Induction of Mucosal Tolerance to E-Selectin Prevents Ischemic and Hemorrhagic Stroke in Spontaneously Hypertensive Genetically Stroke-Prone Rats

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Background and Purpose—Inflammatory and immune mechanisms can precipitate cerebrovascular thrombosis and hemorrhage. Immunologic tolerance can be induced to a specific antigen by intranasal instillation of that antigen. Lymphocytes tolerized in this way provide local immunosuppression on restimulation with the same antigen. This study tests whether tolerization of lymphocytes to E-selectin can suppress local vessel activation and prevent stroke.

Methods—Spontaneously hypertensive genetically stroke-prone rats (n=113) were distributed among the following studies: comparison of ischemic infarcts/intraparenchymal hemorrhages after single or repetitive tolerization schedules with ovalbumin, E-selectin, or PBS; comparison of E-selectin tolerization– and PBS tolerization–induced suppression of delayed-type hypersensitivity in animals subsequently sensitized to E-selectin; and comparison of PBS–, ovalbumin–, and E-selectin–tolerized groups (after intravenous lipopolysaccharide to activate vessels) regarding transforming growth factor-β1–positive splenocyte counts, plasma interferon-γ levels, anti-human E-selectin antibodies, endothelial intercellular adhesion molecule-1, and anti–endothelial cell antibodies.

Results—Nasal instillation of E-selectin, which is specifically expressed on activated endothelium, potently inhibited the cyclic activation and inactivation of brain vessel segments. Superoxide dismutase in brain parenchyma of normal rats reflect occasional perivascular ring patterns of immunoreactive tumor necrosis factor (TNF)-α, heme oxygenase-1, and manganese superoxide dismutase in brain parenchyma of normal rats reflect cyclic activation and inactivation of brain vessel segments.

Conclusions—The novel findings in this study support further investigation of immunologic tolerance as applied to the prevention of stroke. (Stroke. 2002;33:2156-2164.)

Key Words: E-selectin ■ endothelium ■ immune tolerance ■ risk factors ■ rats

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These cycles appear to be more frequent and intense in stroke-prone animals. Stroke risk factors, such as hypertension, diabetes, advanced age, and genetic predisposition to stroke, can prepare rodent brain vessels for thrombosis and hemorrhage in response to a single provocative dose of bacterial lipopolysaccharide (LPS), which induces the release of proinflammatory cytokines in a paradigm related to the local Shwartzman reaction. Conversion of the normally antithrombotic luminal surface of endothelial cells to a prothrombotic and proinflammatory state occurs in response to cytokines such as TNF-α, interleukin (IL)-1, and interferon (IFN)-γ, resulting in fibrin deposition and upregulation of adhesion molecules for platelets and leukocytes.
We found that controlling inflammation in the brain by inducing oral tolerance to myelin basic protein, a central nervous system antigen, decreased infarct size after middle cerebral artery occlusion in the rat. Oral tolerance is a well-established model whereby immunologic tolerance is induced to a specific antigen through the feeding of that antigen. Orally administered antigen encounters the gut-associated lymphoid tissue, which forms a well-developed immune network. Gut-associated lymphoid tissue evolved to protect the host from ingested pathogens and, perhaps by necessity, developed the inherent property of preventing the host from reacting to ingested proteins. The nature of the tolerance depends on the schedule and amount of antigen feeding. Clonal deletion of antigen-reactive T cells can occur after a single feeding of very high dose antigen; active tolerance with the production of regulatory T cells occurs after repetitive feedings of low-dose antigen. On antigen restimulation, T cells subjected to a low-dose regimen secrete cytokines such as transforming growth factor (TGF)-β1 and IL-10, which suppress cell-mediated, or Th1, immune responses. Although activation of these T cells is specific for the tolerizing antigen, the immunomodulatory cytokines secreted in response to activation have 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. Other forms of mucosal tolerance have also been investigated, specifically the administration of antigen via the nasal or aerosol route. The nasal route appears equally efficient and, in some instances, even more effective than the oral route in suppressing autoimmune diseases in animal models.

