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Intranasal administration of E-selectin to induce immunological tolerization can suppress subarachnoid hemorrhage-induced vasospasm implicating immune and inflammatory mechanisms in its genesis

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Abstract

Evidence that inflammatory and immune mechanisms may have a critical role in the development of vasospasm after subarachnoid hemorrhage is accumulating. We examined, therefore, whether induction of immunological tolerance to the adhesion molecule that is uniquely expressed on activated endothelium, E-selectin, could inhibit the vasospasm provoked by subarachnoid blood in a rat subarachnoid hemorrhage model. We found that intranasal instillation of E-selectin every other day for 10 days on a mucosal tolerization schedule suppressed delayed type hypersensitivity to E-selectin confirming tolerance to that molecule and markedly suppressed basilar artery spasm after subarachnoid hemorrhage. The results of this proof-of-concept study suggest that agents that can mimic the local effects of the mediators of mucosal tolerance could have therapeutic potential for the management of post-subarachnoid hemorrhage vasospasm.

Keywords

Vasospasm; Subarachnoid hemorrhage; Immunomodulation; E-selectin; Mucosal tolerance; Basilar artery

1. Introduction

More than 30,000 cases/year of aneurysmal subarachnoid hemorrhage occur in the USA (Ho and Batjer, 1997) with an estimated population-based incidence rate of 7–20 per 100,000 per year (Ho and Batjer, 1997; Ronkainen et al., 1998). Subarachnoid hemorrhage constitutes only 3–8% of all strokes, but accounts for 22–25% of cerebrovascular deaths (Ho and Batjer, 1997). Following a subarachnoid hemorrhage, delayed cerebral vasospasm affects 20–30% of patients clinically and can be identified angiographically in up to 70% of patients (Kassell et al., 1985). Although there have been advances in the diagnosis and treatment of subarachnoid

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hemorrhage and delayed vasospasm, brain infarction occurs in up to 50% of the patients that develop vasospasm (Mayberg et al., 1994).

Evidence is accumulating that subarachnoid blood initiates the production of inflammatory and immune mediators including free radicals that then lead to vasospasm (Aladag et al., 2003; Dumont et al., 2003). On this basis, we decided to conduct a proof-of-principle experiment to test whether immunomodulation targeted to blood vessel segments that have been activated by proinflammatory mediators and are expressing E-selectin can suppress vasospasm in a model of subarachnoid hemorrhage in the spontaneously hypertensive rat.

Nasal instillation of E-selectin, which is specifically expressed on activated endothelium, was previously shown by our group to induce mucosal tolerance to that antigen and to inhibit the development of ischemic and hemorrhagic strokes in spontaneously hypertensive, genetically stroke-prone (SHR-SP) rats with untreated hypertension (Takeda et al., 2002). We have also found that mucosal tolerization to E-selectin provides cell-mediated protection against ischemic brain injury in a middle cerebral artery occlusion model in SHR-SP (Chen et al., 2003). Mucosal tolerance is a well established model by which immunological tolerance is induced to a specific antigen through nasal instillation or feeding of that antigen (Metzler and Wraith, 1996; Weiner et al., 1997). Nasally administered antigen encounters nasal-associated lymphoid tissue that forms a well-developed immune network. Nasal-associated lymphoid tissue evolved to protect the host from invading pathogens and has developed the inherent property of preventing the host from reacting to inhaled proteins that are not themselves pathogenic. The schedule and amount of antigen administration determine the nature of the tolerance. Active tolerance with the production of regulatory T cells occurs after repetitive administrations of low-doses of antigen (Chen et al., 1994; Groux et al., 1997). T cells tolerized with a low-dose regimen become regulatory T cells and secrete cytokines such as IL-10 and transforming growth factor (TGF)- β 1 upon antigen restimulation (Chen et al., 1994). Activation of these regulatory T cells is specific for the tolerizing antigen. The immunomodulatory cytokines secreted in response to regulatory T cell activation have, however, nonspecific effects. Thus, local immunosuppression will occur wherever the tolerizing antigen is present. This phenomenon, known as “active cellular regulation” or “bystander suppression”, leads to relatively organ-specific immunosuppression (Faria and Weiner, 1999).

