Decorin prevents the development of juvenile communicating hydrocephalus

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In post-haemorrhagic and other forms of communicating hydrocephalus, cerebrospinal fluid flow and drainage is obstructed by subarachnoid fibrosis in which the potent fibrogenic cytokine transforming growth factor-β has been aetiologically implicated. Here, the hypothesis that the transforming growth factor-β antagonist decorin has therapeutic potential for reducing fibrosis and ventriculomegaly was tested using a rat model of juvenile communicating hydrocephalus. Hydrocephalus was induced by a single basal cistern injection of kaolin in 3-week-old rats, immediately followed by 3 or 14 days of continuous intraventricular infusion of either human recombinant decorin or phosphate-buffered saline (vehicle). Ventricular expansion was measured by magnetic resonance imaging at Day 14. Fibrosis, transforming growth factor-β/Smad2/3 activation and hydrocephalic brain pathology were evaluated at Day 14 and the inflammatory response at Days 3 and 14 by immunohistochemistry and basic histology. Analysis of ventricular size demonstrated the development of hydrocephalus in kaolin-injected rats but also revealed that continuous decorin infusion prevented ventricular enlargement, such that ventricle size remained similar to that in intact control rats. Decorin prevented the increase in transforming growth factor-β1 and phosphorylated Smad2/3 levels throughout the ventricular system after kaolin injection and also inhibited the deposition of the extracellular matrix molecules, laminin and fibronectin in the subarachnoid space. In addition, decorin protected against hydrocephalic brain damage inferred from attenuation of glial and inflammatory reactions. Thus, we conclude that decorin prevented the development of hydrocephalus in juvenile rats by blocking transforming growth factor-β-induced subarachnoid fibrosis and protected against hydrocephalic brain damage. The results suggest that decorin is a potential clinical therapeutic for the treatment of juvenile post-haemorrhagic communicating hydrocephalus.

Keywords: decorin; fibrosis; hydrocephalus; TGF-β; Smad
Introduction

It is well recognized that fibrosis in the subarachnoid space contributes to the development of many forms of communicating hydrocephalus, especially post-haemorrhagic hydrocephalus, by impairing CSF flow and reducing CSF drainage (Motohashi et al., 1995). Post-haemorrhagic hydrocephalus is a common complication of intraventricular haemorrhage in premature infants, which often requires permanent ventricular shunting to avert the development of severe and debilitating chronic neurological symptoms (Horinek et al., 2003). However, shunting is not an ideal treatment as malfunction after shunt obstruction and infection causes high revision rates (Shooman et al., 2009), contributing to the high frequency of residual neurological deficits (Resch et al., 1996). Accordingly, the introduction of a prophylactic therapy aimed at preventing the development of subarachnoid fibrosis immediately after a haemorrhage would constitute a significant advance in improving outcome for infants at risk of developing hydrocephalus.

Factors released during intraventricular haemorrhage induce inflammatory cell infiltration and subsequent subarachnoid fibrosis (Sajanti et al., 2001), which is characterized by excess production of extracellular matrix molecules (Suzuki et al., 1997; Sajanti et al., 1999; Cherian et al., 2004) and increased collagen turnover (Sajanti and Majamaa, 1999; Sajanti et al., 1999, 2000, 2001). After intraventricular haemorrhage, the fibrogenic cytokine, transforming growth factor-β1 (TGF-β1), is released from activated platelet α-granules (Assoian et al., 1983) and from monocytes (Wahl et al., 1989), to act as a mitogen and chemo-attractant for meningeal fibroblasts, upregulating the local production of fibroblastic extracellular matrix. TGF-β initiates intracellular signalling after binding to TGF-β R1 and R2 receptors (on multiple cell types including meningeal fibroblasts, ependymal and choroid plexus cells), by inducing phosphorylation of the receptor-activated Smad2 and Smad3. Phosphorylated Smad2/3 form a complex with Smad4 that translocates to the nucleus and regulates gene transcription (Wells, 2000; Lan, 2011). The Smad-signalling pathway is essential for inducing endogenous cytokine production and also activation of extracellular matrix gene expression by TGF-β and therefore is important in the induction of fibrosis (Verrecchia and Mauniel, 2002). Thus, studying levels of phosphorylated Smad2/3 in tissues is indicative of the levels of bioactive TGF-β.

Several lines of evidence implicate TGF-β1 in the development of post-haemorrhagic hydrocephalus, including: (i) CSF levels of TGF-β1 are higher in post-haemorrhagic hydrocephalus patients compared with the levels recorded in patients with both non-haemorrhagic hydrocephalus and haemorrhage without hydrocephalus (Kitazawa and Tada, 1994; Whitelaw et al., 1999; Flood et al., 2001; Douglas et al., 2009); (ii) ventriculomegaly is positively correlated with the intensity of TGF-β1 immunostaining in experimental neonatal post-haemorrhagic hydrocephalus (Cherian et al., 2004); and (iii) hydrocephalus develops both in transgenic mice overexpressing TGF-β1 in astrocytes (Galbreath et al., 1995) and after intrathecal injection of human recombinant TGF-β1 (Tada et al., 1994; Kanaji et al., 1997).

