Thrombin-induced TGF-β1 pathway: A cause of communicating hydrocephalus post subarachnoid hemorrhage

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Abstract. The mechanism of communicating hydrocephalus after subarachnoid hemorrhage (SAH) remains unclear. Revealing a signaling cascade may provide significant insights into the molecular etiology of the accumulation of cerebrospinal fluid (CSF) in cerebral compartments during SAH. To investigate the mechanism of the communicating hydrocephalus following SAH, we infused CSF with thrombin (TH), resulting in proinflammatory and proliferative responses in rat meninges of SAH. The effect of TH could be completely blocked by a transforming growth factor β1 (TGF-β1) inhibitor, SB-431542, suggesting that TH-stimulated proliferation of meninges is through the TGF-β1 signaling pathway. The cascade of TGF β1-Smad3 was significantly upregulated by TH, which, in turn, stimulated the proliferation of subarachnoid meninges. TH-induced overexpression of TGF-β1 and activation of its downstream factors might be a mechanism of communicating hydrocephalus after SAH.

Introduction

Subarachnoid hemorrhage (SAH) frequently results in complications including intracranial hypertension, rebleeding and vasospasm. The extravasated blood is responsible for a cascade of reactions involving release of various vasoactive and proinflammatory factors from blood and vascular components in the subarachnoid space. The communicating hydrocephalus following SAH is one of the complex and multifactorial neurological disorders, which arises from fibrosis in the subarachnoid space. Spinal arachnoiditis and periradicular 'inflammation' of the arachnoid membrane and adjacent peridural structures lead to fibrosis within and around the lumbar dural sac and the spinal nerve roots (1). Fibrosis causes cavitas subarachnoidalis stenosis, which in turn blocks the cerebrospinal fluid (CSF) circulation. The mechanism of fibrosis following SAH has yet to be defined.

Thrombin (TH) is an Na^+-activated, allosteric serine protease that plays opposing functional roles in blood coagulation. It is produced in the brain either immediately after a cerebral hemorrhage or after the blood-brain barrier (BBB) breakdown that occurs following several types of brain injury (2). It was reported that TH activity was increased in CSF after SAH and there was a significant correlation between coagulation activity in the subarachnoid space and clearance of SAH (3). Vesey *et al* revealed that TH stimulates proinflammatory and proliferative responses in primary cultures of human proximal tubule cells (PTC), suggesting that the proinflammatory and fibroproliferative actions of TH on human PTC may help explain the extent of tubulointerstitial fibrosis observed in kidney diseases where fibrin deposition is evident (4). Based on these reports, we hypothesized that TH also plays an important role in fibrosis of subarachnoid meninges after SAH.

TGF-β is key in tissue homeostasis and the disruption of the TGF-β pathway has been implicated in numerous human diseases, including cancer, autoimmune, fibrotic, and cardiovascular diseases. TGF-β regulates cellular responses in a positive or a negative way. For example, the anti-inflammatory properties of TGF-β are beneficial in atherosclerosis, while the profibrotic effects contribute to fibrosis in hypertension and cardiac damage. TGF-β is believed to be the most important extracellular matrix (ECM) regulator (5). In vascular smooth muscle cells (VSMCs), endothelial cells, and fibroblasts, TGF-β1 increases the synthesis of ECM proteins, such as fibronectin, collagens and PAI-1. TGF-β reduces collagenase production and stimulates the expression of tissue inhibitor of metalloproteinases (TIMPs), resulting in an overall inhibition of ECM degradation and leading to excessive matrix accu-

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Abbreviations: SAH, subarachnoid hemorrhage; TH, thrombin; CSF, cerebrospinal fluid; CTGF, connective tissue growth factor; ECM, extracellular matrix; TGF-β, transforming growth factor β

Key words: communicating hydrocephalus, subarachnoid hemorrhage, thrombin, TGF-β1, Smad3

mulation (5,6). The mechanisms involved in TGF-β-mediated vascular fibrosis are complex, including activation of Smad proteins, protein kinases, production of mediators and cross-talk between pathways. Further insights into the molecular mechanisms involved in ECM accumulation may contribute to a better understanding of this pathological process and may improve therapeutic strategies.

