Preoperative mucosal tolerance to brain antigens and a neuroprotective immune response following surgical brain injury

Laboratory investigation

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Object. Intracranial surgery causes cortical injury from incisions, hemorrhage, retraction, and electrocautery. The term “surgical brain injury” (SBI) has been developed to categorize this injury inherent to the procedure. Neuroinflammation plays a significant role in SBI. Traditional antiinflammatory therapies are often limited by their immunosuppressive side effects and poor CNS penetration. This study uses mucosal tolerance to develop an immune system that is tolerant to brain myelin basic protein (MBP) so that inflammation can be suppressed in a timely and site-specific manner following surgical disruption of the blood-brain barrier.

Methods. A standard SBI model using CD57 mice was used. Nasopharyngeal mucosa was exposed to vehicle, ovalbumin, or MBP to develop mucosal tolerance to these antigens. Immunological tolerance to MBP was confirmed in vivo through hypersensitivity testing. Neurological scores, cerebral edema, and interleukin (IL)–1β and transforming growth factor (TGF)–β1 cytokine levels were measured 48 hours postoperatively.

Results. Hypersensitivity testing confirmed the development of immune tolerance to MBP. Myelin basic protein–tolerant mice demonstrated reduced neurological injury, less cerebral edema, decreased levels of IL-1β, and increased levels of TGFβ1 following SBI.

Conclusions. Developing preoperative immunological tolerance to brain antigens through mucosal tolerance provides neuroprotection, reduces brain edema, and modulates neuroinflammation following SBI. (DOI: 10.3171/2011.8.JNS11883)

Key Words • surgical brain injury • mucosal tolerance • neuroprotection • traumatic brain injury • mouse

Nearly all intracranial procedures involve an element of cortical incision, retraction, electrocautery, and vascular or microvascular occlusion. In addition, cranial procedures involve inherent surgical risks of intraoperative hypotension, blood loss, and consequent cerebral ischemia. These inherent risks have been acknowledged by neurosurgeons through the development of minimally invasive surgical techniques as well as perioperative neuroprotective therapies. The adaptation of tools such as the endoscope, functional mapping, and the use of stereotactic localization for the purposes of making smaller, more precisely located craniotomies, clearly delineates a recognition of the inherent invasiveness and risk to normal brain tissues involved in cranial procedures.23,26,37 Pharmacological approaches to perioperative neuroprotection, such as the use of osmotic agents and burst suppression, are frequently used with the goal of minimizing complications and improving outcome.32,35 The advancement of therapies in perioperative neuroprotection for cranial surgery has been a recent topic of discussion and experimental investigation.18,19 These investigations have led to the adoption of the term “surgical brain injury” (SBI), which encompasses the damage to the CNS that is inherent to the intracranial procedure itself. Clinically, SBI cannot be easily demarcated from the underlying brain pathology, which makes it difficult, if not impossible, to study SBI as an independent outcome variable. However, animal models have been developed to quantify the impact of SBI on brain physiology as well as neurological outcome.18,19 This experiment investigates the potential role for modification of the CNS inflammatory response as a neuroprotective strategy against SBI using an experimental rodent model.

Abbreviations used in this paper: BBB = blood-brain barrier; IL = interleukin; MBP = myelin basic protein; PBS = phosphate-buffered saline; SBI = surgical brain injury; TGF = transforming growth factor.
Immunomodulation in surgical brain injury

Early investigations involving the CNS and the immune system led to the belief that the brain was an immunologically privileged organ, neither susceptible to nor capable of eliciting an inflammatory response. Although the CNS exhibits some characteristics of immune privilege, it has since been established that inflammation is a common part of CNS physiology and represents a common pathological reaction to almost every neurological disease. Initially considered to only have a pathological role following injury, cerebral inflammation is now known to be a complex system of checks and balances.

Activated leukocytes arrive at the site of CNS injury and release prostaglandins, free radicals, complement cascade factors, and proinflammatory cytokines, which in turn mobilize additional immune and glial cells. This inflammatory upregulation is necessary to mount an adequate response, but its extent is also limited by inherent antiinflammatory mediators that suppress both humoral and cellular immune activation. Ideally a perfect balance between proinflammatory processes and regulation would result in the eradication of the insult without any side effects. However, this is often not the case, and inflammatory processes frequently exacerbate brain injury and worsen neurological outcomes. Consequently, immunosuppressive agents have been investigated as a means of neuroprotection.

