Inhibition of Transforming Growth Factor-β Attenuates Brain Injury and Neurological Deficits in a Rat Model of Germinal Matrix Hemorrhage

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Background and Purpose—Transforming growth factor-β (TGF-β) overproduction and activation of the TGF-β pathway are associated with the development of brain injury following germinal matrix hemorrhage (GMH) in premature infants. We examined the effects of GMH on the level of TGF-β1 in a novel rat collagenase-induced GMH model and determined the effect of inhibition of the TGF receptor I.

Methods—In total, 92 seven-day old (P7) rats were used. Time-dependent effects of GMH on the level of TGF-β1 and TGF receptor I were evaluated by Western blot. A TGF receptor I inhibitor (SD208) was administered daily for 3 days, starting either 1 hour or 3 days after GMH induction. The effects of GMH and SD208 on the TGF-β pathway were evaluated by Western blot at day 3. The effects of GMH and SD208 on cognitive and motor function were also assessed. The effects of TGF receptor I inhibition by SD208 on GMH-induced brain injury and underlying molecular pathways were investigated by Western blot, immunofluorescence, and morphology studies 24 days after GMH.

Results—GMH induced significant delay in development, caused impairment in both cognitive and motor functions, and resulted in brain atrophy in rat subjects. GMH also caused deposition of both vitronectin (an extracellular matrix protein) and glial fibrillary acidic protein in perilesion areas, associated with development of hydrocephalus. SD208 ameliorated GMH-induced developmental delay, improved cognitive and motor functions, and attenuated body weight loss. SD208 also decreased vitronectin and glial fibrillary acidic protein deposition and decreased GMH-induced brain injury.

Conclusions—Increased level of TGF-β1 and activation of the TGF-β pathway associate with the development of brain injury after GMH. SD208 inhibits GMH-induced activation of the TGF-β pathway and leads to an improved developmental profile, partial recovery of cognitive and motor functions, and attenuation of GMH-induced brain atrophy and hydrocephalus. 

Key Words: receivers, transforming growth factor beta ■ transforming growth factor beta
cause the development of hydrocephalus.\textsuperscript{11,12} It has become apparent that TGF-β plays a pivotal role in the modulation of extracellular matrix (ECM) proteins, acting to regulate the synthesis and degradation of their components.\textsuperscript{13,14} ECM protein deposition impairs absorption of cerebral spinal fluid and is an important contributor to the development of the posthemorrhagic hydrocephalus.\textsuperscript{15}

In this study, we examined the effect of collagenase-induced GMH in rats on level of TGF-β1 and on the state of activation of TGFR-I. We hypothesize that increased level of TGF-β1 results in accumulation of ECM proteins and glial fibrillary acidic protein (GFAP), associated with hydrocephalus, brain atrophy, and significant neurological deficits. We also postulate that inhibition of the TGF-β pathway via a novel TGFR-I inhibitor ameliorates GMH-induced brain damage and neurological deficits.

**Materials and Methods**

All experiments were approved by the Loma Linda University Institutional Animal Care and Use Committee. GMH was induced as described.\textsuperscript{16} Pregnant Sprague–Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Ninety-two P7 rat pups were then randomly divided into: six sham-operated (n=20), collagenase-injected vehicle-treated (n=44), collagenase-injected SD208-treated (n=28) animals. Pups of both sexes were used. An aseptic technique was used. For GMH induction, animals were anesthetized with 3% isoflurane and placed onto stereotactic frame. Isoflurane concentration was then reduced to 2%. The scalp area was sterilized, and bregma was exposed. Using bregma as a reference point, the following stereotactic coordinates were measured: 1.8 (rostral) and 1.5 mm (lateral). A burr hole (1 mm) was drilled. A 27-gauge needle was inserted at a rate of 1 mm/min at the depth of 2.8 mm from the dura. Using a microinfusion pump (Harvard Apparatus, Holliston, MA), 0.3 U of clostridial collagenase VII-S (Sigma, St Louis, MO) in 0.5 µL was infused through the Hamilton syringe. The needle remained in place for an additional 10 minutes after injection to prevent back leakage. After the needle was removed, the burr hole was sealed with bone wax and the incision suture closed, and the animals were allowed to recover on a 37°C heated blanket. On recovering from anesthesia, the animals were returned to their dams. Sham operation consisted of needle insertion alone without collagenase infusion. SD208 was purchased from Tocris Bioscience and injected intraperitoneally. Two concentrations of SD208 were tested: 20 mg/kg (low concentration) and 60 mg/kg (high concentration).\textsuperscript{17} Untreated animals received an injection of equal volume of vehicle (0.1% of dimethyl sulfoxide). Two treatment strategies were used: an acute treatment, in which we injected SD208 daily for 3 days starting at 1 hour after induced GMH, and a delayed treatment, in which we injected SD208 daily for 3 days starting at day 3 after induced GMH.