Decorin is a naturally occurring extracellular small leucine-rich proteoglycan whose multiple occurring extracellular interactions with extracellular matrix molecules, including fibronectin and collagen (Bidanset et al., 1992; Hocking et al., 1998; Reese et al., 2013), thrombospordin (Winnefelder et al., 1992), the complement component C1q (Krumdieck et al., 1992), growth factors (Yamaguchi et al., 1990) and growth factor receptors (lozzo and Schaefer, 2010), allow it to influence diverse cellular functions (reviewed by Neill et al., 2012a). Decorin binds to the epidermal growth factor receptor, the Met receptor and the toll-like receptors, suppressing growth (Moscato et al., 1998, 2000), inhibiting angiogenesis (Neill et al., 2012b) and modulating inflammatory responses (Moreth et al., 2012) in tumour and lesion microenvironments. Although in some particular circumstances decorin binding to TGF-β enhances growth factor activity (Takeuchi et al., 1994), inhibition of TGF-β activity after decorin binding is more widely reported and through this mechanism decorin indirectly suppresses inflammatory scarring in fibrotic diseases (Yamaguchi et al., 1990; Border et al. 1992, Kolb et al., 2001). Accordingly, in CNS lesions, decorin reduces the deposition of chondroitin sulphate proteoglycans, fibronectin and laminin, and suppresses astrocytic, microglial and macrophage reactions in and around the wound (Logan et al., 1999a; Davies et al., 2004). Similarly, immuno-neutralization of TGF-β2 suppresses inflammation and scarring in brain lesions (Logan et al., 1999b). These findings suggest that decorin could be a potential therapeutic for inhibiting TGF-β-induced post-haemorrhagic fibrosis.

Here, using a rat model of juvenile communicating hydrocephalus, we have tested the hypothesis that sustained intraventricular decorin infusion both arrests the development of hydrocephalus, by reducing inflammation acutely and inhibiting the development of TGF-β-induced subarachnoid fibrosis, thereby preventing the brain pathology that is induced by hydrocephalus.

Materials and methods

Experimental animals

Three-week-old Sprague-Dawley rats (Charles River) were housed in liters in individual cages, kept under a 12h light/dark cycle with free access to food and water. Animals were monitored for adverse effects of treatments, such as distress, lethargy, weight loss and seizures, and any animals showing severe adverse effects were euthanized. All efforts were made to minimize discomfort and the number of animals used (see ‘Statistical analysis’ section below). The acute 3-day time point experiment was carried out at the University of Birmingham in accordance with the Animals and Scientific Procedures Act 1986, licensed by the UK Home Office and approved by the University of Birmingham Ethics Committee. The chronic 14-day time point experiment was conducted at the University of Utah in accordance with the guidelines of the National Institutes of Health Care and Use of Laboratory Animals and approved by the University of Utah Ethics Committee.

Experimental design

Kaolin (an aluminium silicate) was injected into the basal cistern to induce communicating hydrocephalus. Immediately after kaolin...
injection, osmotic pumps were implanted subcutaneously and connected through a catheter to a cannula placed in the right lateral ventricle for continuous intraventricular infusion of either decorin or PBS vehicle for either 3 or 14 days. For the chronic 14-day experiment, rats were randomly assigned to four groups: (i) intact age-matched controls (‘intact group’, n = 4), with no kaolin injection and no intraventricular infusion to obtain baseline data; (ii) basal cistern kaolin injections only (‘kaolin group’, n = 8) to evaluate the effects of subarachnoid kaolin without intraventricular cannulation and agent delivery; (iii) kaolin injection with intraventricular infusion of PBS (‘kaolin + PBS’ group; n = 8) to determine the effect of vehicle delivery and ventricular cannulation on kaolin-induced subarachnoid fibrosis and ventriculomegaly; and (iv) kaolin injection with intraventricular infusion of decorin (‘kaolin + decorin’ group; n = 8) to measure the effects of decorin on kaolin-induced TGF-β levels, subarachnoid fibrosis, ventricular enlargement and brain damage caused by hydrocephalus. For the acute 3-day experiment, rats were randomly assigned to the same four groups: intact group n = 4, kaolin group n = 5, kaolin + PBS group n = 3 and kaolin + decorin group n = 5. In the acute 3-day experiment rats were sacrificed 3 days after kaolin injection, the brains removed and processed for histology and immunohistochemistry to assess the inflammatory response to treatment. For the chronic 14-day experiment in vivo MRI was conducted after 14 days of treatment to assess the extent of hydrocephalus before sacrifice, followed by immunohistochemical analyses of the brain.

Induction of communicating hydrocephalus

The method of basal cistern injection of kaolin has been described by Li et al. (2008) and used in several subsequent studies (Wagshul et al., 2009; Nagra et al., 2010; Rashid et al., 2012). Anaesthesia was induced with 5% and maintained with 1–3% isoflurane (Vet one, MWI Veterinary Supply) throughout the procedure. Using aseptic techniques, the ventral atlanto-occipital membrane was exposed through a mid-line ventral cervical cutaneous incision, and a 30 gauge needle, angled at 30–45°, inserted into the subarachnoid space of the basal cistern. The needle was advanced 1–2 mm under the occipital bone and 30 μl of 20% kaolin solution (200 mg/ml; Fisher Scientific) in 0.9% sterile saline injected over a period of 15 s. After incision closure, rats were either allowed to recover (for the kaolin group) or transferred to a stereotactic frame (David Kopf Instruments) for osmotic pump and intraventricular cannula implantations.