There is a strong inflammatory reaction surrounding the corticotomy in the SBI model that is characterized by increased neutrophil activation, increased levels of inflammatory cytokines, cell death, and disruption of the BBB. In our experiment we attempt to limit the damage caused by neuroinflammation following SBI by inducing mucosal tolerance to the CNS antigen MBP. Mucosal tolerance is a method of inducing immune tolerance to a specific antigen through chronic exposure of that antigen to the mucosal surfaces of the subject. Tolerance occurs after repetitive low-dose exposure of the antigen to mucosal surfaces (typically oral or nasopharynx surfaces in experimental models). Reexposure of the same or similar antigens to the immune system of tolerant subjects results in a modified immune response that is characterized by specific T-regulatory lymphocytes. This specific T-cell population secretes cytokines, such as TGFβ1, which suppress the cell-mediated immune response at the site of antigen exposure or injury. Mucosal tolerance to MBP has been previously shown to improve outcomes after ischemic stroke. We investigate the role of mucosal tolerance to MBP as a potential neuroprotectant in a rodent model of SBI.

Methods

Experimental Animals

This protocol was approved by the Institutional Animal Care and Use Committee at Loma Linda University. Male CD57 mice weighing 20–25 g were used (Harlan Corporation). Animals were housed in a climate-controlled environment with strict day/night light cycles. Seventy-six animals were subjected to tolerization regimens of vehicle (PBS), ovalbumin, or MBP prior to craniotomy. Divided groups were: sham + PBS (12 mice), sham + ovalbumin (12 mice), sham + MBP (12 mice), SBI + PBS (12 mice), SBI + ovalbumin (14 mice), and SBI + MBP (14 mice). Body weight was measured immediately before, 24 hours after, and 48 hours after surgery. Six randomly selected animals from each group were then killed at 48 hours and their brains were used for molecular analysis; the remaining animals were used to determine brain water content.

Development of Mucosal Tolerance

Before sham surgery or SBI craniotomy, 50 µg of bovine MBP (50 µg/10 µl of vehicle; Sigma Aldrich), 50 µg of ovalbumin (50 µg/10 µl of vehicle; Sigma Aldrich), or 10 µl of vehicle (PBS) was instilled into the nasopharynx (5 µl in each nostril) every other day for 5 treatments over 10 days as previously described. Animals were briefly sedated for the nasal application with isoflurane anesthetic 0.5%~5% in 70% medical air with 30% O2. Surgery was performed 24 hours after the last treatment. Myelin basic protein was chosen as the antigen for the development of mucosal tolerance because it was previously shown to improve outcome in experimental models of stroke in dosages similar to those used in this experiment.

Experimental Craniotomy

A standardized SBI model was used, as described in previous reports. Briefly, prior to surgery, general anesthesia was induced with 80 mg/kg ketamine intraperitoneally and 10 mg/kg xylazine intraperitoneally. Spontaneous ventilation without airway management was permitted by this anesthetic combination. After induction of general anesthesia, mice were placed prone in a Benchmark stereotactic frame under a surgical operating microscope. Scalp fur and skin were cleaned and prepared in a sterile manner. A No. 11 blade was used to incise the skin down to the skull through a single sagittal incision. The periosteum was reflected to expose the right frontal skull. Using the bregma as a landmark, a small square of skull (approximately 4 x 4 mm) was thinned and removed with a bone drill. A durotomy was performed, and the entire right frontal lobe anterior to the bregma was excised by sharp dissection and electrocautery. The resection was carried down to the skull base. Preliminary studies were conducted to demonstrate the consistency of the size of resection based on the weight of the removed specimen. Once the brain tissue was excised, intraoperative packing and saline irrigation along with brief (approximately 1 second) bipolar electrocautery application to the cut surfaces was used to control bleeding. Hemostasis was confirmed by close observation after removal of packing. After hemostasis was assured, the skin was closed with 5-0 silk suture (Ethicon, Inc.). Sham surgery included general anesthesia, skin incision, and craniotomy but no durotomy. A heating pad was used to maintain the body temperature at 36.0° ± 0.5°C during surgery and as the animal recovered from anesthesia. Animals were labeled in a manner that did not reveal their treatment to the surgeon, and the surgeon was unaware of the treatments re-
ceived by the animals prior to and during surgery. Treatments were randomly assigned to the animals by an independent lab assistant and recorded on a spreadsheet.