**Neurological Examination**

The effects of GMH and treatment with SD208 on the development of animals were evaluated using body righting and negative geotaxis tests. Tests were performed in a blinded fashion daily through day 7.\textsuperscript{18} We also tested the effects of both GMH and SD208 treatment on cognitive function using T-maze, water maze, and motor function using foot faults and neurodeficit scales\textsuperscript{16} in a blinded fashion between days 21 and 24 (online-only Data Supplement).

**Western Blots**

The effects of GMH on TGFR-I and TGF-β1 level were evaluated at 3, 6, 12, 24, and 72 hours after GMH by Western blot according to the manufacturer’s recommendations.\textsuperscript{19} The effects of SD208 on the GMH-induced activation of the TGF pathway were evaluated by Western blot on day 3, 1 hour after the last drug injection (online-only Data Supplement).

**Immunohistochemistry and Brain Injury Evaluation**

Brain atrophy was evaluated by calculating brains’ body weight, changes in total brain tissue loss, and brain loss in cortical and subcortical areas at day 24.\textsuperscript{16} The effects of SD208 on GMH-induced accumulation of vitronectin and GFAP were evaluated at day 10 for GFAP and at day 24 for vitronectin\textsuperscript{16} as previously described (online-only Data Supplement).

**Statistical Analysis**

Data were analyzed by 1-way ANOVA followed by Tukey post-test. A P value of <0.05 was considered statistically significant. Significance in differences of the water maze test was estimated using a 95% confidence interval overlap measure. All statistical analyses were performed using SigmaStat. Values are expressed as mean±SD.

**Results**

**Effect of GMH and SD208 on Survival of Animals**

Neither collagenase-induced GMH nor administration of SD208 caused significant mortality in this study. Only 1 GMH vehicle-treated animal died overnight after GMH induction.

**Effect of GMH and SD208 on TGF Pathway**

Although GMH had no significant effect on TGFR-I level (Figure 1A), a significant increase in TGF-β1 level at 3 and 6 days 21 and 24 (online-only Data Supplement).
High-dose SD208 ameliorated GMH-induced neurological deficits. (A) Values are expressed as mean±SD. *P<0.05 vs sham; #<0.05 vs vehicle. Figure 2A). SD208 reduces germinal matrix hemorrhage (GMH)–induced phosphorylation of transforming growth factor receptor I downstream molecules, SMAD 2/3. A, Neither GMH nor SD208 had any apparent effect on SMAD 2/3 level evaluated 3 days after GMH. B, SMAD 2/3 phosphorylation at day 3 was increased in GMH (n=6) animals compared with sham-operated (n=6) animals. Both low (n=6) and high (n=6) SD208 doses decreased GMH-induced phosphorylation of SMAD 2/3. C, Only the high dose of SD208 decreased the ratio of phosphorylated/nonphosphorylated SMAD 2/3 at day 3 (*P<0.05 vs sham; #P<0.05 vs vehicle). Values are expressed as mean±SD.

Effect of SD208 on GMH-Induced Neurological Deficits

At days 1 and 2, we observed significant developmental delay (evaluated by both body righting and negative geotaxis tests) in all vehicle-treated GMH rats compared with sham-operated animals (*P<0.05 versus sham; Figure 3A and 3B). High-dose SD208 ameliorated GMH-induced neurological deficits (#P<0.05 versus vehicle; Figure 3A and 3B); the low dose was ineffective (data not shown). GMH-induced developmental delay resulted in long-term adverse effects on both cognitive and motor function compared with sham-operated animals (*P<0.05 versus sham; Figure 4A–4D). Because only the high dose of SD208 was effective in the short term, this dose was also used for our long-term study.