Materials and methods

Animals. Female Wistar rats (200-300 g) were housed in a room at a temperature of 22±2˚C under a 12-h light/dark schedule and given water and food *ad libitum*. The procedures involving experimental animals adhered to the law and notification of the Chinese Government and were approved by the Laboratory Animal Care and Use Committee of Xinxiang Medical College and Shantou University.

Reagents. Rat α-thrombin and SB-431542, a specific inhibitor of type 1 TGF-β receptor (TβR1), were purchased from Sigma (St. Louis, MO, USA). TGF-β1 and TGF-β immunoassay kit was from R&D Systems Inc., (Minneapolis, MN, USA). Anti-phospho-Smad1 (S465), 2 (S467), 3 (S423 + S425), anti-Smad1, 2 and 3, and anti-β-actin were from Abcam (Cambridge, MA, USA).

Injection of TH into SAH and specimen preparations. Seventy-eight female rats were randomly assigned into 3 experimental groups (Group 1, TH group; Group 2, SB-431542 group; and Group 3, TH + SB-431542 group; n=21 in each group) and the control group (n=15). All rats were anesthetized with 10% chloral hydrate and were punctured at 5 mm below the margo occipitalis with a 22-gauge needle into the cistema magna. Subsequently, Groups 2 and 3 were injected with SB-431542 (0.1 μmol), which was dissolved in 10 μl of vehicle (DMSO:water, 1:2), injected into the cistema magna through a 50 μl microsyringe and a polyethylene tube. One hour after SB-431542 injection, 0.3 ml (10 U/ml) of TH (Groups 1 and 3) or isotonic sodium chloride (control group) was infused into CSF at a rate of 0.1 ml/minute. Animals were kept in the head down position for 30 min after infusion before putting them back into the cages.

At Day 0, 10 and 20 after infusion of TH, 7 rats from the experimental groups (Groups 1, 2 and 3) and 5 rats from the control group were randomly selected and sacrificed, and CSF (100 μl/rat) was drawn respectively. CSF was centrifuged for 20 minutes at 2,000 rpm. The supernatant was collected and maintained at -80˚C for TGF-β1 measurement.

TGF-β1 assay. Concentrations of TGF-β1 in each CSF sample were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Promega). The TGF-β1 levels were measured in 50 μl CSF after 1N HCl was added at 1:3 and incubated for 10 min to activate TGF-β1. Following neutralization with 1 ml 1N NaOH/0.5 M HEPES, the samples were assayed by ELISA. No significant cross-reactivity with other cytokines was observed.

Primary rat meningocyte culture. Rat subarachnoid meninges were removed from the brains of rats. Cells were dissociated and plated in collagen-coated 6-well plates with Eagle's minimum essential medium and 15% fetal bovine serum at 37˚C in 95% air/5% CO_2. Culture of rat meningocytes was grown to 80% confluence and then placed in serum free medium containing 0.1% bovine serum albumin (BSA) for a minimum of 12 h prior to treatment. Specified inhibitor, SB-431542 (10 μM), or vehicle was next added in serum free medium for 30 min prior to the addition of TGF-β1 (2 and 4 ng/ml) or vehicle. TGF-β1 treatments were for either 6 h for RNA analyses or 8 h for protein analyses.

Total RNA isolation and real-time quantitative PCR (qRT-PCR). Total RNA from control and treatments was isolated using TRIzol according to the manufacturer's instructions. RNA obtained was analyzed using Agilent bioanalyzer and quantified via a NinoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA (1 μg) per treatment was reverse transcripted using random primers and the Applied Biosystems reverse transcription kit. Five microliters of each reverse transcription reaction was used for 50 μl real-time PCR using conditional 96-well format. TaqMan probes for CCN2/CTGF, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used in real-time PCR analyses. Real-time PCRs were run in ABI 7700 system at thermal cycler conditions of 50˚C for 2 min, 95˚C for 1 min, and 60˚C for 1 min for 40 cycles. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and CCN2/CTGF mRNA levels were normalized to β-actin and GAPDH mRNAs, and no treatment control.