E-selectin is a glycoprotein cell adhesion molecule that is cytokine inducible and largely restricted to endothelial cells. It mediates the adhesion of various leukocytes, including neutrophils, monocytes, eosinophils, natural killer (NK) cells, and a subset of T cells, to activated endothelium. The expression of E-selectin is induced in human endothelium in response to cytokines IL-1 and TNF-α through transcriptional upregulation. E-selectin becomes expressed on vascular endothelial tissue where vascular segments have become activated.

On the basis of these data, we tested the hypothesis that circulating lymphocytes tolerized by transmucosal administration of E-selectin could target activated vessel segments in the brain that express E-selectin. These lymphocytes would then suppress the activation, prevent local thrombosis and hemorrhage, and, consequently, prevent stroke.

Materials and Methods

A total of 113 male and female offspring (aged 10 to 12 weeks) of spontaneously hypertensive genetically stroke-prone rat (SHR-SP) breeders were used (kind gift of Prof Y. Yamori, Kyoto University, Kyoto, Japan). The National Institute of Neurological Disorders and Stroke Animal Care and Use Committee approved all experiments. Injections were carried out with the animals under brief anesthesia with 4% isoflurane in 30% oxygen/70% nitrous oxide. Littermates were distributed to maintain group blood pressure equivalence. Intranasal instillations were as follows: (1) PBS (Bio-Whittaker), (2) ovalbumin (OVA, consisting of albumin, chicken egg, grade V, Sigma Chemical Co), or (3) recombinant (Chinese hamster ovary expression system) human E-selectin (lectin, epidermal growth factor, complement regulatory-like module-1 (CR1) and CR2 domains, and myc peptide tail, kindly provided by Protein Design Labs, Inc, Mountain View, Calif).

Tolerization Schedule

The tolerization schedule was as follows: (1) single (nonbooster): PBS (20 μL), OVA (2.5 μg/20 μL), or E-selectin (2.5 μg/20 μL) instilled into each nostril every other day for 10 days (total of 5 administrations); (2) repetitive (booster): intranasal instillations of the same substance at the same volume and concentration and on the same schedule as described above, repeated at 3-week intervals for the lifetime of the animal or until the termination of the experiment.

DTH Reaction

For assessing the delayed-type hypersensitivity (DTH) reaction, a single-course tolerization schedule with either PBS or E-selectin was conducted (n=7 and 9, respectively). 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, and afterward, the animals were rechallenged with 50 μg E-selectin/100 μL PBS injected into the ear. Ear thickness increase over baseline was measured with microlipers (Mitsutoyo Co, Ltd) 2 days later.

Immuonassays

The commercial serum cytokine assays used were as follows: OptEIA human TGF-β ELISA (PharMingen) and Quantikine M rat IFN-γ immunoassay (R&D Systems Inc).

ELISA plates (Nunc) were coated overnight (4°C) with 100 μL of 10 μg/mL recombinant human soluble E-selectin solution (sCD62E, R&D Systems Inc), diluted to 10 μg/mL in coating buffer) in carbonate buffer, pH 9.5, and blocked with 5% BSA in PBS (2 hours at room temperature). Plasma samples (100 μL per well) were added, and plates were incubated (2 hours at room temperature). After the washing procedure, 100 μL of biotinylated anti-β1(1:5000 dilution) was added, and plates were incubated (2 hours at room temperature); 100 μL of 1:250 HRP-conjugated streptavidin was then added to each well. Wells were washed (3 times in PBS/0.05% Tween 20) between each step, and plates were covered with adhesive strips during incubations. Anti-human E-selectin monoclonal antibody (biotin-conjugated mouse IgG1, clone BBIG-ES, R&D Systems Inc) confirmed plate coating and generated the standard curve. The standard curve generated for this assay was linear (0 to 100 ng/mL).