Acute treatment improved cognitive and motor functions evaluated by T-maze and water maze as well as by neuroscore and foot fault tests. Delayed treatment significantly improved the cognitive function of treated animals and improved motor functions evaluated by the foot fault test (P<0.05 delayed versus vehicle; Figure 4A). In addition, we showed a strong tendency toward improvement of motor function evaluated by the foot fault test.

Effect of SD208 on GMH-Induced Weight Loss, Brain Loss, and Ventriculomegaly

Twenty-four days after GMH, we observed significant weight loss in GMH animals compared with sham-operated animals (from 119.5±4.6 to 90.5±7.8 g; sham versus GMH animals, respectively). Both acute and delayed treatments with SD208 had a tendency to attenuate GMH-induced weight loss (mean weights, 107.4±3.7 for the acute treatment group and 114.6±6.1
for the delayed treatment group), but this did not reach significance (Figure in the online-only Data Supplement).

We observed a significant decrease of the brain weight in the rats with induced GMH compared with sham-operated animals. High-dose SD208 ameliorated GMH-induced brain atrophy in both the acute treatment group and delayed treatment group (*P<0.05 versus sham; Figure 5A).

Compared with sham animals, GMH caused significant brain tissue loss in both cortical (ipsilateral hemisphere, 83.12±2.74 versus 69.91±3.86 mm³, sham and GMH animals, respectively) and subcortical areas (ipsilateral hemisphere, 72.35±3.96 versus 47.86±1.89 mm³, sham and GMH animals, respectively; contralateral hemisphere, 75.85±1.91 versus 59.28±3.14 mm³, sham and GMH animals, respectively). Total brain tissue loss was also observed in GMH compared with sham animals (ipsilateral hemisphere, 150.25±6.56 versus 116.01±5.24 mm³, sham and GMH animals, respectively; contralateral hemisphere, 157.89±2.84 versus 143.49±5.83 mm³, sham and GMH animals, respectively). Both the acute and delayed treatments significantly attenuated brain tissue loss in both cortical areas (*P<0.05 versus vehicle; Figure 5B).

Effect of SD208 on GMH-Induced Vitronectin and GFAP Accumulation

We have previously shown that in induced GMH, GFAP level peaks on postevent day 10 and vitronectin level peaks on postevent day 24. In this study, we tested the effects of acute and delayed treatment with SD208 on those time points. We demonstrated that TGF-β pathway inhibition by SD208 significantly decreased the level of GFAP (Figure 6A). On day 24, SD208 decreased GMH-associated vitronectin level as measured by Western blot (Figure 6B).

Discussion

GMH is a common consequence of premature newborns. In this study, we examined the effects of collagenase-induced GMH in rats on the activation state of the TGF-β pathway. In addition, we explored the effects of a potent TGFR-I inhibitor on GMH-induced brain injury and associated neurological consequences. The most significant of these neurological sequelae include posthemorrhagic hydrocephalus and brain atrophy which may potentially lead to developmental delay and mental retardation. Hemorrhagic brain injury causes a dramatic increase in TGF-β1 level in the brain, which can subsequently lead to the development of hydrocephalus. Overexpression of TGF-β1 in genetically modified animals and direct injection of the TGF-β1 into the brain of animals also have been shown to cause development of hydrocephalus.

Previously, we described a novel collagenase model of GMH that is able to mimic the motor deficits and ventricular dilation seen in human preterm newborns. The molecular pathways leading to these events remain to be elucidated, and
this is one of the goals of the present study. In this study, we observed a significant increase of TGF-β1 level after induced GMH via collagenase injection. This corroborates with other studies demonstrating increased TGF-β1 level in the central nervous system in response to a hemorrhagic event.4–6 We postulate that activation of the TGF-β pathway is at least partly responsible for the development of brain injury and neurological deficits following GMH.

