, this study investigated the expression of HGF and VEGF in different cerebral tissue sites of adult rats following subarachnoid hemorrhage to evaluate the role of these cytokines in chronic hydrocephalus.

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Key words: hepatocyte growth factor, vascular endothelial growth factor, hydrocephalus, subarachnoid hemorrhage

Materials and methods

Experimental animals and the model of chronic hydrocephalus after subarachnoid hemorrhage. Adult female Sprague-Dawley rats (n=80; 250 to 280 g) were raised and cared for according to the Guide for the Care and Use of Laboratory Animals. The committee for experimental animals of Wuhan University School of Medicine approved all surgical procedures. The rats were randomly divided into experimental (n=60) and control groups (n=20). The 60 rats in the experimental group were randomized into 3 subgroups, A, B and C. Subarachnoid hemorrhage was induced in the experimental animals by injecting 0.4 ml of non-heparinized autologous arterial blood into the cisterna magna on day 0, with a second injection 2 days later, as described previously (19,20). The same volume of saline was injected into the cisterna magna of rats in the control group.

MRI was performed on the experimental rats at 2 weeks (subgroup A), 4 weeks (subgroup B) and 6 weeks (subgroup C). T_2-weighted MRI (T_2WI), diffusion-weighted imaging (DWI), and continuous 1-mm thick coronal section images of the rat brain were obtained. The size of the lateral ventricle in the third ventricle plane was determined from these; lateral ventricle index was calculated (lateral ventricle index = maximum diameter of lateral ventricle/maximum diameter of the brain at that tissue level). MRI was performed at 2 weeks to obtain T_2WI and DWI for the control group. All rats were sacrificed within 24 h of the MRIs, and the brains were excised quickly for hematoxylin and eosin (HE) staining and examination.

Total RNA isolation and reverse-transcriptase polymerase chain reaction. The frozen tissues were homogenized in TRIzol reagent (Gibco, Grand Island, NY, USA), and 1 μg of total RNA from each sample was reverse-transcribed in 20 μl of a reaction mixture containing 2.5 units of M-MLV reverse transcriptase (Gibco) and 50 pM random hexanucleotide at 42˚C for 60 min. RNA integrity and the success of the reverse-transcriptase reaction were monitored by polymerase chain reaction (PCR) amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. For negative control samples, the reverse-transcriptase enzyme was omitted from the complement DNA (cDNA) synthesis. The following primers were used (at 57˚C annealing temperature): For HGF (168 bp) (sense, 5'-CTGGTTCCCCTTCAATAGCA-3' and antisense, 5'-CTCC AGGGCTGACATTTGAT-3') (21). For VEGF (350 bp) (sense, 5'-ATGAACTTTCTGCTGTCTTGGGT-3' and antisense, 5'-TGGCCTTGGTGAGGTTTGATCC-3') (22). For GAPDH (502 bp) (sense, 5'-ATCTTCCAGGAGCGAGATCC-3' and antisense, 5'-ACCACTGACACGTTGGCAGT-3') (21).

Amplified PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and photographed under UV light. The identity of each PCR product was confirmed by sequencing and was observed to be identical with the mRNA sequence of each gene as deposited in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/Entrez). Semiquantitative PCR was performed on the samples to assess differential expression of HGF and VEGF mRNA in the cerebral tissue from different sites in the rat brains. The optimum number of PCR cycles for each cDNA species was determined by plotting the PCR product yield of different cycles on a semilogarithmic graph. The number of cycles yielding exponential amplification was chosen for the final amplification. For quantification of the PCR products of all samples, the expected bands were analyzed using densitometry, and data were expressed as the ratio of HGF or VEGF cDNA to the corresponding GAPDH cDNA.