Implantation of osmotic pumps

Osmotic pumps (model 2002, Alzet, Directer Corporation) were prepared under sterile conditions, filled with either 5 mg/ml human recombinant decorin (Galacorin™, Catalent/Pharma Solutions) or 10 mM phosphate buffered saline (PBS) pH 7.4 (Sigma-Aldrich) vehicle and primed in sterile saline overnight at 37°C. Pumps were adapted for MRI by replacing the metal flow moderator with PEEK tubing and using a customized 5 mm long PEEK brain cannula (Plastics 1) attached to the pedestal. During the operation the surgeon was blinded to the treatment delivered. After placing the rat in a stereotactic frame, the dorsal skull was exposed and a burr hole site in the parietal bone using co-ordinates 1 mm posterior and 1.5 mm lateral to Bregma. The cannula was inserted into the right lateral ventricle and fixed in place with glue and bone cement (Biomet UK Ltd) to a stabilizing screw. The osmotic pump was connected to the cannula and implanted subcutaneously in the neck region. Over the subsequent 14 days, human recombinant decorin was infused at a rate of 2.5 μg/0.5 μl/h. Dosing was based on previous experiments where decorin was infused into the lateral ventricle to inhibit the development of scar-related axon growth inhibitory molecules in the injured spinal cord (Minor et al., 2011).

Tissue preparation for histology

Rats were killed and immediately perfused transcardially with PBS followed by 4% paraformaldehyde (Alfa Aesar) in PBS at pH 7.4. Brains were immersed in 4% paraformaldehyde overnight at 4°C, cryoprotected by sequential immersion in 10%, 20% and 30% sucrose solutions in PBS at 4°C, embedded in optimum cutting temperature embedding matrix (Fisher Scientific), and coronal sections 15-μm thick cut on a Bright cryostat (Bright Instrument) and stored at -20°C before staining.

Antibodies

Extracellular matrix deposition was assessed using antibodies against fibronectin (rabbit, 1:200, Sigma-Aldrich) and laminin (rabbit, 1:200, Sigma-Aldrich, L9393). For analysis of brain pathology, we used the antibodies recognizing glial fibrillary acidic protein (GFAP, mouse, 1:500, Sigma-Aldrich, G3893) for astrocytes, ED-1 (mouse, 1:400, Serotec, MCA341R) and OX-42 (mouse, 1:500, Serotec, MCA275R) for macrophages and microglia, and RECA-1 (mouse, 1:200, Serotec, MCA970R) to label endothelial cells. We investigated decorin’s distribution and action using antibodies against human decorin (mouse monoclonal raised against the recombinant full length protein corresponding to amino acids 1-360 of human decorin, 1:400, Abcam, ab54728), TGF-β1 (rabbit polyclonal raised against a peptide mapping the C-terminus of TGF-β1, 1:200, Santa Cruz Biotechnology, sc-146) and phosphorylated Smad2/3 (pSmad2/3, rabbit polyclonal raised against a short amino acid sequence containing phosphorylated Ser423 and Ser425 of Smad3, 1:200, Santa Cruz Biotechnology, sc-11769-R).

Immunohistochemistry

For fluorescence labelling, sections were washed in PBST (PBS with 0.1% Triton™ X-100), blocked in PBST containing 2% bovine serum albumin and 15% normal goat serum for 20 min at room temperature, and then incubated in primary antibody solution (PBS with 2% bovine serum albumin) either at 4°C overnight or at room temperature for 1 h. After washing in PBST, sections were incubated with appropriate Alexa Fluor® 488 or 594 labelled secondary antibodies (Life Technologies) in secondary antibody solution (PBST with 2% bovine serum albumin and 1.5% normal goat serum) for 1 h at room temperature in the dark. Finally, sections were washed in PBST before mounting in Vectashield™ containing the nuclear stain DAPI (Vector Laboratories). For TGF-β1 and ED-1 co-immunostaining, TGF-β1 was detected using a biotinylated secondary antibody (Vector Laboratories) and streptavidin conjugated to Alexa Fluor® 488 (Life Technologies).

For immunoperoxidase staining, sections were incubated with 0.3% H2O2 (70% methanol in PBS) for 30 min to inhibit endogenous peroxidase. Sections were washed in PBS before blocking in PBS containing 2% bovine serum albumin and 15% normal serum for 1 h at room temperature, and then incubated in primary antibody solution at 4°C overnight. Sections were again washed in PBST before incubation in biotinylated secondary antibody solution (Vector Laboratories) for 30 min at room temperature. Sections were washed in PBST and then...
incubated for 30 min at room temperature in Avidin/Biotin complex (ABC; Vectastain Elite ABC kit, Vector Laboratories) following the manufacturer’s instructions. After rinsing in PBST, sections were treated with 3′3′ diaminobenzidine (DAB) substrate (Vector Laboratories), washed in deionized water, counterstained with Mayer’s haematoxylin, washed in running water before dehydration through an ascending series of alcohol concentrations, cleared in Histo-Clear (Fisher Scientific) and mounted in Vectamount medium (Vector Laboratories). No primary antibody control was performed for each antibody to evaluate background non-specific staining levels. Antibody specificities were confirmed by staining sections with alternative antibodies (mouse monoclonal TGF-β1 antibody, Abcam, ab27969) or by dot blot analysis using immunizing proteins (pSmad2/3 and decorin antibodies). Stained sections were viewed under an Axioplan 2 imaging microscope (Carl Zeiss) and images were captured under identical conditions for each antibody using an AxioCam HRc (Carl Zeiss).

Quantification of ED-1, GFAP, TGF-β1 and pSmad2/3 immunostaining

Images were analysed semiquantitatively using ImageJ analysis software (Abramoff et al., 2004). For evaluation of ED-1 immunofluorescence staining in the 3 day time point experiment, a composite image of the basal subarachnoid space was created using a series of images captured at ×100 from a posterior coronal section of the brain (5.8 mm post-Bregma). A region of interest was drawn around the subarachnoid space and the percentage of pixels that were immunofluorescent above a set threshold was calculated. For evaluation of GFAP immunofluorescence staining, nine regions of interest (three regions × three coronal sections) per rat were selected from images of the corpus callosum and periventricular white matter (Fig. 1A), and the percentage of immunofluorescent pixels above a set threshold calculated. For evaluation of TGF-β1 and pSmad2/3 immunoperoxidase staining in the ependyma, 12 regions of interest (four regions × three coronal sections) per rat were selected from images of the lateral ventricular ependyma (Fig. 1B), and the mean pixel intensity (arbitrary units of pixel intensity) above background threshold (established using the no primary antibody control sections) was calculated.

