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Thrombospondin 2-null mice display an altered brain foreign body response to polyvinyl alcohol sponge implants

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Abstract

Thrombospondin (TSP)-2 is a matricellular protein that participates in the processes of tissue repair and the foreign body response. In addition, TSP2 has been shown to influence synaptogenesis and recovery of the brain following stroke. In the present study we investigated the response following the implantation of polyvinyl alcohol (PVA) sponges in the brain. PVA sponges were implanted into the brain cortex of wild type and TSP2-null mice for a period of 4 and 8 weeks and the response was analyzed by histochemistry and quantitative immunohistochemistry. TSP2 expression was detected in the interstices of the sponge and co-localized with the extracellular matrix and astrocytes. PVA sponge invasion in TSP2-null mice was characterized by dense deposition of extracellular matrix and increased invasion of reactive astrocytes and macrophages/microglia. Furthermore, the angiogenic response was elevated and the detection of mouse serum albumin (MSA) in the brain cortex indicated excessive vessel leakage, suggesting that TSP2 plays a role in the repair/maintenance of the blood brain barrier. Finally, immunostaining demonstrated an increase in the levels of matrix metalloproteinase (MMP)-2 and MMP-9. Taken together, our observations support a role for TSP2 as critical determinant of the brain response to biomaterials.

1. Introduction

Thrombospondin (TSP)-2 is a matricellular protein with anti-angiogenic activity that has been shown to participate in the processes of wound healing, sponge granuloma formation and the foreign body response (Bornstein *et al* 2000, Kyriakides *et al* 1999, 2001). In these studies, TSP2 deposition could be observed several days following the interaction of tissues with biomaterials and observations in TSP2-null mice suggest that it has a role in modulating the ensuing response. Specifically, TSP2-null mice display increased angiogenesis, and deposition of irregularly assembled extracellular matrix within foreign body capsules (Kyriakides *et al* 1999).

In vitro and *in vivo* studies suggest that TSP2 can influence these processes, in part, by modulating the levels of matrix metalloproteinases (MMP)-2 and -9 (Yang *et al* 2000, Agah *et al* 2005, Puolakkainen *et al* 2005). Studies in mice that lack both TSP2 and its close homolog, TSP1, have identified additional roles for TSPs in nerve synaptogenesis and functional recovery after stroke (Christopherson *et al* 2005, Liauw *et al* 2008). In the central nervous system, TSP2 is expressed by immature astrocytes and has been shown to promote the formation of synapses (Lin *et al* 2003, Christopherson *et al* 2005). Furthermore, the expression of TSPs in the brain is dynamically regulated during brain development. Specifically, TSP2 expression is low in late embryonic brain, increases in postnatal brain, and is low or absent in

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the adult brain (Iruela-Arispe *et al* 1993). Moreover, TSP2 can also be re-expressed in reactive astrocyte and microglia after brain injury suggesting that it can participate in brain repair (Lin *et al* 2003, Moller *et al* 1996).

Implantation of biomaterials and devices in the brain is an emerging area of intervention for a variety of conditions including epilepsy and Parkinson's disease (Brown *et al* 2006, Polikov *et al* 2005). In addition, new advancements in neural recording and decoding have shown great promise toward the development of functional brain-machine interfaces. However, the long-term biocompatibility of brain-interfacing biomaterials remains largely unexplored. Furthermore, molecular determinants of the brain response to biomaterials have not been identified. At the cellular level, the foreign body reaction (FBR) in the brain is characterized by the presence of both reactive astrocytes, which form a glial scar, and activated microglia (Kim *et al* 2007, Polikov *et al* 2005, Tian *et al* 2005). It is believed that this response is the main cause of failure of biomaterials and devices in the brain (Fournier *et al* 2003, Polikov *et al* 2005). Based on the upregulation of TSP2 expression in several injury models, its participation in sponge granuloma formation, and its expression in astrocytes we hypothesized that it might play a role in the brain response to biomaterials. In the present study, we explored this possibility by investigating the response to PVA sponges implanted in the brain cortex of wild type and TSP2-null mice. We found that TSP2 is expressed during the invasion of brain tissue into PVA sponges and that TSP2 deficiency results in an altered brain foreign body response characterized by an enhanced inflammation, increased angiogenesis and abnormal blood brain barrier function. These alterations were associated with increased levels of MMP-2 and MMP-9.