Immunohistochemical and Western blot analyses. Immunohistochemical staining was performed using an avidin-biotin-peroxidase technique. For this, tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide in methanol for 10 min to quench endogenous peroxidase activity. After washing in 10 mM phosphate-buffered saline solution (pH 7.4), sections were incubated with 10% normal goat serum to block non-specific binding. Sections were then incubated overnight at 4˚C in a 1:20 dilution of anti-HGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a 1:50 dilution of anti-VEGF (Santa Cruz Biotechnology) primary polyclonal rabbit antibodies. After washing in a phosphate-buffered saline solution, sections were treated with biotinylated goat anti-rabbit IgG and subsequently with avidin-biotin-peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA). To visualize the peroxidase activity, 0.02% diaminobenzidine hydrochloride containing 0.03% hydrogen peroxide was used as a chromogen. Negative immunohistochemical control procedures included omission of the primary antibodies and replacement of the primary antibodies with normal rabbit IgG in appropriate concentrations.

For Western blot analysis, samples frozen in liquid nitrogen were crushed and vigorously vortexed in a buffer solution containing 50 mM Tris hydrochloride (pH 8.0), 150 mM sodium chloride, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Extracted protein (50 μg) was resuspended in sodium dodecyl sulfate sample buffer and boiled for 5 min. Equal amounts of total protein were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon, a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). The blots were reacted with anti-HGF or anti-VEGF antibody in a Tris-buffered saline solution [20 mM Tris-hydrochloride (pH 8.0), 150 mM sodium chloride, 0.05% Triton X-100, and 5% skim milk] for 2 h at 4˚C, and were then reacted with a biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories) in phosphate-buffered saline solution for 1 h. After several rinses with Tris-buffered saline, the membranes were incubated with the avidin-biotin-peroxidase complex. Antibody reactions were detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Chalfont St. Giles, UK), followed by chemiluminescence detection on X-ray film. As an internal control, β-actin expression was analyzed in parallel blots using β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). We quantified the intensity of detected bands with Scion Image Beta 4.0.2 software (Scion Corp., Frederick, MD, USA). Relative intensities of HGF and VEGF signals were obtained by dividing the intensities of the HGF and VEGF signals by the intensities of the β-actin signals.

Statistical analyses. The software used for statistical analyses was SPSS 10.0 for Windows. Data are presented as