The results of this study demonstrate that intranasal instillation of E-selectin on a tolerization schedule generates regulatory T cells, which suppress the Th1 immune responses that mediate delayed type hypersensitivity to E-selectin. E-selectin tolerization also suppresses basilar artery constriction in a subarachnoid hemorrhage model of delayed vasospasm in spontaneously hypertensive rats implicating immune and inflammatory mechanisms in the genesis of vasospasm in this model.

2. Results

2.1. Mucosal tolerization to E-selectin suppresses DTH

Ear swelling occurred after E-selectin sensitization and challenge in the rats that had been previously tolerized with E-selectin (0.005 ± 0.003 mm; mean $\pm$ S.E.M.), and in the rats that had been previously tolerized with PBS (0.035 ± 0.009 mm). The E-selectin tolerized group showed significantly less ear swelling than the PBS tolerized group ($p < 0.01$) (Fig. 1) confirming that intranasal instillation of E-selectin induces suppression of Th1-mediated immune responses and induces tolerization to E-selectin.

2.2. Mucosal tolerization to E-selectin attenuates delayed vasospasm after experimental subarachnoid hemorrhage in SHR

Morphometric analyses were performed on transverse sections of basilar arteries from brains obtained 48 h after intracisternal injection of normal saline (PS and ES groups) or 48 h after intracisternal injection of blood (PB and EB groups). Luminal circumferences measured at each level of the basilar artery in the PS and ES groups showed complete overlapping (Fig. 2A) in contrast to the clear differences in small luminal circumferences found in the PB and EB (subarachnoid hemorrhage) groups (Fig. 2B). We defined two states, i.e. normal and vasoconstricted, for the values of the measured circumferences on the basis of the two different thresholds described above. One threshold was the mean $-(2 \times \text{S.E.M.})$ from the combined PS and ES controls at each slice level (Fig. 3A). On the basis of this threshold, two out of eight rats from the EB group had no basilar vasoconstriction and six out of eight rats from the EB group had basilar vasoconstrictions at a maximum of three slice levels (range of the total number of slice levels per rat: 1–3); one out of eight rats from the PB group had no basilar vasoconstriction and seven out of eight rats from the PB group had basilar vasoconstrictions at multiple slice levels (range: 3–10). A binomial regression was used to test whether there was a statistically significant greater effect of E-selectin than of PBS tolerization on the probability of having circumferences along the basilar artery that were smaller than this threshold. The estimated odds ratio was 0.1974 ($p < 0.0001$) with 95% C.I. (0.0932, 0.4178). This provides strong evidence that E-selectin tolerization statistically significantly alters the probability of vasoconstriction induced by subarachnoid blood along the length of the basilar artery. The estimated odds ratio of 0.1974 indicates that the chance of having basilar artery vasoconstrictions due to subarachnoid blood is about 80% less in the E-selectin tolerized (EB) group than in the PBS tolerized (PB) group. To test whether there was a significant difference in the magnitude of vasoconstriction between the E-selectin tolerized (EB) and PBS tolerized (PB) groups, an exact Wilcoxon two-sample test was performed on averages that were obtained from each rat of all of that rat's basilar artery circumferences that were smaller than the applied threshold. The results showed that the below-threshold sections in the basilar artery in the EB group had significantly larger circumferences than the below-threshold basilar artery constrictions in the PB group ($p = 0.0012$).

Based on the other threshold used, a set of minima each representing the minimal circumference observed in each set of basilar artery sections at each level across rats in PS and ES (Fig. 3B), seven out of eight rats from the EB group had no basilar vasoconstriction and one out of eight rats from the EB group had basilar vasoconstriction at one slice level; one out of eight rats from the PB group had no basilar vasoconstriction and seven out of eight rats from the PB group had basilar vasoconstriction at multiple slice levels (range: 2–8). A binomial regression was performed to test whether E-selectin tolerization statistically significantly reduced below PBS tolerization the probability of having circumferences along the basilar artery smaller than this threshold. The estimated odds ratio was 0.0290 ($p = 0.0006$) with a 95% confidence interval of (0.0039, 0.2174). The odds ratio of 0.0290 indicates that the chance of having basilar artery vasoconstrictions in the EB group is about 97% less than in the PB group. This provides strong evidence that E-selectin tolerization also affects the probability of having vasoconstrictions defined by this threshold along the basilar artery in the subarachnoid hemorrhage groups.

An exact Wilcoxon two-sample test was conducted on the original PB and EB basilar artery circumferences without regard to thresholds. The results showed that the basilar arteries in the EB group (median = 565.66) had significantly larger circumferences than those in the PB group (median 559.50) ($p = 0.016$).