**Neurological Scoring**

A modified Garcia scoring system performed by an independent researcher blinded to the experimental conditions was used to assess neurological function in the mice 24 and 48 hours after surgery as previously described. Briefly, sensorimotor testing was graded on a scale from 0 to 3 in 7 areas: spontaneous activity, side stroking response, vibrissae response, limb symmetry when suspended by the tail, lateral turning when suspended by the tail, symmetry of walking on the forelimbs when partially suspended by the tail, and climbing ability/response. Neurological scores were assigned as follows: 0 = complete deficit, 1 = definite deficit with some function, 2 = mild deficit or decreased response, and 3 = no evidence of deficit/symmetrical responses. The maximum score on this scale is 21 points, representing normal neurological function.

**Brain Water Content**

Brain water content of the cerebral hemispheres, cerebellum, and brainstem was examined by an investigator blinded to treatment to assess brain edema at 48 hours postoperatively. Briefly, mice were killed under anesthesia (isoflurane anesthetic 5% in 70% medical air with 30% O2) 48 hours after SBI, and brains were immediately removed and separated into 4 hemispheric subsections, cerebellum, and brainstem. Samples were immediately weighed on a high-precision balance. Brain samples were then dried for 48 hours at 105°C and then weighed again. Brain water content for each hemisphere was calculated using the formula: ([wet weight – dry weight]/wet weight) × 100%.

**Brain Cytokine Levels**

Standard Western blotting protocol using the antibodies anti-TGFβ1 (Cell Signaling) and IL-1β (Santa Cruz Biotechnology, Inc.) was performed at 48 hours on brain tissue from the hemisphere ipsilateral to the cortical resection. Briefly, animals were killed under anesthesia (isoflurane anesthetic 5% in 70% medical air with 30% O2) 48 hours after SBI, and brains were immediately removed and separated into 4 hemispheric subsections, cerebellum, and brainstem. Samples were weighed on a high-precision balance. Brain samples were then dried for 48 hours at 105°C and then weighed again. Brain water content for each hemisphere was calculated using the formula: ([wet weight – dry weight]/wet weight) × 100%.

Data were expressed as means ± SEMs. One-way ANOVA was used to compare differences among groups and expressed as means ± SEMs using SigmaStat for Windows version 3.5. Data were found to be significant at a p value < 0.05. The Holm-Sidak test for pairwise comparisons was completed following 1-way ANOVA.

Results

**Physiological Parameters**

Seventy-six mice were subjected to a tolerization regimen with MBP, ovalbumin, or treatment with vehicle (PBS) prior to craniotomy. There were no significant differences in temperature, and none of the animals died among the experimental groups. Sham surgery did not result in a significant reduction in postoperative body weight. There were no differences in the body weights among the sham groups (sham + PBS, sham + ovalbumin, and sham + MBP). The mice in the SBI groups lost weight at both 24 and 48 hours following SBI compared with those in the sham groups and preoperative measurements. No significant differences in weight existed among the mice in the SBI groups postoperatively. The differences in the mean values among the groups were statistically significant (p < 0.001). All pairwise multiple comparison procedures were made using the Holm-Sidak method and significance was set at p < 0.05.

**Delayed-Type Hypersensitivity**

The increase in footpad thicknesses of MBP-tolerant mice was significantly less than those of PBS- or ovalbu-
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min-treated mice following the immune challenge with MBP via injection into the footpad (Fig. 1). This measure-
ment represents the severity of the inflammatory reaction against MBP.

Neurological Scores

There were no significant differences in neurological score among any of the sham groups (Fig. 2). All mice re-
ceiving SBI demonstrated a significant reduction in neu-
rological score compared with their corresponding sham
groups at 24 and 48 hours. Animals tolerant to MBP prior
to craniotomy demonstrated improved neurological func-
tion compared with PBS-treated groups 24 hours after
SBI. Forty-eight hours after SBI, animals tolerant to MBP
demonstrated improved neurological function compared
with both PBS- and ovalbumin-treated groups. This ap-
pearance of a statistical difference between the ovalbu-
min and MBP groups appears to be the result of a further
decline in function at 48 hours for the ovalbumin group
(Fig. 2); it is likely related to the progression of cerebral
edema, which peaked at 48–72 hours in this model.

Brain Water Content

Brain water content was not significantly different
among any of the sham groups (Fig. 3). Experimental cra-
niotomy significantly increased brain water content in the
right frontal lobe surrounding the resection site 48 hours
after surgery compared with sham in all groups. Post-SBI
brain water content of the right frontal lobe in animals
tolerant to MBP was significantly reduced in comparison
with vehicle- or ovalbumin-treated groups. The remain-
ing brain regions did not show any significant increases
in brain water content following craniotomy (data not
shown).

