α4 Integrin Is a Regulator of Leukocyte Recruitment After Experimental Intracerebral Hemorrhage

Matthew D. Hammond, BS; William G. Ambler, BS; Youxi Ai, MD; Lauren H. Sansing, MD, MSTR

Background and Purpose—Intracerebral hemorrhage (ICH) is swiftly followed by an inflammatory response. A key component of this response is the recruitment of leukocytes into the brain, which promotes neurological injury in rodent models. However, the mechanisms by which leukocytes transmigrate across the endothelium into the injured brain are unclear. The present study examines leukocyte adhesion molecules (α4 integrin, L-selectin, and αLβ2 integrin) on 4 leukocyte subtypes to determine which are important for leukocyte recruitment after ICH.

Methods—We used the blood injection mouse model of ICH, whereby 25 μL of blood was injected into the striatum. Flow cytometry was used to quantify leukocyte populations and adhesion molecule expression in brain and blood. An α4 integrin–blocking antibody was administered to evaluate the contribution of α4 integrin in leukocyte migration and neurological injury.

Results—α4 integrin was elevated on all leukocyte populations in brain after ICH, whereas L-selectin was unchanged and αLβ2 was increased only on T cells. Antagonism of α4 resulted in decreased leukocyte transmigration and lessened neurobehavioral disability.

Conclusions—α4 integrin is an important cell adhesion molecule involved in neuroinflammation after ICH.

Key Words: cell adhesion molecules • cerebral hemorrhage • inflammation • integrins • monocytes

Intracerebral hemorrhage (ICH) initiates an inflammatory response that is characterized by leukocyte recruitment and elevated cytokine levels. Specific leukocyte populations, including neutrophils, T cells, and inflammatory monocytes, promote secondary injury in models of ICH. It is thought that these cells principally inflict damage through the release of reactive oxygen species, proinflammatory cytokines, and proteases, but the mechanisms used for migration into the central nervous system after ICH are unclear. Although several studies have shown the importance of endothelial cell adhesion molecules, namely vascular adhesion protein-1 and intercellular adhesion molecule-1, for leukocyte recruitment after ICH, no study has examined adhesion molecules on the surface of leukocytes. In the present study, we examined changes in the levels of adhesion molecules on leukocytes in blood and brain. We also blocked α4 integrin function, which resulted in decreased leukocyte recruitment and improved motor function after ICH.

Methods
Protocols were approved by the UConn Health Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. ICH was modeled using 25 μL of autologous blood. Cells were analyzed using an LSRII cytometer (BD). For α4 integrin blocking, mice were injected with isotype control or anti-α4 (clone R1-2; 300 μg/mouse) 2 to 6 hours before ICH. Analysis was performed blinded to treatment. Detailed methods are provided in the online-only Data Supplement.

Results
To determine how ICH affects leukocyte adhesion molecule expression, we performed flow cytometry on blood and brain 2 days after ICH. A mean of 11,128±10,879 leukocytes were isolated from ICH brains versus 4079±305 cells in shams (n=4). The α4 integrin chain was elevated on all leukocyte populations in the ICH brain compared with blood (Figure 1A; Figure I in the online-only Data Supplement). Inflammatory monocytes, which had the highest baseline α4, represented the largest population recruited to the ICH brain at day 2 (Figure II in the online-only Data Supplement). Conversely, L-selectin was decreased on all leukocyte populations examined in the brain except for CD4 T cells, which were unchanged (Figure 1B). αLβ2 was higher on T cells in brain, whereas myeloid cells were unaffected (Figure 1C). Uniformly elevated α4 on all...
leukocyte populations suggests that it may mediate leukocyte recruitment after ICH.

To determine whether α4 is required for entry into the brain, we treated mice with an anti-α4–blocking antibody before ICH. Brain and blood samples were examined using flow cytometry 2 or 7 days later. Concentrations of T cells, neutrophils, and inflammatory monocytes were unchanged in blood by treatment (Figure 2A) as were physiological variables (Table I in the online-only Data Supplement). However, recruitment of T cells and inflammatory monocytes was significantly diminished in day 2 anti-α4–treated brains, suggesting α4 integrin function is a fundamental mechanism by which leukocytes migrate into the hemorrhagic brain (Figure 2B). Leukocyte quantities isolated from isotype control–treated brains were similar to the untreated ICH brains in Figure 1. Importantly, anti-α4–treated mice displayed significantly improved left forelimb use by the cylinder test up to day 2 (Figure 2C). Together, these data demonstrate that α4 is an important cell adhesion molecule involved in acute leukocyte recruitment after ICH.