Fig. 4 presents examples of basilar artery cross-sections in a representative animal from each group. The inflammatory cell infiltrates in the animals subjected to intracisternal injection of blood (particularly the infiltrate encircling the vessel from the PB animal) are noteworthy. It

should also be noted that the perivascular collections of blood in the PS and ES animals represent a postmortem artifact that occurred coincident with decapitation.

3. Discussion

There is growing experimental evidence of a role for local release of immunological and inflammatory mediators. We, therefore, examined in the present studies the possibility that local immunosuppression could attenuate post-subarachnoid hemorrhage vasospasm in spontaneously hypertensive rats. Local immunosuppression was produced by application of mucosal tolerization achieved by intranasal administration of E-selectin. Mucosal tolerization generates E-selectin-specific regulatory T cells that are targeted to activating endothelium. The present results demonstrate that such intranasal instillation of E-selectin produces antigen-specific delayed type hypersensitivity suppression in spontaneously hypertensive rats and validate the development of immunological tolerance to E-selectin in rats exposed to intranasal instillation of E-selectin (Chen et al., 2003; Cher and Mosmann, 1987). Moreover, the E-selectin mucosal tolerization targeted activated basilar arteries and significantly attenuated the delayed vasospasm produced in the basilar arteries of these animals observed 48 h after the intracisternal injection of autologous blood.

Previous studies by our group have provided strong support for the effectiveness of intranasal instillation of E-selectin to induce mucosal tolerization. We previously observed in animals subjected to E-selectin tolerization, increased numbers of splenocytes immunoreactive for TGF-beta, suppression of interferon-gamma levels in plasma and decreased expression of intercellular adhesion molecule-1 on vascular endothelium in animals that had also been exposed to lipopolysaccharide to activate blood vessel endothelium through toll-like receptor-4 stimulation (Takeda et al., 2002). We also observed following E-selectin tolerization augmented production of interleukin-10 in stimulated splenocytes, suppression of CD8+ cytotoxic T lymphocyte accumulation in ischemic brain lesions caused by middle cerebral artery occlusion and robust cytoprotection of ischemic brain by adoptive transfer of splenocytes from E-selectin tolerized donor rats to naïve recipients that were then subjected to middle cerebral artery occlusion (Chen et al., 2003).

Possible mechanisms by which E-selectin tolerization attenuates vasospasm after subarachnoid hemorrhage in spontaneously hypertensive rats can be addressed from the standpoint of the mucosal tolerance model and downstream effects of TGF-beta and interleukin-10 receptor activation since these cytokines have been extensively documented to be key players in mucosal tolerance (Faria and Weiner, 1999, 2005). TGF-beta exerts dramatic and multimodal anti-inflammatory and immunosuppressive effects (Mallat and Tedgui, 2002); suppression of toll-like receptor signaling by Smad3 in the TGF-beta signal transduction pathway may be one aspect of TGF-beta's anti-inflammatory and immunosuppressive activity (McCartney-Francis et al., 2004). Interleukin-10 inhibits a host of inflammatory and immune mediators that include TNF, interleukin-6, a variety of other cytokines, cyclooxygenase 2, and matrix metalloproteinases 2 and 9 (Joyce et al., 1994). It also enhances the release of interleukin-1 receptor antagonist and soluble TNF receptors. Recent evidence suggests that signal transducer and activator of transcription (STAT) 3 is the dominant signal transducer of the majority of interleukin-10 immunosuppressive and anti-inflammatory functions (Williams et al., 2004). Potential mechanisms for STAT3-induced immunosuppression and macrophage deactivation include increased expression of a member of the inhibitor of nuclear factor kappa B family (Bates and Miyamoto, 2004), Bcl-3, that suppresses proinflammatory cytokine expression by nuclear factor kappa B (Kuwata et al., 2003) and increased expression of members of the dual-specificity phosphatase family that dephosphorylate mitogen-associated protein kinases at tyrosine and threonine residues and inactivates them (Ducruet et al., 2005; Lang et al., 2005).