Quantification of inflammatory cells in the subarachnoid space

To evaluate the acute inflammatory response in the subarachnoid space, the number of eosinophils, neutrophils and macrophages were estimated on two posterior coronal sections per rat stained with haematoxylin and eosin. Up to 50 × 1000 images (93.16 × 69.82 µm) were taken at random within the subarachnoid space (the number depending upon the total area of the subarachnoid space in the section) and the number of cells within each image was counted manually. Eosinophils contained bright pink granules in the cytoplasm, neutrophils had multi-lobed nuclei and pale pink cytoplasm and macrophages had an acientic large round or kidney shaped nucleus surrounded by light bluish cytoplasm (Fig. 7C). Macrophages could only be definitively identified at the edge of the kaolin deposits and were therefore probably under-represented in the counts. Also cells that could not be positively identified by these criteria were classed as unidentified. The relative frequency of each cell type was represented as a percentage of the total number of cells.

Magnetic resonance imaging and ventricular measurements

MRI studies were conducted 14 days after injection of kaolin into the basal cistern using a Bruker Biospec 7T scanner equipped with a high performance gradient system (with 600 mT/m maximum gradient amplitude, and 0.25 ms rise time). Animals were anaesthetized using 1–3% isoflurane and their vital signs (respiration, rectal temperature, heart rate and oxygen saturation percentage) were continuously monitored using a magnetic resonance-compatible physiological monitoring system (SA Instruments). Animals were placed in a 72-mm volume coil for signal transmission, and a quadrature surface coil that was placed on the animal’s head was used for signal reception. T2-weighted scans were acquired using rapid acquisition with relaxation enhancement (RARE) sequence with 4000 ms repetition time, 40 ms effective echo time, eight echoes per image, four averages, 30 coronal slices, 0.75 mm slice thickness, 2.5 cm × 2.5 cm field-of-view, and 98 µm × 98 µm in-plane resolution.

The Evan’s ratio (ratio of the greatest width of both lateral ventricles to the greatest width of the brain) was measured in one coronal MRI scan at the level of the foramen of Monro (Fig. 1C). The Evan’s ratio is used clinically to diagnose hydrocephalus and a ratio >0.3 is classed as hydrocephalic (Shprecher et al., 2008). Total ventricular volume was estimated by determining total ventricular area of the lateral, third and fourth ventricles, using ImageJ, in 15 sequential coronal MRI scans (Fig. 1D) and multiplying this value by 0.75 (distance in mm between each scan).

Statistical analysis

The ‘resources equation’ was used to confirm that the appropriate number of animals were included in this study (NC3Rs, 2012). The Shapiro-Wilk test was used to evaluate the normal distribution of the data. This test indicated that TGF-β1 and pSmad2/3 data were normally distributed and therefore were analysed by one-way ANOVA and post hoc Tukey tests. Similarly, after transformation (Log_{10}), ventricular volume, Evan’s ratio and GFAP data were shown to be normally distributed and analysed by one-way ANOVA. Data are reported as group means ± standard error of the mean (SEM). In the chronic 14-day experiment, eight rats were excluded from the final analyses because they either did not develop hydrocephalus or because they died before the end of the study. Details of these are provided in the online Supplementary material. Equal numbers of rats died in decorin and non-decorin treated groups, suggesting that the deaths were not decorin-related. Residual numbers in each group that were included in the analyses were as follows: intact n = 4, kaolin n = 5, kaolin + PBS n = 6, and kaolin + decorin n = 5.

Results

Development of communicating hydrocephalus in juvenile rats

The model used in this study was first characterized in adult rats but had not previously been optimized in neonatal or juvenile rats. In the present study hydrocephalus developed in 82% of juvenile rats in the kaolin and kaolin + PBS groups, which is
comparable to the induction rate reported in adult rats by Li et al. (2008). Non-responders were excluded from further analyses, as a criterion for study inclusion was the induction of hydrocephalus. MRI demonstrated that, in the kaolin and kaolin + PBS groups, the lateral ventricles enlarged bilaterally through all anterior–posterior levels (Fig. 2A) and had Evan’s ratios >0.3. Ventricular volumes in the kaolin and kaolin + PBS groups were similar, indicating that cannulation of the ventricles did not affect the development of ventriculomegaly. Oedema was often observed posteriorly in the corpus callosum and periventricular white matter (Fig. 2A). In all groups the aqueduct of Sylvius and foramina of Luschka were patent, confirming the induction of communicating hydrocephalus and large kaolin deposits could be seen in the basal subarachnoid space in the kaolin and kaolin + PBS groups.

**Magnetic resonance imaging demonstrated that decorin prevented the development of communicating hydrocephalus**

After basal cistern injection of kaolin, ventricular volumes were significantly enlarged ($P < 0.001$) in the kaolin (155.7 ± 31.9 mm$^3$) and kaolin + PBS (198.3 ± 92.9 mm$^3$) groups, compared with normal ventricular volumes measured in the intact group (10.9 ± 1.0 mm$^3$). Also the Evan’s ratio was significantly greater ($P < 0.05$) in the kaolin (0.39 ± 0.02) and kaolin + PBS (0.38 ± 0.04) groups compared with the intact group (0.27 ± 0.02). Decorin treatment prevented ventricular enlargement, so that the ventricular volume and the Evan’s ratio in the
kaolin + decorin group (21.1 ± 5.4 mm³ and 0.27 ± 0.01, respectively) were indistinguishable from the normal values of the intact group (Fig. 2B and C). Thus, continuous decorin infusion prevented the development of hydrocephalus after basal cistern injection of kaolin.