**Discussion**

The present study aimed to understand how adhesion molecules on leukocytes are involved in cell recruitment after ICH. All leukocyte populations examined displayed increased α4 integrin, whereas only T cells showed elevated αLβ2, and no population displayed increased L-selectin in brain. Interestingly, inflammatory monocytes, which were recently shown to worsen ICH injury, represented the largest leukocyte population in brain and had the highest baseline α4 in blood. However, increases in adhesion molecules may not necessarily correlate with the influence of a particular molecule because conformational changes influence ligand affinities and molecules may be downregulated after tissue entry. We therefore confirmed the role of α4 with an antagonist. Treatment with the α4-blocking antibody decreased leukocyte recruitment and reduced early motor deficits, indicating its importance in ICH. α4 integrin heterodimerizes with β1 or β7 integrins. α4β1 is expressed on leukocytes and microglia, whereas α4β7 is found on gut-homing T cells and some vascular endothelium. Because the antibody recognizes the α4 subunit, we cannot attribute the observed benefit to a specific α4 heterodimer. Similarly, we cannot rule out the possibility that the antibody crosses a weakened blood–brain barrier and binds microglial α4 in addition to that on leukocytes or has systemic effects. Nonetheless, these results identify α4 integrin as an important cell adhesion molecule during acute sterile neuroinflammation.
Previous studies using α4-blocking antibodies in ischemic stroke models have shown benefits, both by reduced infarct volumes and by improved neurobehavioral functions. Although these studies mainly attributed improvements to reduced T-cell recruitment, they also showed decreased myeloperoxidase and Gr1 markers common to inflammatory monocytes and neutrophils, indicating myeloid cells were also decreased by treatment. Using flow cytometry, the present study discriminates between inflammatory monocytes, neutrophils, and microglia and identifies monocytes as having the largest decrease during α4 blockade. Interestingly, many leukocytes entered the brain despite α4 blockade, yet there was little disability up to day 2 in treated mice. This suggests that treatment may also inhibit integrin signaling, contributing to altered phenotypes once in tissue. This requires further study.

The study was not intended to evaluate α4 integrin as a therapeutic target. However, our findings suggest that blocking α4 function may provide neurological benefits by reducing acute inflammation. The absence of differences at day 7 is likely because of the single treatment. Although the half-life of the antibody is unknown, it is likely similar to the half-life of the isotype control (4–6 days). In addition, redundant migration mechanisms may compensate for blockade. Additional studies are needed to better characterize α4-mediated leukocyte recruitment using translationally relevant dosing paradigms.

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Disclosures
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References
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Supplemental Methods

Mice
C57BL/6J wild type mice were purchased from the Jackson Laboratory and then bred in-house. Male mice were used for experiments 8-12 weeks after birth. Forty-five total mice were used in these experiments (8 mice for experiments shown in Figure 1 and 37 mice for experiments shown in Figure 2). Mice were randomized to treatment groups (ICH/sham; control/anti-α4 integrin) by coin-flip.

ICH Model
Mice were maintained under 2-5% isoflurane inhalation anesthesia at 37 ± 1.0°C, as 25 µl of autologous blood were injected 0.5-1.0 µl per minute. The blood was injected in two fractions, pausing for 5 minutes to allow injected blood to clot, 2.5 mm right and 3 mm below bregma at a 5° angle toward the midline. This striatal ICH causes a moderate left forelimb deficit. Brains were evaluated for surgical success at the time of sacrifice and included only if the majority of blood was located in the striatum and minimal blood pooled on the surface of the brain. Shams received the same treatment, including needle insertion, but not blood injection. The investigator responsible for surgeries and assessment of surgical success on antibody-treated mice was blinded to the treatment of each mouse. One control mouse died and 1 anti-α4 integrin mouse was excluded due to a failed ICH surgery.

Antibody Treatment
Mice were treated with IgG2b, κ isotype monoclonal control antibody or anti-α4 integrin antibody (clone R1-2; both low endotoxin and azide-free from Biolegend). Injections were given intraperitoneally, 300 µg/mouse in 300 µl sterile PBS 2-6 hours prior to ICH.

Cylinder Test
At the same time each morning mice were placed into a clean glass jar and observed by a blinded scorer. As mice spontaneously reared and placed forelimbs on the wall of the jar, each rear was scored as right, left, or both for 20 rears. A laterality index was calculated to quantify a lateral forelimb deficit as follows: (right – left) ÷ (right + left + both). A score of +1.0 indicates exclusive use of the right forelimb (left forelimb deficit), whereas a score of 0.0 indicates equal use of the forelimbs and no deficit. Mice were either tested on days 1 and 2 or on days 5 and 7. One mouse from each group failed to sufficiently rear in 20 minutes of observation; they were excluded from cylinder test analyses.