2. Materials and methods

2.1. Animal models

All animal studies were approved by the Institutional Animal Care and Committee of Yale University. TSP2-null (C57BL6/129SVJ) and littermate WT (C57BL6/129SVJ) mice were used for all experiments. Grade 3 PVA sponges (M-PACT, Eudora, KS; 0.1 mm thick and 2 mm diameter) were sterilized under UV light and soaked in endotoxin-free phosphate-buffered saline for 48 h. WT and TSP2-null 3-mo old mice were anesthetized via IP administration of 100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine and immobilized in a mouse head holder (921-E David Kopf Instruments). The calvarium was exposed by a midline scalp incision from the nasion to the superior nuchal line, and then the skin was retracted laterally. With a variable speed drill (Ideal Micro-drill), a 2.0 mm burr hole was made 1.0 mm posterior to the bregma and 1.0 mm to the right of midline over the left hemisphere. A sterile piece of PVA sponge was inserted into the brain cortex. The skin incision was closed with a size 6 nylon suture, which was removed at 2 weeks. A total of six mice per genotype per time point were used for implantation.

2.2. Processing of brain tissue

At 4 or 8 weeks post implantation, mice were administered 100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine i.p. to induce relaxation followed by transcardial perfusion with 30 ml 0.1 M PBS and finally fixation with 250 ml of a solution of 4% paraformaldehyde at 4 °C. Brains were removed, post-fixed overnight in 4% paraformaldehyde at 4 °C, and then cryoprotected for 24–48 h in a solution of 30% sucrose in PBS at 4 °C. Tissues were embedded in paraffin and serially sectioned in the horizontal plane across the entire implantation site.

2.3. Histochemistry and immunohistochemistry

Paraffin-embedded sections (5 µm thick) were stained with hematoxylin-eosin (H&E) and various antibodies. For immunohistochemistry, endogenous peroxidase was quenched by 30

min incubation in 3% hydrogen peroxide. Primary antibodies, including F4/80 (1:200, Serotec), anti vWF (1:200, Abcam) and anti GFAP (1:50, R&D systems), were incubated with sections overnight at 4 °C. Immunodetection of F4/80 and vWF required antigen retrieval by citrate buffer heat-induced epitope retrieval and proteinase K treatment, respectively. Biotinylated secondary antibodies were used at a 1:200 dilution. All immunoperoxidase reactions were performed using avidin–biotin method and 3',3' diaminobenzidine as chromogen and all slides were counterstained with methyl green. Analysis of 4 week implants revealed minimal invasion of sponges, which we deemed insufficient for quantification. Thus, detailed analysis was limited to 8 week implants.

2.4. Immunofluorescence and double staining

To examine the deposition of TSP2 and MMPs, sections were incubated with anti-TSP2 Ab (1:50; BD Transduction laboratories), anti-MMP-9 Ab (1:50; R&D systems) and anti-MMP-2 Ab (1:50; Abcam) for 16 h at 4 °C, and the primary antibodies were visualized using a TRITC or FITC conjugated secondary antibodies (1:100).

For double immunofluorescence, sections were incubated with anti-mouse TSP2 Ab as described above and FITC conjugated anti-GFAP (1:100; Sigma-Aldrich, Inc.). After incubation with the primary antibodies for 16 h at 4 °C, sections were incubated with TRITC-conjugated IgG for 2 h, and then washed in PBS. Immunofluorescence was visualized on a Nikon Diaphot 200 microscope.

2.5. Histomorphometric studies

For light microscopy, images were captured with the aid of a Nikon Eclipse 80i microscopy with NIS Elements D 2.30 software. The numbers of inflammatory cells and blood vessels were determined by manual counts from high power digital images ($\times 400$). GFAP distribution and mouse serum albumin (MSA) extravasation into brain tissue were quantified from digital images using image analysis software (Metamorph). Results were expressed as the percentage of GFAP and MSA positive areas. For each immunohistochemical stain, a total of 30 high power images per group were analyzed.

2.6. Statistical analysis

All differences between data sets were determined by the Student's *t*-test. Significance required a *p* value of 0.05 or less.