Immunofluorescence

Postfixed (acetone/methanol [1:1] spleen sections (10 μm) were stained for TGF-β1 as previously described. Polyclonal anti-TGF-β1, a kind gift from Kathy Flanders (National Cancer Institute, Bethesda, Md), was the primary antibody; fluorescein-conjugated donkey anti-rabbit F(ab’2) IgG fragment (1:5000 dilution) was added, and plates were incubated (2 hours at room temperature); 100 μL of 1:250 HRP-conjugated streptavidin was then added to each well. Wells were washed (3 times in PBS/0.05% Tween 20) between each step, and plates were covered with adhesive strips during incubations. Anti-human E-selectin monoclonal antibody (biotin-conjugated mouse IgG1, clone BBIG-ES, R&D Systems Inc) confirmed plate coating and generated the standard curve. The standard curve generated for this assay was linear (0 to 100 ng/mL).

Immunohistochemistry

Freshly frozen rat brain coronal sections (16 μm) were postfixed with cold acetone (15 minutes) and stained for E-selectin or intercellular adhesion molecule (ICAM)-1 as previously described. After a blocking procedure (5% normal donkey serum), antibody-binding sites were visualized (Vector ABC System, Vector Laboratories) with the use of diaminobenzidine as a chromogen. Antibodies were as follows: anti-rat E-selectin (1:500, Protein Design Labs), anti-rat CD4 (1:100, Cedarlane Laboratories Ltd), and biotinylated anti-rat CD54 (1:100, Cedarlane Laboratories Ltd), and biotinylated anti-rat macrophages (Clone:ED2, Biosource International).
donkey anti-mouse IgG F(ab')2 (1:2000; Jackson Immuno Research Laboratories, Inc). Sections were analyzed (Laborlux, Leitz) at ×100 magnification, and vessels expressing immunoreactivity for each antigen were counted in whole sections. The presence of immunoreactive IgG anti-endothelial cell antibodies (AECAs) was examined in frozen rat brain sections (16 μm) that had been postfixed with cold acetone (15 minutes), blocked (5% normal donkey serum), and incubated overnight (4°C) with F(ab')2 biotinylated donkey anti-rat IgG (1:1000; Jackson Immuno Research Laboratories, Inc). Antibody binding was visualized by using the Vector ABC System with diaminobenzidine. For quantification of endothelial IgG expression, images (magnification ×100) of 10 cortical regions in both brain hemispheres were obtained (Axioplan, Zeiss) and analyzed (MetaMorph image processing system, Universal Imaging Corp). IgG immunoreactivity was calculated as percentage of visual field area positive for AECAs.

**Results**

Repetitive Mucosal Tolerization to E-Selectin Prevents Ischemic and Hemorrhagic Stroke in SHR-SP

We administered OVA or E-selectin to 10- to 12-week-old SHR-SP as an intranasal instillation on a single or a repetitive course of tolerizations (Figure 1). We examined the tolerated...
rats at least daily for the duration of their lives to detect signs of stroke or complications of severe hypertension, such as cardiorespiratory failure associated with pulmonary edema. When signs of stroke or cardiorespiratory failure developed, we anesthetized the rats, collected blood samples, and, after perfusion fixation, removed their brains for analysis. Despite the intensive monitoring, 10 of the 40 SHR-SP initially divided among the 4 groups died between observations and were found unsuitable for further examination. We sectioned the remaining 30 perfused brains at 8 predetermined stereotactic levels (240 sections) and stained them with hematoxylin-eosin for blinded quantification of the numbers and areas of infarcts (MetaMorph, Universal Imaging Corp). The E-selectin single-course tolerization (ES) group had 12-fold more infarcts per animal than did the E-selectin booster (ESb) group (3.00 ± 1.9 [mean ± SEM] versus 0.25 ± 0.2, respectively); corresponding stroke numbers for the OVA booster (OVAb) group (4.00 ± 3.4) and OVA single-course tolerization (OVA) group (7.25 ± 3.5) represented 16- and 29-fold increases, respectively, over the ESb group (Figure 2A). There were no hemorrhages in the ESb group, in contrast to 2.3 ± 1.7 (mean ± SEM), 3.2 ± 2.8, and 2.8 ± 2.3 hemorrhages per animal in the ES, OVAb, and OVA groups, respectively (Figure 2C). The average total areas of infarction and hemorrhage per animal totaled from the 8 stereotactic sections were also lowest in the ESb group (0.002 ± 0.001 [mean ± SEM] and 0.0 mm² for infarct and hemorrhage areas per animal, respectively) compared with the ES group (11.4 ± 9.6 and 0.97 ± 0.94 mm², respectively), the OVAb group (6.8 ± 6.7 and 1.04 ± 0.78 mm², respectively), and the OVA group (27.7 ± 22.0 and 0.22 ± 0.16 mm², respectively) (Figure 2B and 2D); we were not able to perform stratified analyses of these covariates because of the limited data set. Lesions in affected animals were readily identifiable (Figure 3A).