We hypothesize that TGF-β pathway inhibition can ameliorate the devastating effects of GMH.16 We used a novel, potent inhibitor of TGFR-I (SD208) because TGFR-I is a last step in the activation of the TGF receptor complex. SD208 displays 100-fold selectively to binding TGFR-I over TGFR-II. After systemic application, SD208 limits the bioavailability of TGF-β in brain by decreasing SMAD2 and 3 phosphorylation.20–22 A therapeutic approach involving inhibition of TGF-β downstream signaling molecules may hold promise for future treatment modalities. We have demonstrated that systemic administration of SD208 decreases phosphorylation of SMADs in the collagenase model of GMH. The observed effect was dose dependent in that only high doses of TGFR-I inhibitor caused a significant decrease of the phosphorylated to nonphosphorylated SMAD 2/3 ratio.

We tested the effects of TGFR-I inhibition on the development of neurological deficits both at early and late time points in juvenile rats. For evaluation of the developmental profile in neonatal animals, we used negative geotropism and righting-reflex tests. Both of these tests have been previously described for early reflex locomotor testing in rats.23,24 In agreement with previous publications, we observed significant delays in the development of GMH animals, with spontaneous resolution of neurological deficits on day 3.16,25 Inhibition of TGFR-I had a dose-dependent effect on amelioration of GMH-induced neurological deficits in the short-term study.

Developmental delay predicts an impairment of neurological function both in premature born human infants and in animal models.26,27 In the long-term study, we also tested whether treatment with SD208 would ameliorate GMH-induced weight...
loss, which has been previously described. In agreement with our previous publication, we observed significant whole-body weight loss in GMH but not sham-operated animals. Both acute and delayed treatments showed a strong tendency to decrease whole-body weight loss. However, the difference between treated and untreated animals was not significant.

We investigated the relationship between the beneficial effects of TGFR-I inhibition observed in the short-term study on motor function and in the long-term study on spatial memory. The effects of TGF pathway inhibition on motor function in juvenile animals were investigated by the neurodeficit scales neuroscore and the foot fault test. GMH caused significant neurological deficits detectable by both of these tests. Both acute and delayed treatment ameliorated GMH-induced impairment of motor function. In addition, we confirmed that GMH caused spatial learning and memory deficits evaluated by water maze and T-maze tests, respectively. In the water maze, no differences during the cued (visible escape platform) learning ability between experimental groups were observed. During the spatial trials (submerged/hidden platform), however, GMH animals performed significantly worse. In addition, GMH animals demonstrated a loss of working memory as evaluated by the T-maze. Both acute and delayed treatments ameliorated GMH-induced learning and memory deficits. Although memory loss is considered the most prominent symptom of hydrocephalus and brain atrophy, there is an indication that TGF-β1 can cause significant disturbance in the learning process without changes in ventricular size. We evaluated brain atrophy by comparison of brain to body ratio between treated and untreated animals. We also used volumetric analyses for calculation of brain tissue loss and ventricular dilation. We demonstrated that TGF-β pathway inhibition results in amelioration of brain atrophy and preservation of brain tissue, as well as decreased GMH-induced ventriculomegaly. Posthemorrhagic hydrocephalus results from dysregulation of cerebral spinal fluid production and reabsorption of ECM proteins. A pathological hallmark of hydrocephalus is overproduction and extensive deposition of these proteins. TGF-β stimulates the synthesis of ECM proteins and results in increased ECM protein accumulation. ECM protein degradation is also blocked by inhibition of the synthesis of proteases and increased synthesis of protease inhibitors. Previously, we demonstrated that collagenase-induced GMH caused significant upregulation of vitronectin at day 17 and 24 after GMH. Differences in GFAP levels between sham-operated and GMH animals reached statistical significance on day 10. We evaluated the effect of TGF pathway inhibition on the level of vitronectin and GFAP during days when level was maximal: day 24 for vitronectin and day 10 for GFAP. We demonstrated that TGF inhibition significantly decreased GMH-induced levels of both vitronectin and GFAP proteins. We did not evaluate the effect of TGF-β pathway inhibition on cerebral spinal fluid absorption directly, and this will remain a goal for future studies.