3. Results

3.1. TSP2 expression and distribution after PVA sponge implantation

Immunohistochemistry was performed to investigate the possibility that TSP2 participates in the brain FBR. Sections of brain without implants revealed no immunoreactivity (not shown). In contrast, TSP2 deposition was observed within the interstices of the sponge at 8 weeks and was associated with both cells and ECM (figure 1(A)). Double staining, with anti-TSP2 and GFAP (figure 1(B)) revealed extensive co-localization between TSP2-positive cells and astrocytes (figure 1(C)). Analysis of sponges from TSP2-null mice revealed no TSP2 deposition and served as negative control (figure 1(D)). The observed immunoreactivity (red color) in figure 1(D) is due to non-specific binding of antibodies to the PVA sponge, which can also be observed in figures 1(A–C).

3.2. Increased matrix deposition in TSP2-null mice

Examination sections stained with H&E from wild type and TSP2-null mice revealed minimal cell invasion at 4 weeks (figure 2). In contrast, sponges at 8 weeks showed cellular invasion

and matrix deposition, which was excessive in TSP2-null mice (figure 2). Specifically, the interstices of the sponges in TSP2-null mice were occupied with densely packed ECM and displayed cellular invasion. In contrast, minimal matrix deposition was observed in sponges implanted in wild type mice.

3.3. Enhanced neuroinflammation in TSP2-null mice

The invasion of macrophage/microglia into PVA sponges was evaluated by immunohistochemical analysis with the F4/80 antibody. Sponges in wild type and TSP2-null mice were found to contain inflammatory cells but appeared more numerous in the latter (figure 3(A, B)). Enumeration of immunoreactive cells from digital images revealed that their increased presence in the TSP2-null mice was significant (figure 3(C)). Microglia/macrophage is the major glial cell type involved in the brain's inflammatory response (Polikov *et al* 2005). Therefore, we conclude that the increased presence of F4/80-positive cells in the TSP2-null mice suggests the presence of a more pronounced inflammatory response.

3.4. Enhanced astrocyte reaction in TSP2 KO mice

Immunohistochemical detection of GFAP was performed to detect astrocytes and to determine the extent of 'reactive gliosis', the term used to describe the activation, hypertrophy and proliferation of astrocytes in response to injury. In comparison to wild type (figure 4(A)), sponges in TSP2-null mice contained more reactive astrocytes (figure 4(B)). Determination of the percent area occupied by GFAP-positive cells revealed a significant increase in TSP2-null samples (figure 4(C)).

3.5. Increased angiogenesis in sponges implanted in TSP2-null mice

Anti-vWF antibody, a marker for endothelial cells, was used to analyze the angiogenic response to PVA implants. Consistent with the anti-angiogenic activity of TSP2, a large number of vessels were observed in TSP2-null mice (figure 5(B)). Furthermore, the majority of vessels in wild type mice were observed at the interface between PVA and brain tissue (not shown). On the other hand, blood vessel distribution was observed throughout the sponge in TSP2-null mice. Enumeration of vessels per high power field confirmed the significant increase over wild type (figure 5(C)).

3.6. Increased blood brain barrier permeability in TSP2-null mice

Increased permeability of the blood brain barrier could explain the excessive neuroinflammation in TSP2-null mice. Thus, we detected extravascular MSA, which has been shown to be a reliable marker of the integrity of the BBB (Blakemore 1969, Wolman *et al* 1981, van Vliet *et al* 2007). No MSA immunoreactivity was detected in the cerebral cortex contralateral from the implant site in both groups, suggesting the presence of structurally intact BBB (not shown). Extravascular MSA was detected in the interstices of the sponge indicating that the foreign body response is associated with disruption of the blood brain barrier (figure 6). Quantitative immunohistochemistry revealed a ten-fold increase in leakage in TSP2-null mice (figure 6(C)).

3.7. Increased MMP-2 and MMP-9 deposition in TSP2-null mice

MMP-2 and MMP-9 deposition was detected by immunofluorescence and was present in both groups following implantation (figure 7). In comparison to wild type, MMP-2 and MMP-9 deposition was increased in the interstices of the sponges implanted in TSP2-null mice. MMP-9 was detected in both cells and extracellular matrix (figure 7(B)) whereas MMP-2 was primarily associated with the latter (figure 7(D)). Overall, the distribution pattern of MMP-9 is consistent with its well-documented expression by inflammatory cells.