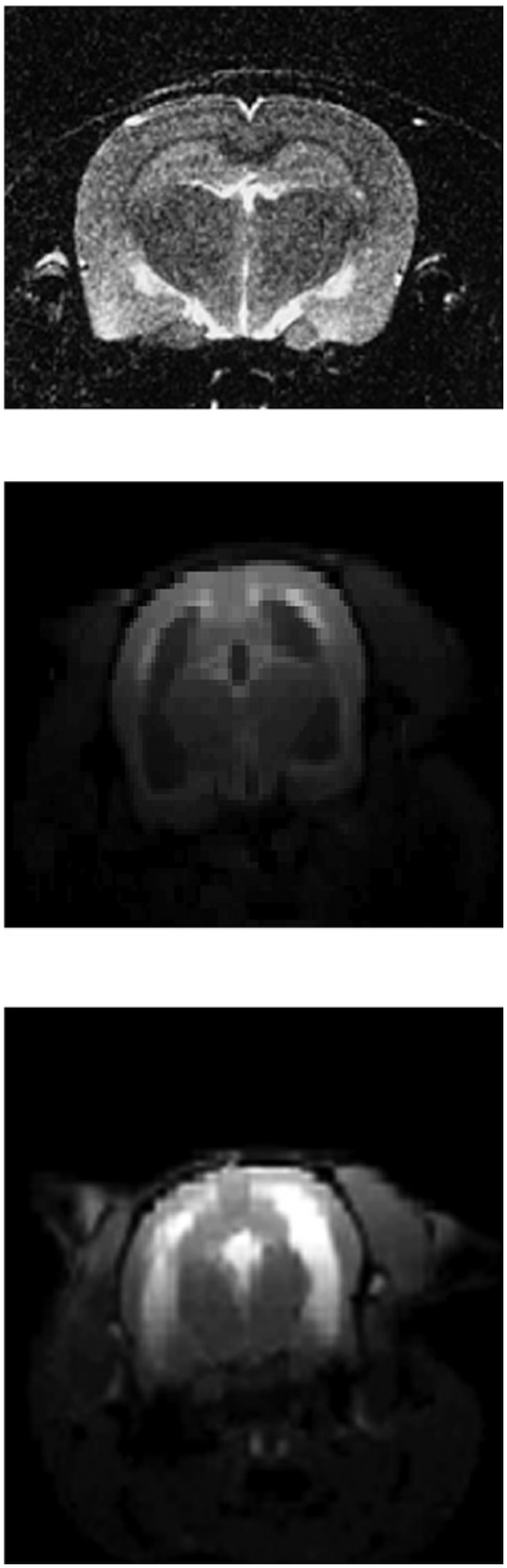

Fig. 1. MRIs of cerebral tissue of rats with chronic hydrocephalus. (A) T_2-weighted brain MRI of rats in the control group showing no ventricular system dilation. (B) Diffusion-weighted brain MRIs of the rats in the experimental group showing ventricular dilatation and periventricular white matter edema in varying degrees. (C) T_2-weighted brain MRI of rats in the experimental group with ventricular dilatation and periventricular white matter edema in varying degrees.

Table I: Comparison of lateral ventricular sizes between the control and experimental groups.

Group	n	Ventricular size
Control	17	0.004±0.001
Experimental	21	
A	8	0.435±0.128
B	7	0.558±0.145
C	6	0.636±0.245

the means ± SD. The statistical significance of differences between groups was evaluated using the t-test, and the level of significance was set at $P<0.05$.

Results

MRI images, general specimens and HE staining of cerebral tissue of rats with chronic hydrocephalus. In the control group, 1 rat died from anesthesia, 2 died from surgical trauma, and 17 rats survived. In the first few days following control surgery, the rats displayed reduced food intake and activity, with significant weight loss, but recovered within 5 to 7 days. Their MRIs exhibited no ventricular dilatation (Fig. 1A).

In the experimental groups, 3 rats died from anesthesia, 3 died from surgical trauma, and 4 died within 2 weeks of surgery, but prior to MRI. Fifty rats (group A, n=17; group B, n=17; group C, n=16) completed the entire course of the experiment. Initially after surgery the animals showed decreased dietary intake, significant weight loss, and reduced activity and aggression. After approximately 1 week, some rats exhibited gait instability, trembling, and some even exhibited convulsions. Within 2 weeks, the weight of most rats began to increase. A few rats exhibited no, or only slow, increases in weight associated with ongoing impairment of activity. In 21 of the 50 surviving rats (group A, n=8; group B; n=7; group C, n=6), MRI revealed ventricular dilatation and periventricular white matter edema in varying degrees (Fig. 1B and C). Comparisons of the size of ventricles showed significant ($P<0.05$) differences between the experimental animals and the controls as well as between the subgroups (Table I).

Control animals exhibited no expansion of the ventricular system. Experimental animals displayed significant expansion in the body and anterior horn of the lateral ventricle, midbrain aqueduct, and fourth ventricle after 2 weeks (Fig. 2A). Compared to the HE-stained brains in the control group (Fig. 2B), HE-stained rat brains in the experimental group showed a thin cortex and a compressed and deformed hippocampus (Fig. 2C). Pathological analysis of the experimental animals after 2 weeks revealed that the ventricular system was significantly expanded, most significantly in the anterior horn of the lateral ventricle (Fig. 2A and 2C). The dorsal cortex was compressed, nerve cells were compacted, and the space surrounding the cells in the white matter was enlarged due to edema. After 3 to 4 weeks, the ventricular system in experimental animals was further expanded. In the periventricular white matter, especially in the corpus callosum, fiber was distorted, shrunken and torn, with