In conclusion, GMH increased the level of TGF-β1 and activated the TGF-β pathway in brain tissue. The activation of TGF-β pathway contributed to the development of brain injury after GMH. Inhibition of GMH-induced activation of the TGF-β pathway led to significant cognitive and motor improvement and attenuation of GMH-induced brain atrophy and development of hydrocephalus.

Disclosures
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I. Supplemental Material

Animals and Drugs

Pregnant Sprague–Dawley rats were purchased from Harlan Laboratories, (Indianapolis, IN). 92 P7 male and female rat pups were then randomly divided into the sham-operated (n=20), collagenase-injected vehicle-treated (n=44), collagenase-injected SD-208 treated (N=28) animals. For GMH induction, animals were anesthetized with 3% isoflurane and placed onto a stereotaxic frame. Isoflurane concentration was then reduced to 2%. The scalp area was sterilized and bregma was exposed. Using bregma as a reference point, the following stereotactic coordinates were measured: 1.8 mm (rostral), 1.5 mm (lateral). A burr hole (1mm) was drilled. A 27 gauge needle was inserted at a rate of 1 mm/min at the depth of 2.8 mm from the dura. Using a microinfusion pump (Harvard Apparatus, Holliston, MA) 0.3 units of clostridial collagenase VII-S (Sigma, St Louis, MO) in 0.5µl was infused through the Hamilton syringe. The needle remained in place for an additional 10 min after injection to prevent “back-leakage”. After the needle was removed, the burr hole was sealed with bone wax and the incision suture closed, and the animals were allowed to recover on a 37 °C heated blanket. Upon recovering from anesthesia, the animals were returned to their dams. Sham operation consisted of needle insertion alone without collagenase infusion.

Neurological Examination

The developmental profile was assessed for seven days after GMH induction by negative geotropism and righting reflex tests. For negative geotropism, the time needed to turn 180° after being placed head down on a 20° angle slope was recorded1. For righting reflex, the time required for the rat pups to completely rollover onto all four limbs after being placed on their backs was measured1. The maximum allotted time was 60 s per trial (3 trials/day) for these tests. The average value of all three trials was used for further evaluation.

Cognitive function

The animals were assessed using the water maze and T-maze tests.
The water maze was performed as described previously\textsuperscript{2, 3}. The apparatus consisted of a metal pool (110 cm in diameter), filled with water to within 15 cm of the upper edge, containing a small platform (11 cm in diameter) for the animal to climb onto, and swim path length was digitally analyzed by Noldus Ethovision tracking software (with a maximum of 60 s/trial). The cued trials, with the escape platform obviously visible from above the water's surface, measured general associative learning, sensorimotor abilities, and motivation to escape the water. The platform's location changed every other trial. The spatial trials measured spatial learning, with the platform submerged just below the water's surface, hidden but discoverable. The probe trials measured spatial memory by recording time spent in the target quadrant once the platform was removed.

The T-maze assessed short-term (working) memory ability\textsuperscript{4}. For each trial, the rat was placed into the stem (40 cm×10 cm) of the T-maze and allowed to explore until either the left or right arm was chosen. Following a sequence of 10 trials, the rate of spontaneous alternation (0\%=none and 100\%=complete; alternations/trial) was calculated. Test was performed on day 21.

**Motor function**

For motor function evaluation, neurodeficit scales and foot fault tests were performed on day 24.

- Neurodeficit scales quantifies the neurodeficit using a series of six tests measuring functional deficits: 1) proprioceptive limb placing, 2) lateral limb placement, 3) forelimb placement, 4) postural reflex, 5) back pressure towards edge, and 6) lateral pressure towards edge. Score 10 is considered as severe, 5 as moderate and 0 as no neurological deficits\textsuperscript{3}.
- Foot-fault testing. The number of complete limb missteps through wire-grid openings was documented per limb over 120 s while the animal explored an elevated wire (3 mm) suspension (20 cm×40 cm) grid\textsuperscript{5}.