**Decorin was delivered throughout the entire ventricular system and basal subarachnoid space**

MRI located the cannula in the frontal horn of the right lateral ventricle (Fig. 2A). There was no positive human decorin staining in the brains of intact, kaolin, kaolin + PBS and kaolin + decorin groups, as illustrated in the intact group (Fig. 3A). By contrast, in the kaolin + decorin group, decorin staining was observed throughout the ventricular system on the apical surfaces of the choroid plexus epithelium and lateral ventricle ependymal cells (Fig. 3B and C). Decorin staining was also abundant in the basal cisterns through the entire rostral-caudal axis of the brain, especially in the basement membranes of subarachnoid vessels and in the meninges over the cortex (Fig. 3D). The wide distribution of decorin throughout the subarachnoid space confirmed successful delivery.

**Decorin reduced TGF-β1 staining intensity in the ependyma**

In the intact group, there was weak cytoplasmic TGF-β1 staining of the ependymal and choroid plexus epithelium cells in the lateral ventricles (Fig. 4A), and strong staining of meningeal cells and cells of the ependyma in the third ventricle (not shown). In the kaolin and kaolin + PBS groups, there was more intense TGF-β1 staining in ependymal and sub-ependymal cells (Fig. 4B and C), macrophages and fibroblasts within sites of subarachnoid fibrosis (Fig. 6) and in occasional astrocytes and oligodendrocytes in the corpus callosum (data not shown). The intensity of ependymal TGF-β1 staining was also reduced in the kaolin + decorin group (Fig. 4C).
staining (arbitrary units of pixel intensity) in the kaolin (40.5 ± 4.2) and kaolin + PBS (44.0 ± 5.6) groups was statistically greater (P < 0.05) compared with the intact group (21.6 ± 2.3). The intensity of TGF-β1 staining in the ependyma in the kaolin + decorin (23.3 ± 1.2) group was indistinguishable from that in the intact group, indicating widespread antagonism of the cytokine by decorin (Fig. 4D and E). There was also a high frequency of TGF-β1-positive macrophages in areas of subarachnoid fibrosis in the kaolin and kaolin + PBS groups, but numbers were much lower in the kaolin + decorin group (Fig. 4F). Thus, decorin prevented both the increase in ependymal TGF-β1 and the ingress of TGF-β1-positive macrophages into the subarachnoid space after kaolin injection into the basal cistern.

Decorin reduced Smad2/3 phosphorylation in the ependyma

The widespread bioactivity of intraventricular decorin against TGF-β1 was also reflected by differences in elements of the TGF-βR signalling cascades. Accordingly, patterns of pSmad2/3 staining of meningeal, choroid plexus epithelial and ependymal cells were similar to those of TGF-β1 staining. Thus, ependymal cell pSmad2/3 staining was weak and cytoplasmic in the intact group and more intense, especially at apical surfaces of ependymal cells in the lateral ventricles of the kaolin + decorin (37.7 ± 2.7) and kaolin + PBS (36.8 ± 0.8) groups was statistically greater (P < 0.001) compared with the intact group (15.2 ± 3.5). The intensity of pSmad2/3 staining in the ependyma in the kaolin + decorin (19.0 ± 3.0) group was indistinguishable from the intact group (Fig. 5E). This finding suggests that decorin suppressed TGF-β receptor activation and the induction of downstream Smad2/3 signalling.

Decorin reduced fibrosis in the subarachnoid space

In the intact group, laminin immunostaining was present in thin strands of extracellular matrix in the subarachnoid space and

Figure 3 Distribution of human decorin in the brain and subarachnoid space in the kaolin + decorin group. No human decorin was detected in the lateral ventricles of intact rats (A). In the kaolin + decorin group, decorin staining (red) was observed on the apical surfaces of the choroid plexus epithelial and ependymal cells in the ipsilateral (B) and contralateral ventricles (C). Decorin staining was also detected in the basal subarachnoid space (D), with intense staining in the basement membranes of the arachnoid vasculature (arrow). DAPI (blue) was used as a generic nuclear marker. Scale bar = 100 μm. CP = choroid plexus; E = ependyma; BV = blood vessel.
vascular basement membranes. Laminin and GFAP co-immunostained the glial limitans externa (Fig. 6A). In both the kaolin and kaolin + PBS groups, the deposition of laminin extended throughout the basal subarachnoid space into which GFAP-positive astrocytic processes infiltrated (Fig. 6A) where the glial limitans externa was disrupted. The laminin staining in the subarachnoid space of the kaolin + decorin group was similar to that seen in the intact group, largely confined to the glial limitans and vascular basement membranes. Laminin deposits were also present on the apical surface of ependymal cells in the kaolin and kaolin + PBS groups (data not shown), but not in the intact or kaolin + decorin groups.

A dense disorganized network of fibronectin was deposited in the fibrosed subarachnoid space of kaolin and kaolin + PBS groups but was absent from the intact and kaolin + decorin groups (Fig. 6B). Thus, decorin prevented extracellular matrix deposition and fibrosis in the subarachnoid space of kaolin-treated rats.