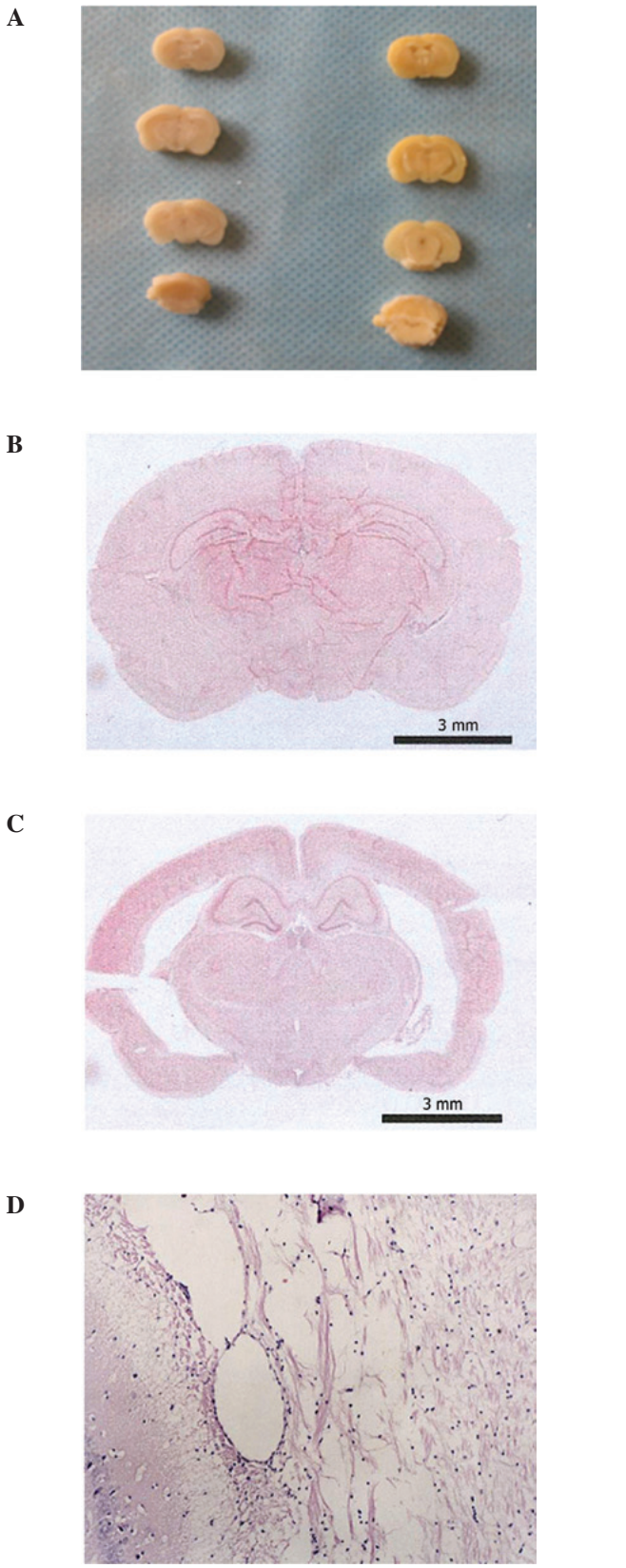

Fig. 2. General specimens and HE staining of cerebral tissue of rats with chronic hydrocephalus. (A) Consecutive coronal sections of rat brain. Left, Control group specimens showing no expansion of the ventricular system. Right, Experimental group with significantly expanded lateral ventricle anterior horn, body, midbrain aqueduct and fourth ventricle. (B) HE staining of rat brain of the control group. (C) HE staining of rat brain of the experimental group, showing a thin cortex and a compressed and deformed hippocampus. (D) Photomicrograph of the white matter area in a rat brain with severe hydrocephalus, showing the distortion, avulsion and compaction of corpus callosum fibers (HE staining, magnification x100).

widened gaps between fibers (Fig. 2D). Cortical neurons were very dense compared to the controls, but the absolute number of neurons was not significantly reduced. The hippocampus was deformed by compression.