Protein isolation and western blotting. Cells were homogenized in a lysis buffer [25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 25 μg/ml leupeptin, 1 mM DTT, 135 mM NaCl, 25 mM NaF, 0.5% Triton X-100] and centrifuged. The supernatant, each containing 100 ng of total protein, was separated on a sodium dodecyl sulphate (SDS)-10% polyacrylamide gel coupled to a 4% polyacrylamide stacking gel. After transferring the separated proteins to polyvinylidene difluoride (PVDF) membranes, the membranes were soaked in 20 ml of 4% BSA in Tris-buffered saline Tween-20 (TBST) buffer for 1 h at room temperature, and were then probed 1 h with either anti-CTGF, anti-phospho-Smad1, 2 and 3 primary antibody, and then with HRP-conjugated secondary antibodies. Antigens were detected using the Pierce chemiluminescent substrate system (ECL). Following anti-phospho antibody analyses, the same membranes were used with anti-Smad1, 2 or 3 analysis, i.e. after blotting with anti-phospho-Smad1, 2 and 3, the membranes were stripped, and re-detected using ECL for background check. These membranes were then used for anti-Smad1, 2 and 3 analyses, respectively. The actin protein was analyzed for all the blots at final stripping steps.

Histological evaluation. After obtaining CSF at Day 10 and 20 post-infusion, rat brains were fixed with 5% paraformaldehyde and then sectioned transversely with a thickness of 5 mm at the frontal lobe, apical lobe and end-lobe. Specimens were embedded in paraffin wax and 5-μm sections were stained with Masson's method. The sections were examined with microscopy. The thickness of the meninges was analyzed with Fuji micro-image analysis system. The section of each sample

Table I. Time course of TGF-β1 in rat CSF induced by TH infusion.

Group	Mouse ID	Day 0	Day 10	Day 20
Controls	1	44.58	30.83	46.03
	2	34.55	38.74	36.56
	3	36.91	64.71	34.51
	4	31.20	27.48	21.88
	5	31.34	23.17	29.11
	Mean ± SD	35.72±5.50	36.99±6.51	33.62±8.97
TH (3 U/0.3 ml)	11	36.44	426.58	286.42
	12	39.75	264.71	276.91
	13	41.34	339.63	406.99
	14	36.42	286.42	191.87
	15	41.86	291.20	281.13
	16	31.00	286.42	302.69
	17	41.34	268.55	236.14
	Mean ± SD	38.31±3.62	309.1±57.31[a]	283.2±66.22[a]

Values are TGF-β1 (pg/ml); [a]p<0.01 between TH infusion and control.

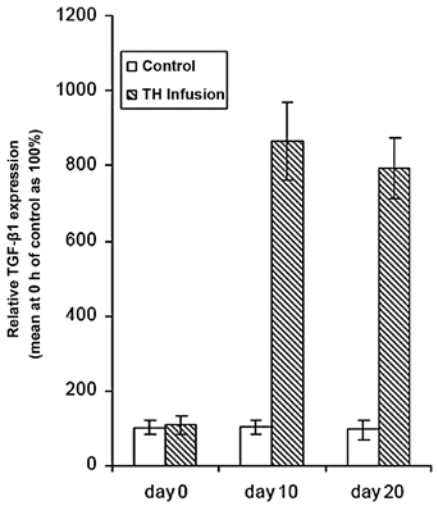

Figure 1. Time course of TGF-β1 release induced by TH in rat CSF. TGF-β1 activity in rat CSF was analyzed with ELISA. Thrombin stimulated TGF-β1 in a time-dependent manner (P<0.001). Control, infused with isotonic sodium chloride, 0.3 ml, and absence of thrombin; TH, infused with thrombin, 3 U/0.3 ml (see Materials and methods for details). Results are expressed as relative expression level (control was set as 100%) of pg/mg cell protein. Values are the means ± SD of four wells.

was taken from at least 3 separate subarachnoid meninges individually.

Statistical analysis. Analysis of variance (ANOVA) was used to assess the comparison (paired) of values. The LSD method was used for multiple comparisons between groups. Linear correlation analysis was used to compare the concentration of TGF-β1 in CSF with the average thickness of subarachnoid meninges. P<0.05 was considered to indicate a statistically significant difference.