Expression of IL-1β and TGFβ1

Western blot analysis of the right hemisphere showed
that IL-1β levels in sham animals were not affected by
treatment (Fig. 4 left). Surgical brain injury increased the
expression of IL-1β in PBS- and ovalbumin-treated
groups (p < 0.001, 1-way ANOVA), while MBP-tolerant
animals did not demonstrate a significant increase in the
expression of this proinflammatory cytokine.

The levels of TGFβ1 in sham animals were not af-
 affected by treatment (Fig. 4 right). Craniotomy decreased
the expression of TGFβ1 in PBS- and ovalbumin-treated
groups by approximately 50%, whereas preoperative lev-
el levels were preserved with MBP tolerance. The expression
of TGFβ1 was significantly elevated in the MBP-treated
groups compared with PBS- or ovalbumin-treated groups.

Statistical Power

The power of all tests performed with an alpha level
of 0.05 was > 0.800, except for the testing of IL-1β ex-
pression, which had a power of 0.542.

Discussion

Inflammatory mediators likely play a significant role
in the traumatic (cortical incision) and ischemic (brain
retraction) injury associated with SBI. Previous experi-
ments in SBI models have found significant cerebral
edema and inflammatory pathology surrounding the cor-
ticotomy. Additionally, experiments in traumatic and
ischemic brain injury have found that delayed deteriora-
tion is associated with an elevation of proinflammatory
mediators. Our experiment has demonstrated that tol-
erance to MBP attenuates SBI, as animals in the treat-
ment group had less postoperative edema, and improved
postoperative neurological function (Figs. 2 and 3). This
neuroprotection is associated with the maintenance of
inherent levels of the immunosuppressant cytokine TGFβ1
(Fig. 4 right) and inhibition of the proinflammatory cyto-

cine IL-1β (Fig. 4 left).

Mucosal tolerance to MBP resulted in significant re-
ductions in postoperative IL-1β (Fig. 4 left), which is a
proinflammatory cytokine that orchestrates systemic in-
flammatory responses, and is a major contributor to neu-
roinflammation. It is expressed at relatively low levels
in the brain under physiological conditions and regulates
many important physiological functions within the CNS. However, under pathophysiological conditions there is
a marked elevation in the level of IL-1β and its recep-
tor. Given the significant changes in IL-1β expression
following brain injury, it has been a target of numer-
ous investigations and is one of the most studied proin-
flammatory cytokines today. Therefore, we selected this
cytokine as a means of quantifying the degree of inflam-
mation following SBI in our experiment. Microglia are
the earliest major source of IL-1β after experimental CNS
injury, infection, or inflammation, whereas neurons, astro-
cytes, and oligodendrocytes are likely secondary sources
of IL-1β. The proinflammatory cytokine IL-1β augments
T-cell responses to mitogens, leads to the recruitment of
leukocytes through adhesion molecule expression, and
amplifies other proinflammatory cytokines. The experi-
mental inhibition of IL-1β has provided further evidence
of its importance in orchestrating neuroinflammatory re-
sponses. Yamashita et al. reported that intraventricular
Injection of anti–IL-1β antibodies to rats reduces ischemic brain damage. Additionally, mice lacking the gene for the enzyme caspase-1, which is required to activate IL-1β, exhibited reduced brain damage in several models of ischemia. Increases of IL-1β levels are known risk factors for poor outcome after stroke or brain injury, and high circulating levels of inflammatory markers are predictive of poor clinical outcome in stroke patients. Our data supports these findings by demonstrating that the attenuation of postoperative IL-1β is associated with improved surgical outcomes.