mRNA expression of HGF and VEGF. Reverse-transcriptase PCR assays were performed to detect the presence of HGF and VEGF mRNA in different sites in the cerebral tissue of adult rats with chronic hydrocephalus after subarachnoid hemorrhage. As shown in Fig. 3A, PCR products of the expected sizes for HGF (168 bp) and VEGF (350 bp) were detected in the cerebral cortex, hippocampus, midbrain and white matter of the rat brains. These showed 100% homology with published sequences. These results indicate that the cerebral tissue of different brain sites, including the cerebral cortex, hippocampus, midbrain and white matter, expresses HGF and VEGF mRNA in adult rats with chronic hydrocephalus after subarachnoid hemorrhage. The relative abundances of HGF and VEGF genes with respect to GAPDH were significantly increased in the cerebral cortex, hippocampus, midbrain and white matter of the experimental groups compared to the control animals. The expression of HGF and VEGF mRNA in the experimental groups was significantly increased after 2 weeks and the expression of HGF and VEGF mRNA in the white matter of the experimental groups was significantly increased (Fig. 3B, $P<0.05$).

Immunohistochemical and Western blot analysis for HGF and VEGF protein. The expression of HGF and VEGF in the cerebral cortex, hippocampus, midbrain and white matter was examined at 4 weeks in group B rats using immunohistochemistry. Positive staining was mostly cytoplasmic, homogeneous and brown (Fig. 4). Western blot analysis of the cerebral tissue of different sites in the control group and the experimental rats with chronic hydrocephalus revealed that the expression of HGF and VEGF proteins was significantly increased in the cerebral cortex, hippocampus, midbrain and white matter of the experimental groups compared to the control group (Fig. 5, $P<0.05$).

Discussion

Chronic hydrocephalus in humans is defined as clinically and radiographically demonstrated hydrocephalus lasting 2 weeks or longer following the original hemorrhage, and which requires shunting. There is a high clinical correlation found between chronic hydrocephalus and the female gender, distal posterior circulation location of the ruptured aneurysm, increasing age, more extensive bleeding, and Hunt and Hess and Fisher grades at the time of hospital admission (23-25). Almost 46% of the patients who presented with intraventricular hemorrhage required placement of a ventriculoperitoneal (VP) shunt (25). Various researchers have observed that chronic hydrocephalus is relatively common (19%) in patients with anterior communicating artery aneurysms and is present in the majority of patients (53%) with an aneurysm of the vertebrobasilar system (26).

In the present study, subarachnoid hemorrhage was induced in adult female Sprague-Dawley rats by injection of 0.4 ml of non-heparinized autologous arterial blood into the