**Western Blot**
Animals were euthanized with isoflurane and perfused transcardially with 100 ml of cold PBS. Hemispheres were isolated and stored at -80°C until analysis. The whole-cell lysates were obtained by gently homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Inc., sc-24948) and centrifuging (14,000 g at 4°C for 30 min). The supernatant was collected and the protein concentration was determined using a detergent compatible assay (Bio-Rad, Dc protein assay). Equal amounts of protein (30 µg) were loaded and subjected to electrophoresis on an SDS-PAGE gel. After being electrophoresed and transferred to a nitrocellulose membrane, the membrane was blocked and incubated with the primary antibody overnight at 4°C. The following primary antibodies were used:

- Rabbit polyclonal to TGFR1 1/500 (Millipore ABF17)
- Rabbit polyclonal to TGFβ1 1/500 (Abcam ab9758)
- Rabbit polyclonal to SMAD2+3 TGFβ1 1/200 (Abcam ab65847)
- Rabbit polyclonal to phosphorylated SMAD2+3 TGFβ1 1/1000 (Abcam ab63399)
- Rabbit monoclonal [EP873Y] to Vitronectin 1/20000 (Abcam, ab45139)
- Rabbit polyclonal to GFAP 1/50000 (Abcam, ab7260)

The same membrane was probed with an antibody against β-actin (Santa Cruz, 1:1000) for an internal control. Incubation with secondary antibodies (Santa Cruz Biotechnology) was done for 1 h at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham Biosciences, Arlington Heights, IL) and visualized with an imaging system (Bio-Rad, Versa Doc, model 4000). Data was analyzed using Image J software.

**Brain injury evaluation**

After neurological testing, animals were euthanized with isoflurane and perfused transcardially with 100 ml of cold PBS following 50 ml of 4% PBS. The brains were removed and the quantification of weight was performed using an analytical microbalance (model AE 100; Mettler Instrument Co., Columbus, OH) capable of 1.0 µg precision. Brains were post-fixed in 10% paraformaldehyde for 24 hours. Then brains were dehydrated in 30% sucrose for 3 days. Histopathological analyses used 10 µm thick coronal sections, caudally cut every 600 µm on a cryostat (Leica Microsystems LM3050S), then mounted and stained on poly-L-lysine-coated
slides. Morphometric analysis of cresyl violet slides involved computer-assisted (ImageJ 4.0, Media Cybernetics, Silver Spring, MD) hand delineation of the ventricle system and hemisphere (cortex, subcortex). The borders of these structures were based on criteria derived from stereologic studies using optical dissector principles\textsuperscript{3,6}. The volumes were calculated: \[ \text{Volume} = \text{Area of coronal section} \times \text{Interval} \times \text{Number of sections} \].

**Immunohistochemistry**

Samples were collected and brains were cut as described above.

Antigens were retrieved by 15 minutes incubation in 95º C. After blocking, the following primary antibodies were applied overnight by 4º C:

- Rabbit monoclonal [EP873Y] to Vitronectin 1/250 (Abcam, ab45139)
- Rabbit polyclonal to GFAP 1/500 (Abcam, ab7260)

Secondary antibodies were applied for one hour by room temperature.

*Imaging was performed in the LLUSM Advanced Imaging and Microscopy Core with support of NSF Grant No. MRI-DBI 0923559 (SM Wilson) and the Loma Linda University School of Medicine.*
II. Supplemental Figure

![Bar chart showing body weight comparison across different groups.]

- **Sham (N=8)**
- **Acute Treatment (N=8)**
- **Vehicle (N=8)**
- **Delayed Treatment (N=8)**

Each group is represented by a different pattern. The chart indicates the body weight in grams, with standard deviation bars.
III. Supplemental Figure Legend: SD 208 ameliorated GMH-induced body weight loss

24 days after GMH induction animals ( ) shown significant loss of body weight compared to sham-operated animals ( ). (* P<0.05 vs. sham) SD 208 acute treatment ( ) and delayed treatment ( ) ameliorated GMH-induced loss of body weight, however the difference in body weight between treated and untreated animals did not reach statistical significance.
IV. Supplemental References