In addition to preclinical animal models in which a multitude of inflammatory mediators have been implicated in vasospasm after subarachnoid hemorrhage, many inflammatory and immune mediators have been observed to increase in subarachnoid hemorrhage patients as compared with controls (Dumont et al., 2003). For instance, soluble E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 are elevated in the cerebrospinal fluid of subarachnoid hemorrhage patients (Polin et al., 1998). Inflammatory cytokines and platelet-activating factor have been found to be elevated in jugular venous blood of patients with subarachnoid hemorrhage (Hirashima et al., 1997). Activated mono-nuclear cells from the cerebrospinal fluid of subarachnoid hemorrhage patients can synthesize and release endothelin-1 providing a link between inflammation and the development of cerebral vasospasm (Fassbender et al., 2000). Other clinical studies have found that during subarachnoid hemorrhage vasospasm, increased levels of immunoglobulins and complement components are present in serum and vessel walls (Kasuya et al., 1989). Such studies combined with findings in preclinical models raise the strong possibility that inflammatory and immune responses may “represent a critical common pathway in the pathogenesis of cerebral vasospasm pursuant to subarachnoid hemorrhage” (Dumont et al., 2003).

In the present experiments, with this rat subarachnoid hemorrhage model, buprenorphine was administered to all rats to control pain as is customary in the medical care of subarachnoid hemorrhage patients (MacDonald and Weir, 2004). Pilot studies showed that in animals subjected to intracisternal injection of blood, buprenorphine administration did not affect the degree of vasospasm (data not shown). Of 57 different published animal models of subarachnoid hemorrhage, each model has advantages and disadvantages so that none has universal acceptance (Megyesi et al., 2000). In this study, we fixed the vessels and brains by immersion fixation rather than by perfusion fixation because we focused on vascular constriction due to smooth muscle contraction or vascular remodeling rather than on passive constriction due to vascular elasticity (Ceviker et al., 1995). Basilar arteries that have developed vasospasm from SAH have been clearly shown to retain the properties that give rise to vasospasm when they are excised and studied by means of isometric tension recordings in a Krebs–Henseleit bath (Macdonald et al., 2006).

In summary, the results of this proof-of-concept study provide clear support for immune and inflammatory mechanisms in the vasospasm observed this rat model of subarachnoid hemorrhage. Neither immunomodulatory nor anti-inflammatory approaches are a mainstay of current clinical treatment of cerebral vasospasm after subarachnoid hemorrhage. Our results suggest that small molecules directed at the signal transduction pathways for the cytokines that mediate mucosal tolerance could be effective in this disorder.

4. Experimental procedures

4.1. Animal preparation

All procedures performed on animals were in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee. Normal adult (10- to 12-week-old, 280–320 g) male Spontaneously Hypertensive Rats (SHR) were obtained from Charles River Laboratories (Wilmington, MA) and maintained in a climate-controlled room on a normal 12-h light/dark cycle with food and water available ad libitum. To study whether induction of mucosal tolerance to E-selectin can suppress cerebral vasospasm caused by subarachnoid hemorrhage, rats were divided into the following four groups based on the agents that were instilled intranasally (phosphate buffered saline (PBS) or E-selectin) and injected intracisternally (normal saline or blood): (1) normal controls pretreated with PBS intranasally and injected intracisternally with normal saline (PS group, $n=9$); (2) controls pretreated with E-selectin intranasally and injected intracisternally with normal saline (ES group, $n=10$); (3) experimental animals pretreated with PBS

intranasally and injected intracisternally with blood (PB group, $n=8$); (4) experimental animals pretreated with E-selectin intranasally and injected intracisternally with blood (EB group, $n=8$).

4.2. Tolerization schedule

The tolerization schedule was as follows: PBS (20 μ L) or recombinant human E-selectin (Novavax, Rockville, MD) (2.5 μ g/20 μ L) instilled into each nostril every other day for 10 days (total of 5 administrations).

4.3. Monitoring of physiological variables

Mean arterial blood pressure (MABP) was monitored with a blood pressure analyzer (Model 300; Digi-Med, Louisville, KY) that had been calibrated with an air-damped mercury manometer. Arterial blood $p\text{CO}_2$, $p\text{O}_2$, and pH were measured with a blood gas analyzer (RapidLab 860; Bayer, Norwood, MA). The rats were kept normocapnic, and adequately oxygenated while anesthetized. Body temperature was continuously monitored by a rectal probe and maintained at 37 °C throughout the experimental and postoperative periods by a thermostatically controlled infrared lamp (Model 73A; Yellow Springs Instrument Co., Yellow Springs, OH).