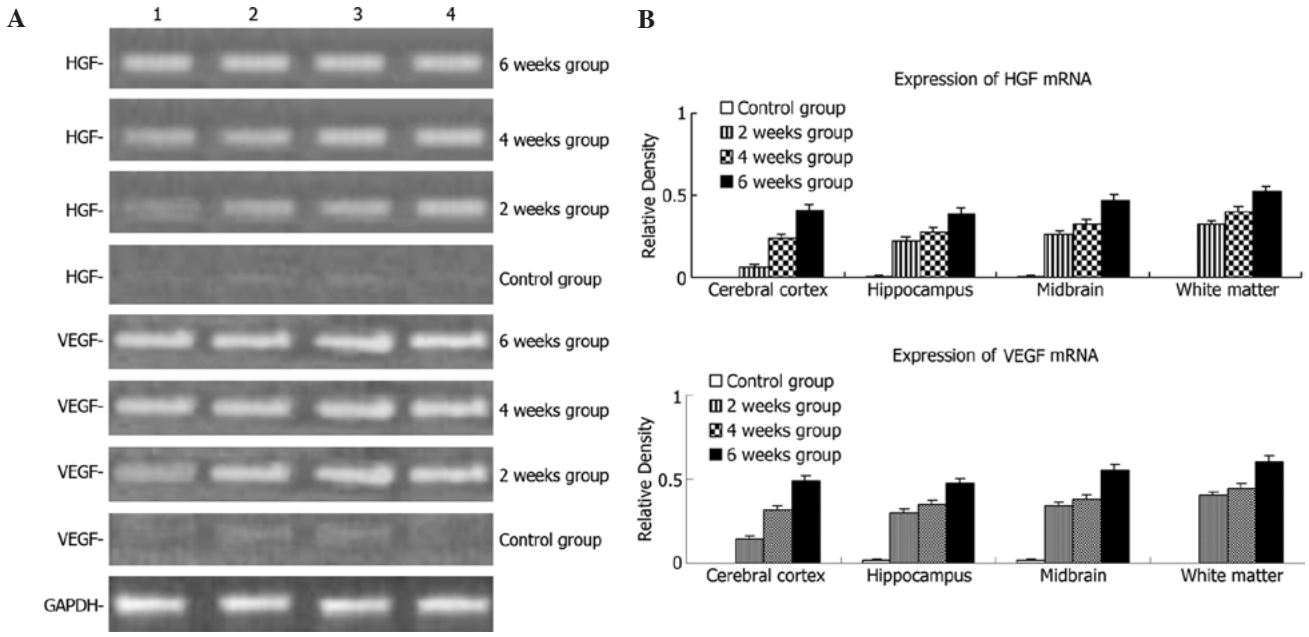

Fig. 3. Gene expression of HGF and VEGF analyzed using semiquantitative reverse-transcriptase polymerase chain reaction. (A) HGF and VEGF mRNA were detected in the cerebral cortex (Lane 1), hippocampus (Lane 2), midbrain (Lane 3) and white matter (Lane 4) of the experimental groups compared to the control group. (B) The bands were quantified using densitometric scanning, and the relative amount of each gene was calculated by dividing by the internal control. GAPDH, glyceraldehyde-e-phosphate dehydrogenase.