Decorin suppressed the inflammatory response in the subarachnoid space

At Day 3, the basal subarachnoid space in the kaolin and kaolin + PBS groups contained thick kaolin deposits associated with
a considerable accumulation of inflammatory cells including neutrophils, macrophages and eosinophils (Fig. 7A and C). In the kaolin + decorin groups, the subarachnoid space appeared smaller, containing fewer inflammatory cells and had smaller kaolin deposits (Fig. 7B) than that in the kaolin and kaolin + PBS groups. The relative percentages of eosinophils, neutrophils and macrophages in the subarachnoid space were similar between the kaolin (1.7 ± 0.6%, 22.2 ± 1.9% and 13.7 ± 0.9%, respectively), kaolin + PBS (3.2 ± 1.3%, 23.5 ± 2.1% and 20.1 ± 2.6%, respectively) and kaolin + decorin groups (5.4 ± 1.7%, 17.0 ± 4.3% and 14.8 ± 1.1%, respectively; Fig. 7E). However, the extent of ED-1-positive staining (expressed as the percentage of immunofluorescent pixels in the subarachnoid space) was lower in the kaolin + decorin group (10.6 ± 1.0%) compared with the kaolin (19.3 ± 3.7%) and kaolin + PBS groups (18.6 ± 3.5%; Fig. 7F–H). At Day 14 in the kaolin and kaolin + PBS groups, the basal subarachnoid space contained smaller residual kaolin deposits and the main inflammatory cells present were macrophages, with few scattered accumulations of eosinophils and neutrophils. In the kaolin + decorin group the subarachnoid space was largely devoid of kaolin deposits and inflammatory cells (data not shown). These findings at Day 14 are consistent with the observation of reduced acute phase inflammation in the decorin-treated brains and demonstrate that decorin treatment suppressed the kaolin-induced inflammatory response from as early as Day 3.

Decorin protected against hydrocephalus-induced brain damage

After induction of hydrocephalus, the levels of GFAP immunostaining (percentage of immunofluorescent pixels per area) in both the corpus callosum and periventricular white matter were higher in the kaolin (7.0 ± 2.4% and 10.7 ± 1.9%, respectively) and kaolin + PBS (11.6 ± 4.7% and 12.8 ± 4.40%, respectively) groups compared with the intact group (1.7 ± 0.7% and 1.9 ± 0.4%, respectively). The levels of GFAP immunostaining in the kaolin + decorin group (1.0 ± 0.3% and 1.4 ± 0.4%, respectively) were similar to the normal levels observed in the intact group (Fig. 8A and B). In the corpus callosum, perivascular basement membrane laminin staining was increased in the kaolin and kaolin + PBS groups compared with the kaolin + decorin group (Fig. 9A). Also, in the kaolin and kaolin + PBS groups, OX-42-positive microglia accumulated in areas adjacent to ependymal rupture in the walls of the expanded lateral ventricles (Fig. 9B) demonstrating a distinctive amoeboid morphology with shortened, thicker processes characteristic of activated microglia. Numerous ED-1-positive haematogenous macrophages accumulated in areas of fibrosis in the subarachnoid space in the kaolin and kaolin + PBS groups but very few macrophages immigrated into the subarachnoid space of the kaolin + decorin group (Fig. 6B). These findings are consistent with the hypothesis that decorin protects against ventriculomegaly and the development of hydrocephalus-associated pathology.

Discussion

We have shown that continuous intraventricular delivery of human recombinant decorin prevented the development of ventriculomegaly in a kaolin model of juvenile communicating hydrocephalus. Moreover, decorin treatment was associated with reduced laminin and fibronectin deposition, inflammation and...
astrogliosis, showing that decorin prevents the development of fibrosis and protects against hydrocephalic brain damage. Furthermore, we have demonstrated in our hydrocephalic animals that the kaolin-induced increase in subarachnoid fibrosis was associated with raised levels of TGF-β/Smad2/3 signalling, whereas decorin treatment suppressed a key stimulus for the development of subarachnoid fibrosis, maintaining normal levels of TGF-β-Smad2/3 signalling. These findings support our hypothesis that the arrested development of subarachnoid fibrosis, hydrocephalus and associated brain pathology are to some extent causally linked with decorin-mediated blockade of TGF-β signalling, despite the range of decorin influences described in the introduction.

MRI analysis confirmed that a basal cistern injection of kaolin induced hydrocephalus in 3 week old rats, and our immunohistochemistry analyses demonstrated that fibronectin and laminin extracellular matrix accumulated in the subarachnoid space. Comparison of the kaolin and kaolin + PBS group data confirmed that the implanted intraventricular cannula did not ameliorate ventricular enlargement by shunting CSF subcutaneously. The combined incidence of induction of hydrocephalus in the kaolin and kaolin + PBS groups was 87%, giving a projected incidence of induction of hydrocephalus in the kaolin + decorin group (n = 5) of 4.3 rats. Thus, we attribute the normal ventricular volume in the kaolin + decorin group to decorin-induced suppression of