4.4. Experimental procedure

Animals were anesthetized with isoflurane (5% for induction and 1–1.5% for maintenance) in a 70% nitrous oxide/30% oxygen mixture. The region of the femoral triangle was shaved and wiped with Betadine followed by alcohol. A polyethylene catheter (PE 50, Clay-Adams, Parsippany, NJ) was inserted into the femoral artery for collecting arterial blood samples and blood-pressure monitoring. The rats were then placed in prone position with the neck flexed, and the suboccipital and cervical regions were shaved and sterilized as done for the femoral catheterization. A 10 mm of midline suboccipital incision was made, and the nuchal ligament was identified. The underlying tissue was mobilized with midline blunt dissection and separated with a retractor to reveal the atlanto-occipital membrane. Animals in the two subarachnoid hemorrhage experimental groups (PBS pretreated and E-selectin pretreated) received an infusion of blood into the cisterna magna. A 27-gauge needle was used to penetrate the atlanto-occipital membrane, and a silastic catheter (0.012-in. inner diameter $\times$ 0.025-in. outer diameter) (Read Plastic, Rockville, MD) was inserted into the cisterna magna. After 0.1 mL of cerebrospinal fluid was withdrawn over a 30-s period, either normal saline or blood was injected into the cisterna magna. In the two groups (PB and EB) that were injected intracisternally with blood, 0.3 mL of autologous, nonheparinized blood, which had been collected from the femoral artery, was injected into the cisterna magna over a 2-min period. The rats in the two control groups (PS and ES groups) received an injection of 0.3 mL of normal saline into the cisterna magna in the same manner as in the intracisternal blood injection groups. In order to make the control groups injected intracisternally with normal saline comparable to the experimental groups injected intracisternally with blood, 0.3 mL of blood was also withdrawn from the femoral artery. The rats were then placed in a 30° head-down position to direct the flow of blood or saline toward the intracranial cisterns. After 30 min in this head-down position, the catheter was removed from the femoral artery, and the leg and scalp incisions were treated with 5% (w/w) lidocaine ointment and sutured. To minimize postoperative pain and distress, we injected an analgesic dose of buprenorphine (0.03 mg/kg) subcutaneously with a 27-gauge needle at the end of the surgery and again every 8 h for the first 24 h and then every 12 h until 48 h post-surgery. The entire surgical procedure was completed within approximately 45 min. The animals were then allowed to recover from anesthesia before being returned to their cages. All animals were monitored daily, postoperatively.

4.5. Morphometric analyses

Forty-eight hours after the injections into the cisterna magna the rats in each group were reanesthetized and euthanized and their brains were immediately removed and placed in ice-cold 4% paraformaldehyde in 0.1 molar phosphate buffer for 3 days at 4 °C. The basilar arteries and brain stem were then excised en bloc and cryoprotected in 20% sucrose in 0.1 molar phosphate buffer for 3 days at 4 °C. After snap-freezing in isopentane cooled to -40 °C to -50 °C, the specimens were stored at -70 °C until processed as follows. The specimens were embedded in tissue freezing compound, mounted in a (Leica CM3050S) microtome cryostat, and cut transversely into 20 µm sections at 200 µm intervals beginning at the cephalad end of the basilar artery. The sections were then stained with standard hematoxylin and eosin and mounted on glass coverslips with Permount medium. To quantify the luminal circumference of the basilar artery, images (magnification $\times 200$) of 26 sequential transverse sections, each separated by 200 µm were obtained (Axioplan, Zeiss and Meta-Morph image processing system, Universal Imaging Corp.). The luminal circumferences of the basilar artery visualized in the images were measured by means of a computerized image analysis system (NIH Image 1.62). These measurements were made blindly with respect to the experimental group to which the animal belonged. We then plotted measured circumferences of each basilar artery from all the analyzable slice levels (i.e. slices that were not lost, obliquely cut, or folded). Two different thresholds for minimal normal circumferences at each section level were then determined and these thresholds were used to categorize in a binary fashion the measured circumferences as normal or constricted.

4.6. DTH reaction

In order to assess the delayed-type hypersensitivity (DTH) reaction, a single-course tolerization schedule (5 intranasal doses of either PBS or E-selectin, each administered every other day over a 10-day period), was carried out ($n=4$ in each group) as described above. Fourteen days later, the animals were immunized (hind footpad) with 75 µg E-selectin/200 µL PBS plus 50 µL complete Freund's adjuvant (Sigma). Fourteen days later, ear thickness was measured as a baseline before the rats were rechallenged with 50 µg of E-selectin/100 µL PBS injected into the ear. Ear thickness increase over baseline was measured with microcalipers (Mitsutoyo, Tokyo) 2 days later.