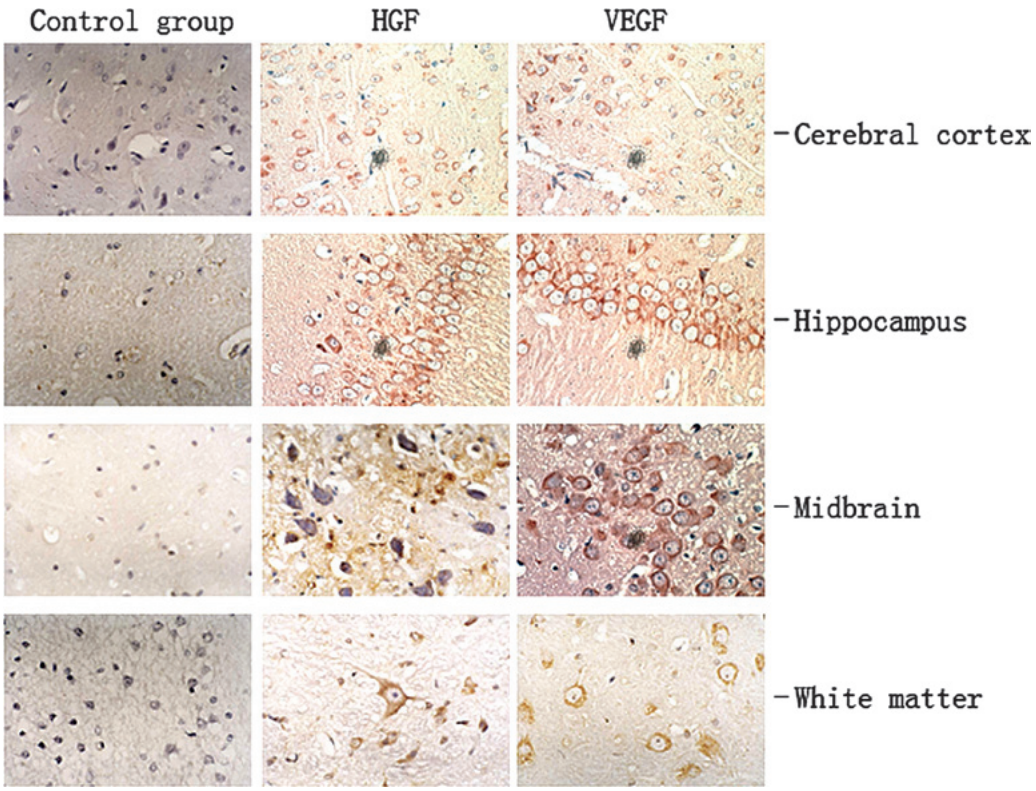

Fig. 4. Immunohistochemical localization of HGF and VEGF in the cerebral cortex, hippocampus, midbrain and white matter of the experimental animals at 4 weeks compared to the control group (original magnification x400).

cisterna magna on day 0, with a second injection 2 days later. In 21 of the 50 rats (42%), MRI revealed ventricular dilatation and periventricular white matter edema in varying degrees.

Hydrocephalus caused by TGF-β_1 overexpression is a significant and notable finding. Severe hydrocephalus has been observed by 2 research groups in transgenic animals overexpressing TGF-β_1 in astrocytes (27,28). In support of these results, intrathecal injection of human recombinant TGF-β_1 leads to the development of communicating hydrocephalus in mice (29). TGF-β_2 and TGF-β_3 expression may

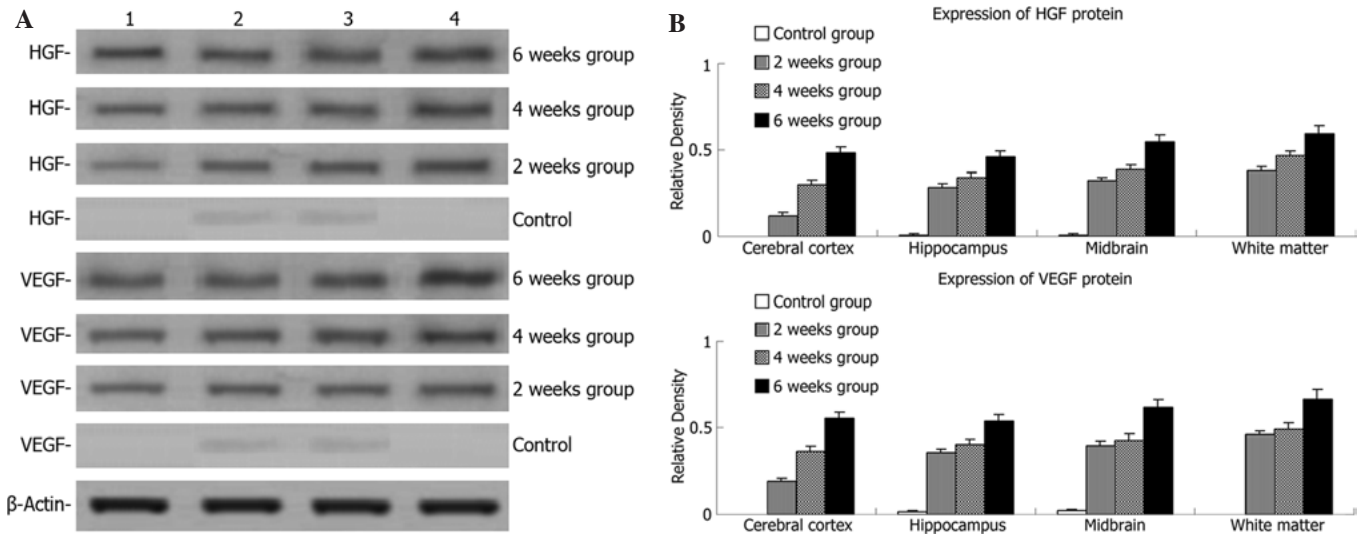

Fig. 5. Expression levels of HGF and VEGF protein analyzed using Western blot and densitometry. (A) Western blot analysis of HGF and VEGF in the cerebral cortex (Lane 1), hippocampus (Lane 2), midbrain (Lane 3) and white matter (Lane 4) of the experimental groups compared to the control group. (B) Densitometric analysis of relative HGF and VEGF expression levels.