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**Figure 6** Fibrosis in the subarachnoid space. Images of coronal sections illustrating areas of fibrosis in the subarachnoid space. In (A) and (B), the upper rows show sections taken at the level of the Bregma, lower rows at a level 5 mm posterior to the Bregma (inserts); boxed areas in the inserts indicate the areas of subarachnoid space selected for analysis and illustrated in each row. (A) Representative images showing more laminin (red) staining in areas of fibrosis in the basal subarachnoid in the kaolin and kaolin + PBS groups compared with the intact and kaolin + decorin groups. GFAP-positive (green) astrocytic processes (arrows) penetrated fibrotic areas. (B) Fibronectin (green) staining was increased in the fibrosed subarachnoid space of kaolin and kaolin + PBS groups compared with the intact and kaolin + decorin groups. ED1-positive macrophages (red) amassed in fibrotic areas in the kaolin and kaolin + PBS groups but few invaded the subarachnoid space in the kaolin + decorin group. DAPI (blue) was used as a generic nuclear marker. Scale bars = 100 μm. BV = blood vessels; ON = optic nerve; SAS = subarachnoid space.
The acute inflammatory response 3 days after kaolin injection. Representative composite images of the basal subarachnoid space in the kaolin + PBS (A) and kaolin + decorin (B) groups (haematoxylin and eosin). The subarachnoid space in the kaolin and kaolin + PBS groups contained thicker kaolin deposits and greater accumulations of inflammatory cells compared with the kaolin + decorin group. (C) At 3 days after kaolin injection, eosinophils (white arrow), neutrophils (white arrowhead) and macrophages (black arrow) could be identified in the subarachnoid space. Phagocytosis of the kaolin granules (black arrows) by macrophages had already begun at this early time point (D). (E) Histogram of the percentage of eosinophils, neutrophils and macrophages in the subarachnoid space of kaolin, kaolin + PBS and kaolin + decorin groups showing that the distribution of each cell type was similar between the three groups. (F) Histogram of the mean ± SEM percentage of ED-1-positive staining in the subarachnoid space in the four groups. ED-1 staining was increased in the kaolin and kaolin + PBS groups compared with the kaolin + decorin group. Representative composite images of ED-1 staining (red) in the subarachnoid space of the kaolin + PBS (G) and kaolin + decorin (H) groups. DAPI (blue) was used as a generic nuclear marker. Scale bars: A, B, G and H = 500 µm; C = 20 µm; D = 10 µm. BV = blood vessels; CP = basal cerebral peduncle; SAS = subarachnoid space; 3 N = oculomotor nerve.
hydrocephalus by blockade of subarachnoid fibrosis and not to either shunting effects or failure of our kaolin injection technique.

The relevance of the kaolin model of communicating hydrocephalus to clinical post-haemorrhagic hydrocephalus has been demonstrated by observations that intraventricular or subarachnoid injection of blood generates a pattern of ventriculomegaly similar to that induced by kaolin (Suzuki et al., 1977; Cherian et al., 2004). After a haemorrhage or blood injection, the cisternal subarachnoid space is filled with erythrocytes embedded within a fibrin matrix containing TGF-β-rich platelets that evoke an inflammatory response characterized by the recruitment of leukocytes (Dumont et al., 2003), the invasion of cytokine expressing macrophages supplemented by the transformation of pial cells into macrophages that phagocytose erythrocytes and debris (Jackowski et al., 1990), and the production of extracellular matrix by meningeal fibroblasts (Sajanti et al., 1999). Our study corroborates the findings of Slobodian et al. (2007), demonstrating that kaolin injection causes acute inflammatory cell infiltration into the subarachnoid space, with subsequent phagocytosis of the kaolin granules by macrophages and deposition of extracellular matrix. The suppressive effect of decorin on the inflammatory response is seen as early as Day 3, with reduced numbers of inflammatory cells populating the subarachnoid space. This is in agreement with a study by Logan et al., (1999b) demonstrating that after a cerebral lesion, continuous decorin treatment reduces the number of ED-1-positive macrophages in and around the

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**Figure 8** Astrogliosis in hydrocephalic brains (A) Representative images of GFAP-positive staining (green) in the corpus callosum in the four treatment groups (taken in the area highlighted in Fig. 1A). (B) Histogram of the mean ± SEM percentage GFAP positive staining in the corpus callosum and periventricular white matter in the four groups. Above normal levels of GFAP were observed in the kaolin and kaolin + PBS groups, whereas normal constitutive levels were maintained in the kaolin + decorin group. DAPI (blue) was used as a generic nuclear marker. Scale bar = 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 9  Tissue damage in hydrocephalic brains. (A) In the corpus callosum of the kaolin and kaolin + PBS groups there was intense perivascular laminin staining (red) in RECA-1 positive (green) vessels (white arrows) compared with the weak laminin staining seen in the vasculature of the kaolin + decorin group. (B) Increased OX-42 (red) staining reflecting microglia activation, especially in areas where the ependyma was lost in the expanding lateral ventricles of the kaolin and kaolin + PBS groups, was not apparent in the brains of the kaolin + decorin group. DAPI (blue) was used as a generic nuclear marker. Scale bars: A = 100 μm; B = 50 μm.
wound site. In addition, Xaus et al. (2001) and Comalada et al. (2003) reported that decorin inhibits macrophage proliferation through p27kip1 and increases their activation by preventing TGF-β induced inhibition. Together, these results suggest that the anti-inflammatory actions of decorin are achieved by both reducing the number of ED-1-positive macrophages and increasing their efficiency to clear debris from the subarachnoid space.