4.7. Statistical analyses

The effectiveness of E-selectin mucosal tolerance in suppressing subarachnoid hemorrhage vasospasm was assessed as follows. The basilar arteries from the two experimental groups (PB and EB) were assigned to one of two dichotomous categories (i.e. normal or vasoconstricted) based on their circumferences. The categories were defined on the basis of threshold values for the circumferences obtained independently from the two control groups (PS and ES) that received only normal saline in their subarachnoid space. First, we tested whether there were significant differences in the circumferences of the PS and ES groups by a multivariate analysis of variance model (MANOVA), assuming a compound symmetric covariance structure to reflect possible correlations of the observations within a given rat. There was no significant difference between these two control groups ($F(1,17)=0.48$, $p=0.4975$). In the absence of significant differences, we combined the data from PS and ES groups to define the circumferences of normal basilar arteries.

Two threshold levels were then derived from the data obtained from the 19 rats in the combined PS and ES control groups and used to classify each basilar artery section as normal or constricted on the basis of its circumference. The first threshold was the mean-(2 $\times$ standard error of the mean (S.E.M.)) for each set of circumferences from the first to the 26th levels of basilar artery sections in a cephalad to caudad direction. The second threshold, also derived from the data of the PS and ES controls, was a set of minima each representing the minimal

circumference observed at each level, first to 26th, cephalad to caudad, of the basilar artery sections obtained from the PS and ES groups. Based on the first or the second threshold, the total number of basilar artery sections smaller than the applied threshold was determined and, for each rat with any basilar artery sections below that threshold, the average of the circumferences of those basilar artery sections was calculated. A binomial regression was used to test whether there was a statistically significant effect of pretreatment on the probability of having vasoconstriction along the basilar artery in the subarachnoid hemorrhage groups. We also analyzed differences in the below threshold basilar artery circumferences between the two experimental groups, i.e. the EB and PB groups, by means of an exact Wilcoxon two-sample test. In addition, an exact Wilcoxon two-sample test was conducted based on original basilar artery circumferences without taking into account section information and without applying thresholds obtained from both PS and BS. SAS version 9.1 was used for conducting the MANOVA, binomial regression and the exact Wilcoxon two-sample test. For assessing the delayed-type hypersensitivity (DTH) reaction, ear thickness of the treatment group was analyzed by an unpaired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

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Fig. 1. Delayed-type hypersensitivity to E-selectin suppression test. The time course of the procedure is schematized and the degree of ear swelling in PBS- and E-selectin-tolerized groups is shown.

Fig. 2.

Luminal basilar artery circumferences in the PS and ES groups (A) and PB and EB groups (B) at 26 successive levels of the artery each separated by 200 μm moving from cephalad to caudad. Circumferences of each basilar artery were measured from each of the analyzable slices (i.e. slices that were not lost, obliquely cut, or folded) at each of the slice levels; $n=2-8$ (median=6) for PS, $n=2-8$ (median=5) for ES, $n=1-7$ (median=5) for PB, $n=3-8$ (median=5) for EB. Open squares designate luminal circumferences from animals tolerized to PBS and filled circles designate luminal circumferences from animals tolerized to E-selectin.

Fig. 3.

The mean-(2×S.E.M.) thresholds (A) and minimal circumference thresholds (B) derived from the combined PS and ES data are shown for each level, i.e. first to the 26th cephalad to caudad sections of each set of basilar artery sections at each level across rats. In panel A, the mean-(2×S.E.M.) thresholds are superimposed on the PB and EB data sets. In panel B, minimal circumference thresholds are superimposed on the PB and EB data sets. At each basilar artery level in each group in each plot, the range and median of the number of analyzed slices is the same as indicated in the legend for Fig. 2. Open squares designate luminal circumferences from animals tolerized to PBS and filled circles designate luminal circumferences from animals tolerized to E-selectin.

Fig. 4. Cross-sections of basilar arteries of animals from each of the 4 groups displayed at 200 $\times$ and 400 $\times$. Perivascular blood and leukocyte accumulation is apparent in the PB and EB groups. The perivascular blood that is present in the PS and ES control groups resulted from postmortem bleeding at the time of decapitation.