We subjected additional animals (n = 4) to nasal instillation of PBS and followed them until they developed a stroke or...
cardiorespiratory failure. Regarding infarct number (7.8±3.0 [mean±SEM]), infarct area (12.4±4.4 mm²), and hemorrhage number (5.0±2.5), PBS data closely resembled OVA, OVAb, and ES group data listed above. Hemorrhage area in PBS was dominated by a huge 38.36-mm² hemorrhage in a single outlier and averaged 9.6±9.6 mm² (Figure 2A through 2D inserts). Comparison of ESb and PBS data (unpaired t test) revealed significant differences in infarct number (P=0.004), infarct area (P=0.002), and hemorrhage number (P=0.02).

Effective stroke prevention in the ESb group occurred despite persistent untreated hypertension. The initial and final blood pressures by the tail-cuff method (which generally underestimates catheter-recorded values by at least 10 mm Hg) were not different among the groups. The initial systolic blood pressures were (group mean±SD) 166±17 mm Hg for ESb, 176±23 mm Hg for ES, 164±8 mm Hg for OVAb, and 174±21 mm Hg for OVA. For the same 4 groups, the final systolic blood pressures were 192±26, 202±38, 189±22, and 211±36 mm Hg, respectively.

The animals lived for variable periods of time ranging from 14 weeks to the termination of the experiment at 56 weeks. Deaths were from heart failure secondary to severe hypertension in the ESb group and from strokes and heart failure in the OVAb, OVA, and ES groups. Differences in average age at time of death among the groups did not reach statistical significance with this sample size. At the 56-week termination point, Kaplan-Meier survival curves estimated that 50% of the ESb group, 40% of the OVAb group, and 30% of the ES and OVA groups would survive the 56th week (Figure 3B).

Figure 4. Comparison of the effect of E-selectin or PBS tolerization on DTH. The experimental paradigm and the differential effects on ear swelling are shown (for PBS, 0.36±0.18 mm; for E-selectin, 0.12±0.06 mm).

Mucosal Tolerization to E-Selectin Suppresses DTH
We demonstrated that intranasal instillation of human E-selectin does produce tolerance to a DTH paradigm described in Materials and Methods (Figure 4). Tolerance was antigen specific, inasmuch as intranasal administration of OVA had no significant effect on DTH (results not shown).

Mucosal Tolerization to E-Selectin Produces Regulatory T Cells and Suppresses Vessel Activation
We harvested the spleen from 14-week-old SHR-SP that had undergone a single-course tolerization with PBS, OVA, or E-selectin (n=3 to 4) and intravenous injection of 0.45 mg/kg LPS 2 weeks after tolerization to stimulate proinflammatory activation of blood vessels with E-selectin expression and to stimulate tolerized lymphocytes to produce TGF-β. Eight hours after LPS injection, we obtained 10-μm frozen spleen sections and stained for immunoreactive TGF-β. The ES group had a significant increase in the number of TGF-β-positive cells, mainly in the periarteriolar lymphatic sheaths (Figure 5, top panel). TGF-β–positive cells colocalizing CD4, CD8, and ED2 (monocyte/macrophage) were present, but no single marker colocalized with a majority of positive cells. Serum levels of TGF-β after LPS injection did not differ among the groups. In addition, animals exposed to single-tolerization PBS, OVA, and E-selectin produced detectable plasma IFN-γ in response to LPS (1175±978, 827±959, and 967±153 pg/mL, respectively; n=3 or 4); no IFN-γ response was observed in the booster ES group (3 tolerizations).
We instilled PBS or E-selectin intranasally in 3 SHR-SP per group on an initial tolerization schedule, followed by 2 booster tolerizations. Two weeks after the second booster, we injected 0.45 mg/kg LPS to activate the vessel endothelium and processed the brains for ICAM-1 and E-selectin immunohistochemistry. ICAM-1 expression was significantly reduced in E-selectin–tolerized animals (Figure 5, bottom panel).