Transforming growth factor–β1 is a pleiotropic cytokine, and in the CNS it has been shown to promote the survival of neurons and inhibit microglial and astrocyte proliferation. It is recognized as the hallmark cytokine secreted by T-regulatory lymphocytes, following the induction of mucosal tolerance. These lymphocytes, through the action of their cytokines such as TGFβ1, markedly inhibit local proinflammatory cells. Given the prominence of TGFβ1 secretion by T-regulatory lymphocytes, we selected this cytokine as a marker for the effectiveness of our mucosal tolerance treatments. The use of this cytokine has correlated well with the induction of mucosal tolerance in other experiments. Transforming growth factor–β1 is expressed in the normal adult brain by parenchymal microglial cells, exerting a trophic anti-inflammatory effect in the adult CNS. Transforming growth factor–β1 is likely a significant contributor to the partial immunological privilege of the brain, which is demonstrated by the brain’s increased tolerance of foreign tissue grafts and resilience to developing inflammatory reactions in comparison with other body tissues. We demonstrated a baseline expression of TGFβ1 in sham animals, which was significantly reduced following SBI (Fig. 4 right). However, treatment with mucosal tolerance to MBP preserved endogenous levels of TGF-β1 (Fig. 4 right), and was associated with decreased cerebral edema (Fig. 3) and improved neurological score (Fig. 2). We believe that TGFβ1 provides these neuroprotective effects through its anti-inflammatory properties. Our findings are consistent with other experiments in that endogenous levels of TGFβ1 are present in the cortex at detectable levels. Experiments by Becker et al. and Gee et al. in ischemic stroke models also support our findings. Becker et al. found that mucosal tolerance to MBP before middle cerebral artery occlusion reduced infarct size. Gee et al. demonstrated that lymphocytes collected 1 month after surgical brain injury resulted in significant loss of neurological function 24 and 48 hours after surgery. The MBP-tolerant groups show a significant preservation of neurological function 24 and 48 hours after SBI. The Holm-Sidak test for pairwise comparisons was performed and the p values were less than the critical levels indicated in the figure (statistically significant).
middle cerebral artery occlusion in animals that had undergone MBP immune tolerance expressed higher levels of TGFβ1. The elevation of TGFβ1 was associated with improved neurological outcome and reduced brain atrophy following ischemic stroke.

Our experiment provides evidence for a unique anti-inflammatory therapy against SBI that potentially avoids many of the pitfalls of other antiinflammatory approaches. Previous trials and experiments testing systemically administered antiinflammatory therapies for brain injury have been met with variable success. The use of steroids in traumatic brain injury has proven to be detrimental to outcome.34 Steroid use in spinal cord injury is still highly debated, but it is no longer a standard of care in many hospitals.30 In ischemic stroke, antiinflammatory treatments have been shown to improve outcome in animal models,5,14 whereas human trials have failed to show clinical benefit.11 Systemic antiinflammatory agents are frequently limited by side effects such as infection, pneumonia, or sepsis.11,30 Mucosal tolerance to brain antigens can overcome these disadvantages by providing antiinflammatory therapy in a timely and site-specific fashion. Through mucosal tolerance it is possible to modulate the immune system’s interaction with specific antigens as opposed to suppressing the entire system. We believe that further investigation into its protective mechanisms is warranted given the findings in this study as well as other studies in models of ischemic stroke and multiple sclerosis.4–6,33

Conclusions

These findings provide evidence for the efficacy and safety of mucosal tolerance to MBP, and open the door for through the mitigated delayed hypersensitivity reaction (Fig. 1). In addition, the cytokine profile of the neuroinflammatory reaction was significantly changed, with increases in TGFβ1 and decreases in IL-1β (Fig. 4). This change in cytokine profile is suggestive of changes in the T lymphocyte subtype that is dominating the inflammatory reaction, as TGFβ1 is characteristic of T-regulatory lymphocytes’ reactions. The presence of T-regulatory lymphocytes is associated with neuroprotection through their local inhibition of cell-mediated immunity.5,6,29,49

The association between the altered cytokine profiles, reduced cerebral edema, and improved neurological outcomes suggests that these cytokines may be responsible for the clinical improvement of the animals. Our experiment also demonstrated clinical safety, because the animals did not demonstrate toxicity to the treatment. The animals treated with MBP had no deleterious changes in weight or neurological function following treatment. In the future, long-term neurological outcomes and histological examination of brains to determine side effects will be required. However, the efficacy of mucosal tolerance to MBP is currently undergoing testing in humans for the treatment of rheumatoid arthritis and multiple sclerosis, and no toxicities or autoimmune reactions have been found.38,43

**Fig. 4.** Graph showing the effects of mucosal tolerance on cerebral cytokine levels following SBI among 6 groups of mice (6 mice per group). Left: Levels of IL-1β were equal among all sham groups. Levels were significantly increased in the SBI + PBS and SBI + OVA groups. The MBP-tolerant mice did not demonstrate an increase in IL-1β after SBI. Levels of IL-1β were significantly higher in the SBI + PBS and SBI + OVA groups compared with the SBI + MBP group. Right: Levels of TGFβ1 were equal among all sham groups. Levels of TGFβ1 were reduced by approximately 50% in the SBI + PBS and SBI + OVA groups. There was no reduction in TGFβ1 in MBP-tolerant mice after SBI. Post-SBI levels of TGFβ1 were significantly higher in the MBP-tolerant mice than in the SBI + PBS or SBI + OVA groups. Expression levels of each protein using Western blots are expressed as a ratio of β-actin levels for normalization. The Holm-Sidak test for pairwise comparisons was performed and the p values were less than the critical levels indicated in the figure (statistically significant).
Disclosures

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