be modulated differently in the hydrocephalus, and TGF-β_3 may contribute to the development of hydrocephalus in this rat model (30). It is known that VEGF is highly expressed in the rat brain folowing reversible global cerebral ischemia produced by cardiac arrest and resuscitation (15), and HGF is also highly expressed in the rat brain following ischemia (16). The MR spectroscopic results of some researchers have indicated compromised energy metabolism and suggested the occurrence of cerebral ischemia in experimental hydrocephalus (17). Certain researchers have noted that VEGF was highly expressed in the CSF of premature infants with posthemorrhagic hydrocephalus (18), and VEGF and TGF-β_1 are highly expressed in the CSF of premature infants with posthemorrhagic hydrocephalus (31).

HGF is a multipotent growth factor that has mitogenic and morphogenic effects on various epithelial cells. It is produced by mesenchyme, acts upon epithelial tissues in the liver, kidney and lung, and counteracts the fibrosis-inducing effect of TGF-β_1 (32). Neutralization of HGF by antibody leads to the acceleration of renal failure/fibrosis, while the administration of exogenous HGF leads to marked attenuation of renal failure/fibrosis (33). The balance between HGF and TGF-β_1 plays a key role in both the pathogenesis and therapeutics of chronic renal failure. Certain researchers have examined the effect of exogenous HGF (30 mg of human recombinant (hr) HGF intraventricularly for 7 or 14 days) in a model of hr TGF-β_1-induced communicating hydrocephalus in C57BL/6 mice. They observed that HGF treatment resulted in a reduction of ventriculomegaly, as demonstrated by magnetic resonance imaging. They also recorded improved spatial memory and ink passage test scores, demonstrating normalized CSF flow in mice receiving HGF treatment, as opposed to delayed CSF flow in the hydrocephalic mice at baseline. Finally, they noted that histological examination of hydrocephalic mice undergoing HGF treatment revealed reduction of collagen fibers in the meninges and normalization of their structures. These results indicate that exogenous HGF may be useful in the treatment of hydrocephalus in humans (34).

The two principal actions of VEGF on endothelial cells, angiogenesis and increased vascular permeability, have been observed in the brain (35). Increased vascular permeability results in albumin leakage from plasma into CSF, and VEGF levels were indeed found to be related to CSF protein concentrations in children with hydrocephalus, although the association was rather modest. VEGF-mediated neovascularization may enhance the oxygen supply, and topical VEGF application has been shown to reduce ischemic brain damage (36). However, edema formation and the breakdown of the blood-brain barrier associated with VEGF may further increase intracranial pressure and indirectly elicit inflammation (37,38). Antagonizing VEGF activity has been shown to reduce the development of edema and tissue damage after ischemia and reperfusion (39). In addition to acting on endothelial cells, VEGF has been reported to be mitogenic for astrocytes and to enhance survival of neurons (40). Thus, VEGF appears to exert beneficial and detrimental effects on the brain. Its precise role in children with increased intracranial pressure secondary to hydrocephalus remains to be determined.

In the present study, which is the first comparative analysis of the expression of HGF and VEGF in hydrocephalic brain tissue, elevation of HGF and VEGF mRNA and protein expression in the cerebral cortex, hippocampus, midbrain and white matter of adult rats with chronic hydrocephalus after subarachnoid hemorrhage, was observed. We speculate that HGF and VEGF expression may be modulated differently in hydrocephalus. This study improves our understanding of the roles played by HGF and VEGF in the CNS under both physiological and pathological conditions.

Acknowledgements

This study was partially supported by grants from the National Natural Science Foundation of China (no. 30901535), the Research Fund of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (no. 10XHJT01) and

the New One Hundred Person Project of Shanghai Jiao Tong University of School of Medicine (no. 10XBR01).

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