Results

TH induces expression of the TGF-β1. A high level of TH was present in patient CSF after SAH (7). Kasuya *et al* reported that the TH activity in CSF after SAH was correlated with the degree of SAH (8). To elucidate the mechanism and cascade reaction of CSF after SAH, we first infused TH into SAH. All animals treated with TH infusion showed a significant increase in total TGF-β1 levels in the CSF. Ten days after TH infusion, TGF-β1 expression increased 7.6-fold. At Day 20 after TH infusion, TGF-β1 expression was slightly lower than that at Day 10. There were no significant differences between Day 10 and 20 (P>0.05) (Fig. 1 and Table I). The results suggested that the TGF-β1 pathway may be evoked by accumulated TH in CSF.

TGF-β1 induces expression of the CTGF in primary meningocytes. Several studies have indicated that CTGF could be selectively stimulated by TGF-β1. We investigated whether this cascade reaction also occurs in the tissues or cells of the subarachnoid space. We isolated meningocytes from subarachnoid meninges for a primary culture. The expression of CTGF mRNA in the culture of meningocytes was increased gradually in response of TGF-β1 treatment. The responses were both dose and time dependent (Table II and Fig. 2A). The expression of CTGF proteins was also increased gradually (Fig. 2B). Pre-treatment of SB-431542 for 30 min blocked expression of CTGF protein which was induced by TGF-β1 (Fig. 2C). Since CTGF was known for its role as a mediator of the chronic fibrotic effects, our results suggested that TH-induced TGF-β1-CTGF cascade may also play a role in CSF after SAH.

Smad3 is critical for CTGF expression induced by TGF-β1 in primary meningocytes. TGF-β1 signaling was mediated by Smads in several cell types. Increasing evidence shows that TGF-β1 acts by activating its downstream mediators, Smad2/3,

Table II. TGF-β1 stimulated CTGF mRNA expression.

Time course (h)	0	2	4	6
Controls	82±16	84±14.7	83±15.5	85±16.1
TGF-β1 (2 ng/ml)	84±17.1	274±12.1	496±14.5	602±13.3
TGF-β1 (4 ng/ml)	86±18	416±12.5	661±13.2	945±15.3
SB-431542 (10 μM) + TGF-β1 (2 ng/ml)	88±10	90±9.7	93±5.9	89±11.1

Values are CTGF mRNA (ng/ml) (mean ± SD); [a]p<0.05 and [b]p<0.01 between TGF-β1 treatment and control.

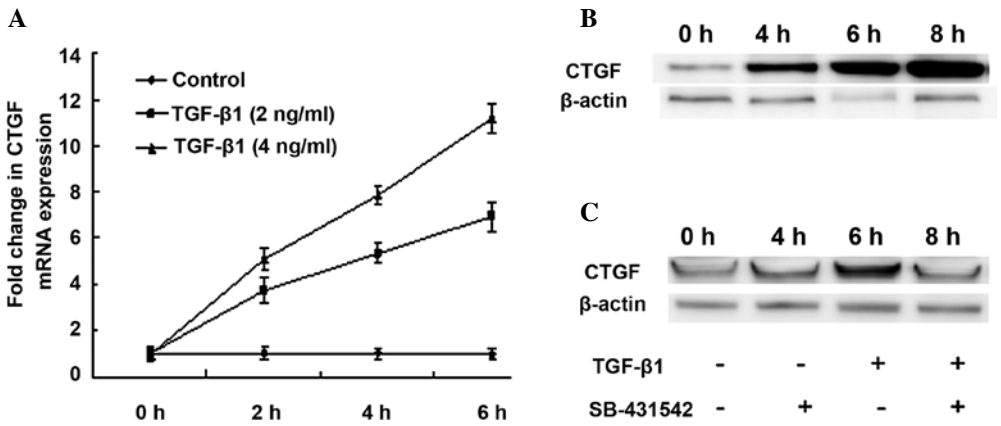

Figure 2. Both mRNA and protein expressions of CTGF are induced by TGF-β1 in rat primary meningocytes. (A) Quantitative real-time RT-PCR of rat primary meningocytes showed that the mRNA expression of CTGF was dose and time dependent. Values are the means ± SD of four parallel RT-PCR reactions. (B) Rat primary meningocytes were plated in 6-well plates, serum starved overnight and treated with TGF-β1 (4 ng/ml) or vehicle treatment for 30 min. CTGF protein was analyzed by western blotting. (C) Rat primary meningocytes were treated with SB-431542 (10 μM) or diluent alone (control) for 0.5 h prior to TGF-β1 (2 and 4 ng/ml) or vehicle treatment for 30 min. CTGF protein was analyzed by western blotting. The same membranes were used for β-actin analyses after stripping the previous signals. Each experiment was repeated three times with similar results.