4. Discussion

In the present study we expanded on our previous observations regarding the participation of TSP2 in the foreign body response by analyzing its expression following the implantation of PVA sponges in the brain cortex. Furthermore, we compared the response between wild type and TSP2-null mice and observed differences in ECM deposition, neuroinflammation, angiogenesis, BBB permeability and MMP-2 and MMP-9 deposition. Taken together, these observations indicate that TSP2 plays a critical role in the brain FBR by modulating ECM remodeling, angiogenesis and repair of the BBB. Analysis of sponge implants at 4 weeks revealed little or no invading response in TSP2-null and wild type mice, respectively. Previously, robust invasion of PVA sponges has been shown to occur within 2–3 weeks when implanted in the subcutaneous (SC) space, indicating that the response of the brain cortex is slower (Kyriakides *et al* 2001). However, direct comparisons of tissue-specific biomaterial responses in the brain and other tissues have not been reported. Invasion of sponges in the SC space is driven by inflammation and angiogenesis, which might occur to a less extent in the brain. In addition, unlike our observations in sponges implanted SC, we did not observe formation of foreign body giant cells in the brain foreign body response. It is possible that the lack of these cells, which have been shown to secrete pro-inflammatory and pro-fibrotic factors such as interleukin-1 α and transforming growth factor- β , respectively (Hernandez-Pando *et al* 2000), contributes to the reduced invasion at 4 weeks. Because of the lack of measurable invasion of sponges at 4 weeks, we extended the implantation time to 8 weeks.

Both TSP1 and TSP2 have been shown to be upregulated in the ischemic brain but are synthesized by different cells and exhibit distinguishable spatiotemporal expression patterns (Lin *et al* 2003). Specifically, TSP1 co-localized with the astrocytic marker S100 in the ischemic cortex at day 1 after unilateral occlusion of the distal middle cerebral artery and the common carotid artery. TSP2 on the other hand, co-localized with the astrocytic marker GFAP in the ischemic cortex at day 7 after unilateral occlusion (Liau *et al* 2008). Additional evidence for a role for TSP1 and TSP2 in the CNS includes the observation that they promote synaptogenesis (Christopherson *et al* 2005). Consistent with this finding, both TSP1 and TSP2 expressed in the developing nervous system during synaptogenesis and their expression decreases with maturation (Adams and Tucker 2000). Moreover, a recent study has shown that TSP 1 and 2 are necessary for synaptic plasticity and functional recovery after stroke (Liau *et al* 2008). In the present study, we observed significant TSP2 deposition in the interstices of the sponge and double immunostaining revealed extensive association with GFAP-positive astrocytes. Our results are consistent with previous studies and suggest that astrocytes are a major source of TSP2.

TSP2 has been linked to ECM remodeling and modulation of cell-matrix interactions and a large number of studies have demonstrated alterations in these processes in TSP2-null mice (Kyriakides *et al* 1998). Thus, the alteration in ECM deposition within the interstices of the sponges in TSP2-null mice is not surprising. However, little is known regarding a potential role for TSP2 in inflammation. An anti-inflammatory property has been proposed for TSP2 in a study where it was shown that TSP2 deficiency led to an accelerated and increased influx of T-cells and monocytes/macrophages within the renal cortex (Daniel *et al* 2007). Similarly, increased inflammation in TSP2-null mice has also been reported in a skin delayed-type hypersensitivity reaction model (Lange-Asschenfeldt *et al* 2002). In a rheumatoid arthritis model, TSP2 suppressed the production of the pro-inflammatory mediators IFN- γ and TNF- α and induced the depletion of tissue-residing T cells (Park *et al* 2004). Moreover, recent studies have shown that the anti-inflammatory function of TSP2 is modulated through its interaction with CD47 on T cells (Lamy *et al* 2007). However, the lack of TSP2 has not been shown to influence injury-induced inflammation and inflammatory cells have not been shown to express TSP2. Thus, the increased neuroinflammation observed in this study represents a novel

observation. In general, such an increase might be due to enhanced microvascular leakage and/or altered regulation of pro-inflammatory mediators (Park *et al* 2004).