Low TGF-β levels occur in the CSF and brain parenchyma of healthy humans (Mogi et al., 1995; Krupinski et al., 1996; Flood et al., 2001) and rats (Unsicker et al., 1991; Logan et al., 1992). TGF-β1 levels are increased in the meninges, subarachnoid macrophages and choroid plexus after kaolin injection into the cisterna magna (Hatta et al., 2006), in the CSF and choroid plexus of post-haemorrhagic hydrocephalus patients, (Kitazawa et al., 1994; Whitelaw et al., 1999; Flood et al., 2001; Heep et al., 2004; Douglas et al., 2009) and in experimental intraventricular haemorrhage (Cherian et al., 2004). In this study, the kaolin and kaolin + PBS groups showed upregulation of the TGF-β/Smad signalling throughout the ventricular system including the choroid plexus, suggesting that raised CSF levels of TGF-β mediate the progression from meningeal inflammation through to subarachnoid fibrosis. Although the Smad intracellular signalling pathway is central to the actions of the TGF-β family, TGF-β also signals through the mitogen-activated protein kinase (MAPK), Rho-like GTPase and phosphatidylinositol-3-kinase (PI3K)/AKT signalling pathways, which mediate a wide range of other cellular functions including epithelial-mesenchymal transition, activating fibroblasts and promoting extracellular matrix production (Zhang, 2009; Nakera and Trojanowska, 2012). For example, in an experimental model of intraventricular haemorrhage, phosphorylated MAPK levels are increased in the ependymal and sub-ependymal glia (Cherian et al., 2004) providing a possible alternative supplementary fibrogenic signalling pathway in hydrocephalus. In the studies described here, decorin suppression of subarachnoid deposition of fibronectin and laminin after kaolin injection was linked to the maintenance of constitutive levels of TGF-β1 and downstream Smad signalling. As decorin sequestration of TGF-β into extracellular matrix blocks all forms of TGF-β signalling, suppression of activation of non-Smad pathways also probably occurred in these studies.

Aquilina et al. (2008) and Hoque et al. (2011) have reported no protection against ventriculomegaly in a neonatal rat model of intraventricular haemorrhage after administration of the TGF-β antagonists perifenidone losartan, and colchicine (administered by gavage) and decorin (injected into the lateral ventricle 1 and 6 days after the induction of hydrocephalus). One explanation for these findings could be that, since the former three reagents target TGF-β transcription, they were ineffective in neutralizing translated TGF-β stored in platelet granules and released on haemorrhage. Platelet release accounts for the initial phase of the biphasic TGF-β surge in the CSF (Flood et al., 2001; Douglas et al., 2009), which initiates TGF-β-mediated subarachnoid fibrosis. Macrophages and monocytes subsequently immigrate into the CSF and release a second wave of TGF-β that is supplemented by a sustained supply from ependymal, choroid plexus epithelial and meningeal cell sources (Wahl et al., 1989; Flood et al., 2001).

The two-bolus injection regime of decorin, used by Hoque et al. (2011), was unlikely to have blocked sustained high levels of TGF-β production, as our findings indicate that continuous delivery of decorin was required for effective neutralization of TGF-β signaling in our kaolin model of communicating hydrocephalus. The results presented here clearly suggest that initiation of decorin delivery immediately after basal cistern kaolin injection suppressed the development of hydrocephalus by blocking TGF-β signaling from the outset as a prophylactic treatment and thus maintained normal ventricular volumes.

Hydrocephalic brain damage developing from multiple insults, including compression, stretch, ischaemia and hypoxia, ultimately leads to periventricular oedema, demyelination, axonal degeneration, metabolic impairments, accumulation of metabolic waste products, gliosis and inflammation (Silva, 2004; McAllister, 2012). In the thinned corpus callosum and periventricular white matter of our hydrocephalic rats, MRI detected oedema and immunohistochemistry demonstrated increased perivascular laminin and astrocytic GFAP staining. Similar increases in perivascular laminin staining have been seen in hydrocephalic transgenic mice overexpressing TGF-β1 (Wyss-Coray et al., 1995) and in blood vessels local to brain lesions (Szabo and Kalman, 2004), suggesting similar mechanisms may affect the integrity of the blood–brain barrier and contribute to the development of oedema in both these conditions. Meningeal inflammation and progressive reactive astrogliosis and microgliosis in the periventricular matter are prevalent features of hydrocephalus (Fukumizu et al., 1996; Khan et al., 2006; Del Bigio, 2010a; McAllister, 2012).

Reactive astrogliosis in the brain is reflected by an upregulation of GFAP and hypertrophy of the astrocytic processes. After CNS injury, activated astrocytes may be beneficial in the acute stages but detrimental in the later stages (Pekny and Nilsson, 2005). In hydrocephalus, ongoing reactive astrogliosis makes the brain less compliant, affecting shunt success, and could prevent axon regeneration and remyelination (McAllister, 2012). In the current study, GFAP-positive staining increased in the corpus callosum and periventricular white matter after the induction of hydrocephalus and correlated with the magnitude of ventricular enlargement irrespective of treatment. Our results extend previous research findings showing a positive correlation between the severity of ventriculomegaly and the messenger RNA and protein levels of GFAP (Yamada et al., 1992; Deren et al., 2010; Eskandari, et al., 2011; Xu et al., 2012). Unlike sustained intraventricular infusion of decorin that normalizes gliosis and prevents oedema, shunting reduces but does not restore GFAP to normal levels (Miller and McAllister, 2007; Eskandari et al., 2011). Ependymal disruption in the enlarged ventricles of our hydrocephalic rats was similar to that described by Del Bigio et al. (2010b) in post-haemorrhagic hydrocephalus and associated with underlying accumulation of reactive microglia.

In summary, continuous intraventricular decorin infusion prevents the development of kaolin-induced hydrocephalus by blockade of acute inflammation and TGF-β-mediated subarachnoid fibrosis, protecting against the development of hydrocephalic brain damage. As post-haemorrhagic hydrocephalus develops after a readily diagnosed haemorrhagic insult, initiating decorin delivery immediately after the bleed will block the effects of
TGF-β signalling from the outset as a prophylactic rather than a curative treatment. Therefore, our findings demonstrate that sustained intraventricular decorin may be a potential therapeutic treatment for preventing the development of acute juvenile post-haemorrhagic hydrocephalus and associated morbidity. Further studies are required to investigate whether decorin is also effective as a treatment of established hydrocephalus.

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Supplementary material

Supplementary material is available at Brain online.

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