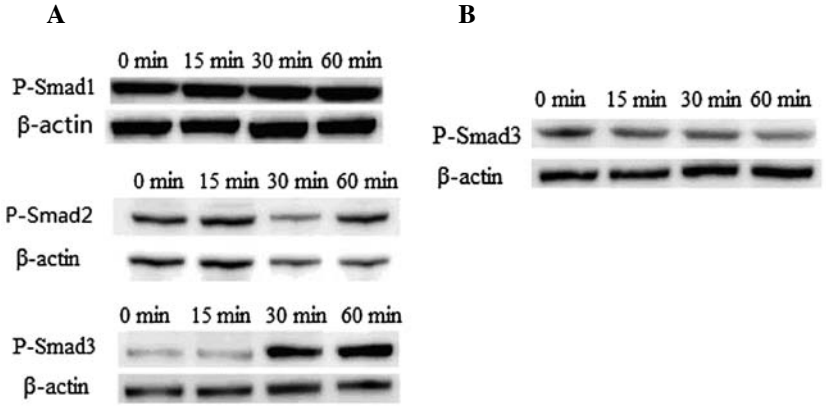

Figure 3. Activation of Smad3 is required by TGF-β1. (A) Rat primary meningocytes were plated in 6-well plates and treated with TGF-β1 (4 ng/ml) for 0, 15, 30 and 60 min. Smad1, 2 and 3 activation (phosphorylation) was assessed by western blotting. (B) Rat primary meningocytes were plated in 6-well plates, serum starved overnight and treated with SB-431542 (10 μM) for 0.5 h prior to TGF-β1 (4 ng/ml) treatment for 0, 15, 30 and 60 min. Smad3 activation (phosphorylation) was analyzed by western blotting.

to mediate renal fibrosis (9,10). To determine whether the Smads also play a role in mediating meningocyte fibrosis, we assessed the activation of Smad signaling by TGF-β1. Western blot analysis revealed that phosphorylation of Smad3 was initiated by TGF-β1 when primary meningocytes were treated with TGF-β1 at 15 min post-treatment and persisted through 60 min. By contrast, phosphorylation of Smad-1 and Smad-2 were not altered by TGF-β1 (Fig. 3A). Pre-treatment of SB-431542 for 30 min blocked signaling of Smad3 completely (Fig. 3B). We also determined protein expressions of Smad1, 2 and 3 to ascertain whether they are also altered by TGF-β1. Our results indicated that protein expression levels were