**Antibody Formation**

We did not detect anti-human E-selectin antibody in animals tolerized to E-selectin in either the stroke prevention or the DTH studies. In animals that received either single or repetitive E-selectin tolerization (3 courses) followed 2 weeks later by intravenous LPS to activate endothelial expression of E-selectin (n=3 or 4 per group), a detectable anti-human E-selectin antibody response did occur. Serum levels of anti–E-selectin antibodies were significantly elevated (P<0.0001) in animals tolerized by intranasal E-selectin treatment compared with PBS or OVA treatment. The elevated levels of anti–E-selectin antibody seen after booster E-selectin did not differ from those observed after single-tolerization E-selectin. However, immunoreactive IgG AECAs on luminal endothelium were suppressed in single-tolerization ES animals relative to OVA and PBS single-tolerization animals that received LPS (n=3 per group) (Figure 6).

**Discussion**

E-selectin booster tolerization potently inhibited the development of ischemic and hemorrhagic strokes in SHR-SP rats with untreated hypertension. Tolerization to E-selectin also suppressed the DTH response to that antigen. ESb animals exposed to intravenous LPS differed from animals in the control groups; they had increased numbers of splenocytes positive for immunoreactive TGF-β, undetectable plasma levels of IFN-γ, and suppression of LPS-stimulated ICAM-1 expression. Immunoreactive IgG binding to luminal endothelium was also suppressed in ESb animals. E-selectin tolerization...

**Figure 5.** TGF-β-positive splenocytes (top) and adhesion molecule expression (bottom). Top, Quantification (mean±SEM) of TGF-β-positive splenocytes (as count per 10 fields) in PBS (278±22), OVA (288±15), and E-selectin (575±36) single-tolerization groups that received LPS. Bottom, Quantitative comparison (mean±SEM) of immunoreactive ICAM-1– and E-selectin–positive vessels per section, respectively, in PBS (485±62 and 138±15) and E-selectin (285±32 and 101±19) single-tolerization groups that received LPS (n=3 per group).

We instilled PBS or E-selectin intranasally in 3 SHR-SP per group on an initial tolerization schedule, followed by 2 booster tolerizations. Two weeks after the second booster, we injected 0.45 mg/kg LPS to activate the vessel endothelium.

**Figure 6.** AECAs on luminal endothelium of vessels. Top, Quantification (mean±SEM) of AECAs-positive vessels per section in PBS (8.6±0.4), OVA (3.6±0.9), and E-selectin (1.7±0.3) single-tolerization groups that received LPS (n=3 per group). Bottom, Photomicrographs of immunoreactive AECAs in brain vessels from the PBS (a), OVA (b), and ES (c) groups. Bar=100 μm.
tion was not associated with the formation of anti–E-selectin antibodies except in response to LPS stimulation.

The protection against thrombosis and hemorrhage in the present study was antigen specific and required that tolerization of lymphocytes be maintained as occurred in the ESb group. On this basis, vessel activation with E-selectin expression can be inferred to precede the development of thrombosis or hemorrhage. Furthermore, early stages in the development of thrombosis or hemorrhage involve local inflammatory and immune mechanisms that can be aborted by tolerization of lymphocytes to a locally expressed antigen. Applicability of these findings to clinical stroke could be influenced by the possibility that hypertension and immune function in SHR-SP and humans differ to some degree.

Evidence from our prior studies, from studies of lymphocyte trafficking, and from studies of the effector phase of mucosal tolerance supports the following model for the observed stroke prevention. Circulating antigen-specific (tolerized) lymphocytes undergo a process of tethering, rolling, activation by chemokines, arrest with firm attachment, and diapedesis in vessel segments that have become activated by proinflammatory cytokines. Various adhesion molecules and paracrine signaling molecules mediate this process, and their assorted combinations guide selective migration of antigen-experienced cells to specific nonlymphoid tissues.