Detection of extravascular MSA in TSP2-null mice indicated the presence of a leaky BBB, which could explain the presence of excess inflammatory cells. Prolonged and enhanced vascular leakage phenotype has also been observed in TSP2-deficient mice during skin inflammation (Lange-Asschenfeldt *et al* 2002). In addition, increased vessel permeability has also been reported in Akt1-null mice, which was associated with reduced TSP-1 and TSP2 expression and could be rescued by re-expression of TSP2 (Chen *et al* 2005). We have recently discovered that the implantation of biomaterials in the brain cortex leads to prolonged leakage of the blood brain barrier that requires 4–8 weeks for complete repair (Tian and Kyriakides submitted). Furthermore, it has been shown that both MMP-9 and MMP-2 are critical for the repair/maintenance of the BBB (Asahi *et al* 2001, Mun-Bryce and Rosenberg 1998). The fact that both of these enzymes are elevated within sponges implanted in TSP2-null mice suggests that conditions exist that might delay the repair of the BBB leading to enhanced and prolonged inflammation. In turn, the persistent presence of inflammatory cells, which express MMPs, can influence both matrix production and the repair of the BBB.

Consistent with previous observations in a number of injury models, we detected elevated levels of MMPs (Kyriakides *et al* 2001, Agah *et al* 2005, Kokenyesi *et al* 2004). *In vitro* studies suggest that TSP2 modulates the extracellular levels of MMP-2 and MMP-9. It is unclear if such a mechanism is also present in astrocytes or other cell types present within the interstices of the sponge. However, based on our *in vivo* observations following the implantation of PVA sponges SC, we speculate that the brain phenotype develops in a similar manner. Collectively, our findings demonstrate that TSP2 participates in the brain response to implants and the phenotype of TSP2-null mice suggests that TSP2 plays a critical role in its proper resolution.

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Figure 1.

TSP2 deposition in PVA sponges. Representative images from PVA sponges implanted in wild type (A–B) and TSP2-null mice (D) and stained with anti-TSP2 (A, D) and GFAP (B) are shown. Sponge is denoted by an asterisk. (C) Merge of images A and B. Arrows denote overlap between TSP2 and GFAP immunoreactivity.

Figure 2. Increased ECM deposition in TSP2-null mice. Representative images of sections from PVA sponges (*) implanted in WT (A) and TSP2-null mice (B) for 4 weeks (A, B) and 8 weeks (C, D) and stained with H&E. Scale bar = 100 μ m.

Figure 3.

Enhanced brain neuroinflammation in TSP2-null mice. Representative images of PVA sponges (*) from WT (A) and TSP2-null (B) mice stained with F4/80 antibody and visualized with the peroxidase reaction (brown color) are shown. (C) F4/80+ cells were enumerated and expressed as cells per high power field. * p value < 0.01.

Figure 4. Increased astrocyte reaction in TSP2-null mice. Representative sections of PVA sponges (*) implanted in WT (A) and TSP2-null (B) mice stained with GFAP and visualized with the peroxidase reaction (brown color) are shown. Histomorphometric analysis was performed to determine the percent GFAP-positive area. * p value < 0.01.

Figure 5. Enhanced angiogenic response in TSP2-null mice. Representative sections of PVA sponges (*) implanted in WT (A), TSP2-null (B) mice and stained with anti-vWF Ab and visualized with the peroxidase reaction (brown color) are shown. (C) Angiogenesis was enumerated and expressed as number of vessels per high power field. **p* value < 0.01.

Figure 6. Blood brain barrier permeability in TSP2-null mice. Representative images of PVA sponges (*) implanted in WT (A) and TSP2-null (B) mice stained with anti-MSA Ab and visualized with the peroxidase reaction (brown color) are shown. (C) Histomorphometric analysis was employed to determine the percent of extravasated MSA. * p value <0.01.

Figure 7. Enhanced MMP-9 and MMP-2 deposition in TSP2-null mice. Representative images of PVA sponges (*) implanted in WT (A, C) and TSP2-null (B, D) mice stained with anti-MMP-9 antibody (A, B) and anti-MMP-2 antibody (C, D) are shown.