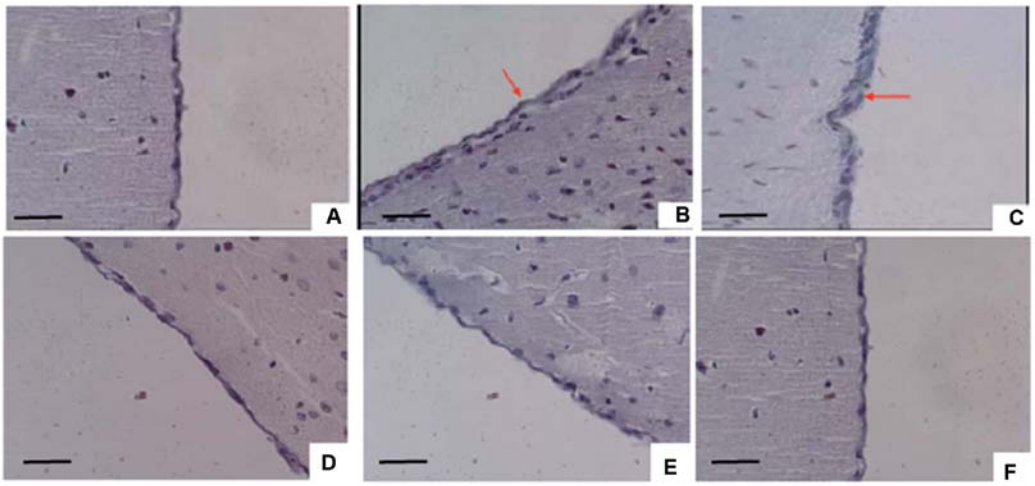

Figure 4. Rat subarachnoid meningeal fibrosis induced by TH. Masson's trichrome staining of rat subarachnoid meningeal sections (magnification, x400). Rats were infused with TH at (A) Day 0 (before TH infusion), (B) Day 10, and (C) Day 20. Arrows show the thickness and fibrosis of subarachnoid meninges. Pre-treatment of SB-431542 (0.1 μmol/10 μl) for 1 h prior to TH infusion at (D) Day 0 (before TH infusion), (E) Day 10, and (F) Day 20. The experiment was repeated four times with similar results. Bar=100 μm.

Table III. TH induces a significant collagen accumulation at rat subarachnoid meninges.

Days after TH infusion	0	10	20
Isotonic sodium chloride	3.43±1.10	3.13±0.17	3.53±0.25
TH[a]	3.68±0.43	4.29±0.52[c]	5.67±0.77[d]
SB-431542[b]	3.57±0.64	3.61±1.01	3.59±0.75
SB-431542[b] + TH[a]	3.45±1.23	3.47±0.47	3.61±0.39

[a]TH, 3U, dissolved in 0.3 ml isotonic sodium chloride. [b]SB-431542, 0.1 μmol, dissolved in 10 μl of vehicle (DMSO:water, 1:2). Values are the means of thickness (μm) ± SD. [c]$p<0.05$ and [d]$p<0.01$ between TH infusion and non-infusion.

not affected by TGF-β1 in the period of treatment (data not shown), suggesting that only activated Smad3 is essential for CTGF induction by TGF-β1 in our experimental conditions.

Pathological changes of subarachnoid meninges by TH infusion. Rat subarachnoid meninges showed marked inflammatory changes in the meningeal collagen fibers following TH infusion at Day 10 and 20 of the experiment (Fig. 4, Tables III and IV), irrespective of the concomitant application of isotonic sodium chloride (data not shown). Mice exposed to TH demonstrated meningeal inflammation by Day 10, which was resolved completely by Day 20. Masson's trichrome staining revealed slight collagen within inflammatory areas of subarachnoid meninges in TH-treated rats at Day 10, whereas there was significant collagen accumulation in the rats at Day 20. To elucidate whether TH is a cause of proliferation in subarachnoid meninges and its possible route, we pre-injected SB-431542 to block the TGF-β1 signaling pathway. Our results indicated that there was no obvious inflammation or collagen accumulation at Day 10 and 20 following TH infusion at pre-treated groups (Fig. 4). This strongly suggested that TH could be one cause of subarachnoid meningeal fibrosis following SAH, and the TGF-β1 signaling pathway could be critical for this pathological process.

Discussion

Alterations in the CSF circulation may follow SAH; however, the frequency and extent to which this occurs is unknown. A previous report revealed that leptomeningeal fibrosis is the pathoanatomic basis of increased resistance to CSF outflow (11). Fibrosis of the arachnoid villi has been suggested as the cause for obstruction of CSF flow (12). Accurate definition of these abnormalities is important since in some instances they result in symptomatic communicating hydrocephalus. The origin of chronic communicating hydrocephalus following SAH is not well understood. Genetic studies revealed that numerous cytokines, growth factors or related molecules in the cellular signal pathways play an important role in the development of hydrocephalus. To establish the mechanism of the communicating hydrocephalus following SAH, we infused CSF with TH, resulting in proinflammatory and proliferative responses in rat meninges of SAH. The effect of TH could be completely blocked by a TGF-β1 inhibitor, SB-431542, suggesting that TH-stimulated proliferation of meninges is through the TGF-β1 signaling pathway.

Shirato *et al* discovered that TH stimulates kidney endothelial cells to secrete both cellulose protein and TGF-β1 (13). TGF-β1 is an important cytokine and growth-signaling

Table IV. Time course of thickness of rat subarachnoid meninges in different experimental groups.