The initial steps in adhesion, tethering, and rolling are mediated by selectins and $\alpha_\beta$ integrins. Chemokines, C5a (complement protein cleavage product), platelet-activating factor, and leukotriene B4 are mediators of lymphocyte activation. Integrins on lymphocytes bind to endothelial immunoglobulin superfamily members to mediate arrest. Once arrested on an activated vessel segment or having migrated into vessel wall, antigen-specific lymphocytes can be restimulated. Antigen presentation through the trimolecular complex (major histocompatibility complex class II molecule, antigen, and T-cell receptor) mediates restimulation. Endothelial cells that have been activated by IFN-γ (perhaps from CD4+CD28+ NK-T cells) express major histocompatibility complex class II molecules and can serve as antigen-presenting cells. Endocytosis of E-selectin expressed on vessels may facilitate presentation of that antigen. Pericytes contact endothelial cells through fenestrations in the basal lamina and, along with perivascular macrophages, can also serve as antigen-presenting cells in the vessel wall. Specific antigen presentation to T-regulatory cells stimulates the release of immunomodulatory cytokines such as TGF-β and/or IL-10, which provide active cellular regulation locally. These cytokines have broad immunosuppressive effects on lymphocytes and macrophages, inhibit inducible NO synthase, suppress superoxide anion generation, and reduce the expression of E-selectin. The net effect decreases thrombogenicity and preserves vessel integrity.

Several alternative mechanisms could contribute to the stroke prevention described in the present study. Although activation of endothelium with LPS did stimulate the production of anti–E-selectin antibodies in E-selectin–tolerized animals, anti–E-selectin antibody was not detected in the ESb group. This finding renders it unlikely that neutralizing anti–E-selectin antibodies prevented stroke in the ESb group by blocking that adhesion molecule in activated vessel segments. Additionally, E-selectin–deficient mice display no significant change in the trafficking of leukocytes because of the functional redundancy with P-selectin. Alternatively, overall reduction of AECAs, as shown by suppressed immunoreactive luminal rat IgG in the ESb group, could have reduced local activation or apoptosis of endothelial cells and prevented strokes. E-selectin tolerization may also have suppressed local activity of a functional T-cell subset associated with ischemia in unstable angina, the IFN-γ–secreting CD4+CD28+ NK-T cells.

Although the precise molecular mechanisms for the strong stroke prevention conferred by mucosal tolerization to E-selectin remain to be clarified, the overall effect of this intervention is to target immunosuppression to activated vessel segments. After a single course of antigen exposure, lymphocyte tolerance lasts for a period of weeks. Long-term maintenance of the tolerant state requires repeated booster exposures to the antigen. The novel findings in the present study support further investigation of immunologic tolerance as applied to the prevention of stroke.

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References

Immune Tolerance and Stroke: A Turning Point

In this issue of Stroke, an exciting report by Takeda and colleagues suggests a novel approach to prevent ischemic and hemorrhagic stroke by induction of mucosal immune tolerance to a specific physiological antigen: E-selectin. Mucosal tolerance describes a state of lymphocyte hypo-responsiveness to protein antigens applied across mucosal surfaces by oral or nasal instillation.\(^1\) Inducing specific mucosal immune hypo-responsiveness to self-antigens associated with autoimmune syndromes has been suggested as an appealing therapy to autoimmune diseases.\(^2\) Strikingly, Takeda et al demonstrate that induction of mucosal tolerance to E-selectin, by repeated nasal instillation of low levels of this antigen, markedly prevents the development of ischemic and hemorrhagic strokes in spontaneously hypertensive stroke-prone rats.

Previous reports have demonstrated that administration of specific antigens via the enteric route or application on the nasal mucosa leads to specific systemic suppression of cellular and/or humoral immune responses termed mucosal or oral tolerance, respectively.\(^3\) The tolerance is revealed when attempts are made to immunize the host to the same antigen that was previously administered orally or nasally. Mucosal tolerance induced by low doses of antigen triggers “active suppression” via activation of antigen-specific regulatory T cells that secrete immunomodulatory cytokines. Specifically, T-cell subsets that have been associated with mucosal immune hypo-responsiveness include the Th2, Th3, T regulatory 1, Th0, and CD4+ CD25+ T regulatory cells.\(^3\)

Although the “bystander suppression” by regulatory T cells is nonspecific, the activation of the regulatory T cells is mediated by specific tolerizing antigens. Intriguingly, the investigators demonstrate a preferential effect of E-selectin, mediated by specific tolerizing antigens. Intriguingly, the activation of the regulatory T cells is nonspecific, the activation of the regulatory T cells is mediated by specific tolerizing antigens. Intriguingly, the investigators demonstrate a preferential effect of E-selectin, mediated by specific tolerizing antigens.


tolerance in prevention of stroke. Several lines of evidence have indicated that E-selectin may play an important role in the pathophysiology of an ongoing ischemic stroke. However, this work further raises the possibility that E-selectin also plays a role in the predisposition of brain tissue to ischemic and hemorrhagic events. Thus, understanding the mechanisms that predispose the host to the development of stroke will help to identify other candidate antigens for which mucosal tolerance induces neuroprotection.

Major unanswered issues include the discrete molecular and cellular events that link mucosal tolerance to neuroprotection from stroke. The authors speculate that E-selectin antigen is presented via MHC class II on endothelial cells to T-cell receptors on antigen-specific regulatory T-cells, upon their attachment to an activated vessel segment. Consequently, these regulatory T-cells are presumably activated and release immunomodulatory cytokines that act on the endothelium to elicit anti-inflammatory and antithrombotic properties. This interesting hypothesis, however, remains largely unexplored. Because endothelial cells are not "professional" antigen-presenting cells, it will be crucial to demonstrate their role in E-selectin antigen presentation to specific regulatory T cells in this stroke-prone rat model.

An interesting observation in this article that might have deserved more attention is the generation of E-selectin antibodies in response to lipopolysaccharide (LPS) in rats apparently tolerized to E-selectin. This indicates that nasal administration of E-selectin results in positive selection of B cells with B-cell antigen receptor specificity to E-selectin. These E-selectin–reactive B cells are anergic under normal conditions of mucosal tolerance. However, stimulation with LPS activates these B cells in T-cell independent manner to differentiate and secrete antibodies to E-selectin. Although the authors indicate that the rats did not have circulating anti–E-selectin antibodies during the duration of the study, other studies by the same group have shown that a single administration of LPS increases the incidence of paralysis and death resulting from cerebral infarcts in stroke-prone rats. Interestingly, anti–E-selectin antibodies were shown to prevent tissue injury after cerebral ischemia. Activation of E-selectin antibodies will prevent tissue damage if ischemic, and hemorrhagic stroke coincides with infections in genetically prone subjects. Thus, humoral immune responses mediated by antigen-specific mucosal tolerance may provide another potential mechanism for neuroprotection in stroke-prone subjects.

In the current calendar year, strokes are expected to kill 150 000 Americans, and 500 000 more will suffer moderate to severe physical and cognitive disabilities. To date, at least 14 major clinical trials of drugs that targeted stroke amelioration have failed because of lack of efficacy, at a direct cost of perhaps $500 million plus significant impact on future investment decisions in this disease area.

The results presented by Takeda et al call for a paradigm shift in stroke research—to stroke prevention as a goal—and to a role for the immune system, and to nasal inhalation-induced tolerization to specific antigens, in building brain blood vessel resistance to stroke-triggering events. The promise of this shift, given the national statistics of stroke incidence, damage, and victim care costs, is so great as to warrant an extraordinary response to the ideas and data in Takeda et al. Those with the relevant knowledge and facilities should move quickly to confirm or refute these results, using a dedicated Web site to optimize communication. Once the results are confirmed, the research community should aggressively explore the underlying mechanisms, pursuing theory and application concurrently. The power of a parallel concerted effort on the part of dozens of laboratories and hundreds of researchers will dramatically advance the suggestive ideas presented here, transforming them into mature research programs.

**References**

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