Group	Mouse ID	Day 0	Day 10	Day 20
TH	1	4.40	3.69	6.20
	2	3.23	4.16	4.96
	3	4.11	4.58	6.63
	4	3.67	4.88	5.46
	5	3.47	3.54	6.79
	6	3.61	3.27	6.40
	7	3.61	5.41	6.23
	Mean ± SD	3.73±0.4	4.22±0.77[a]	6.11±0.67[a]
SB-431542 + TH	11	3.32	3.18	3.81
	12	3.72	3.06	3.33
	13	2.82	3.40	3.40
	14	3.84	3.04	3.24
	15	3.43	4.96	3.53
	16	2.96	3.02	3.28
	17	3.25	3.36	3.45
	Mean ± SD	3.34±0.42	3.43±0.69	3.48±0.22

Values are thickness (μm) of rat subarachnoid meninges. [a]$p<0.01$ between TH infusions and non-infusion (Day 0).

molecule in the brain. TGF-β1 expresses at a high level after SAH (7). In mouse models, severe hydrocephalus has been observed in transgenic mouse overexpression of TGF-β1 in astrocytes (14,15). Furthermore, several studies indicated that TGF-β1 plays a central role in the response to brain injury, and is involved in a variety of disorders of the central nervous system such as stroke (16,17), ischemia (18,19) and abscess (20). Under various pathological conditions, TGF-β1 expression is found in neurons, suggesting a role as a neuronal crisis cytokine. The mechanism of how TGF-β1 promotes the collagen protein production is complex. At the level of transcription and translation, TGF-β1 participates with nuclear factor and activates type 1 collagen gene promoter, then stimulates the expression of collagen junctura fibrosa protein gene by reporter gene, and increases the composition of collagen protein. However, it also upregulates the expression of proteinase inhibitor through restraining the breakdown of proteins such as metalloprotease, collagenase, stromelysin, and elastase.

To elucidate the cascade reactions of the TGF-β1 signaling pathway in subarachnoid meningeal fibrosis, we investigated several downstream factors of TGF-β1. Our results revealed that one downstream factor of TGF-β1, CTGF, was selected to be stimulated in primary subarachnoid meningocytes treated by TH. CTGF is well known for its role as a downstream mediator of the chronic fibrotic effects of TGF-β1. Activated by TGF-β1, CTGF induces fibroblasts to become myofibroblasts that deposit layers of collagen, which occurs faster than the natural rate of collagen breakdown. The affected organs become stiff and cannot perform functions essential to life and health. FibroGen studies have demonstrated that TGF-β1 alone only causes a transient increase in fibrosis and that scar formation is persistent only when CTGF is present (http://www.fibrogen.com/rd/ctgf/fibrosis.html). Previous studies have demonstrated that the levels of CTGF correlate with the degree and severity of fibrosis in several diseases, including diabetic nephropathy, glomerulosclerosis, IgA nephropathy, diabetic retinopathy, advanced macular degeneration, cirrhosis, biliary atresia, congestive heart failure, lung fibrosis and scleroderma. This prompted us to investigate the molecular mechanisms that might link changes in subarachnoid meningeal fibrosis, TGF-β1 and CTGF gene expressions. In this study, we demonstrated that TH-induced overexpression of TGF β1-signaling is one of the mechanisms of communicating hydrocephalus after SAH.

In general, TGF-β1 signals through a generic Smad-mediated pathway. However, the signaling pathways that mediate TGF-β1 induction of CTGF have been shown to vary depending on the cell type being examined (21) and, to date, there have been no studies on subarachnoid meningocyte. The Smad family is divided into three subclasses: receptor regulated Smads, activin/TGF-β receptor regulated (Smad2 and 3) or BMP receptor regulated (Smad1, 5, and 8). We selected anti-phospho and non-phospho-Smad1, 2 and 3 in this study. Smad1 was used to eliminate the non-specific induction of TGF-β1. We elucidated that activation of Smad3 is critical for stimulation of CTGF expression. Both phosphorylation of Smad3 and CTGF expression could be blocked by SB-431542. Our results strongly suggest that the proliferative responses in rat meninges induced by TH are due to TH-triggered activation of the TGF-β1 signaling pathway. This cascade reaction might be a mechanism of communicating hydrocephalus post SAH. To our knowledge, this is the first study to explore the TH-TGF β1-Smad3-CTGF signaling pathway associated with subarachnoid meningeal fibrosis and communicating hydrocephalus.

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