Flow Cytometry
Brain preparation: two or seven days following ICH, mice were sacrificed and intracardially perfused with 40 ml ice-cold PBS. Brains were removed and the ipsilateral hemisphere was mechanically and enzymatically (collagenase/dispace and DNase; Roche) digested into a single-cell suspension. The resulting homogenate was filtered through a 70 µm cell strainer and suspended on a 30%/70% isotonic Percoll gradient (GE Healthcare) and centrifuged at 500 × g at room temperature. The interphase was harvested and washed for staining.
Blood preparation: 150 µl of blood were lysed with two room temperature incubations in an ammonium chloride hypotonic solution and centrifuged at 450 × g. The resulting pelleted leukocytes were washed for staining.

Cell staining: all samples were incubated for 10 minutes in Fc block (eBioscience) with rat IgG to block Fc receptors and minimize non-specific antibody staining. Samples were then incubated for 15 minutes with monoclonal antibodies against the following cell-surface markers: CD45, αLβ2/LFA-1 (Biolegend), Ly6G/1A8, Ly6C (BD Biosciences), CD4, CD8, CD62L/L-selectin, CD11b (Tonbo Biosciences), and CD49d/α4 integrin (eBioscience). Dead cells were identified in each sample by their excessive uptake of Alexa Fluor 350-carboxylic acid, succinimidyl ester (CASE; Life Technologies). Counting beads (10,000 per sample; Life Technologies) were added to determine the portion of each sample that was analyzed by the cytometer. The number of beads collected was then used to calculate the total number of cells present in each brain sample and to back-calculate the concentrations of leukocyte populations in the blood. Fluorescence-minus-one (FMO) negative controls were used for each antibody and were made freshly each preparation day. Single-stain positive controls were used for cytometer compensation and an unstained negative control was used to determine background.

Data collection & analysis: cells were evaluated using an LSRII flow cytometer running Diva software (BD Biosciences) equipped with 5 laser lines (355, 405, 488, 561, and 640 nm). Data were analyzed using FlowJo software (Tree Star). As shown in Supplementary Figure I below, only single cells found to be alive by CASE staining were used in analyses. CD45<sup>hi</sup> cells were considered blood-derived leukocytes in the brain. Cell classification is as follows: CD45<sup>hi</sup>, CD4<sup>+</sup> (CD4 T cells); CD45<sup>hi</sup>, CD8<sup>+</sup> (CD8 T cells); CD45<sup>hi</sup>, CD4<sup>-</sup>, CD8<sup>-</sup>, Ly6G<sup>+</sup>, Ly6C<sup>+</sup> (neutrophils); and CD45<sup>hi</sup>, CD4<sup>-</sup>, CD8<sup>-</sup>, Ly6G<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>hi</sup> (inflammatory monocytes). In Figure 1 these populations were then analyzed for their surface levels of specific adhesion molecules by the mean fluorescence intensity (MFI), a measure of molecule abundance. The MFI was then multiplied by the proportion of each population that stained positive for that specific adhesion molecule to find the integrated MFI (iMFI) displayed in the Figure 1 bar graphs, which is a combined measure of molecule frequency and surface density.

Statistical Analyses
Data were tested for normality using the Shapiro-Francia test at α=0.05. Distributions found to be approximately normally distributed were tested for differences using an unpaired t test, whereas distributions found not to be normally distributed were tested using the Mann-Whitney U test. P values less than 0.05 were considered statistically significant. All statistical tests were performed using Stata software.
Supplemental Table

**Supplemental Table I:**
Mouse physiological variables during ICH surgery were unaffected by pre-treatment with an α4 integrin blocking antibody.

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>p value temperature</th>
<th>Respiratory Rate (mean ± SD)</th>
<th>p value respiratory rate</th>
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<tr>
<td>Isotype Control</td>
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<tr>
<td>Anti-α4 Integrin</td>
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<td></td>
<td>94.76 ± 15.23</td>
<td></td>
</tr>
</tbody>
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Supplemental Figures

**Supplemental Figure I.** Flow cytometry gating strategy in a day 2 ICH brain. **A,** Cells staining low or negative for CASE were considered live cells. **B,** Single cells, as assessed by forward scatter height (FSC-H) and forward scatter width (FSC-W) were gated for further analysis. **C,** A “leukocyte gate” was drawn on the forward scatter area (FSC-A) by side scatter (SSC) plot to isolate all leukocytes and microglia. **D,** CD45 intensity was examined to separate microglia from blood-derived leukocytes. **E,** CD45\textsuperscript{low} cells were classified as CD11b\textsuperscript{+} microglia. **F,** CD45\textsuperscript{hi} cells were further interrogated for the T cell markers CD4 and CD8. **G,** Blood-derived non-T cells were examined by Ly6G and Ly6C to find double-positive neutrophils. **H,** The remaining cells were then analyzed by CD11b and Ly6C to locate the Ly6C\textsuperscript{hi} inflammatory monocytes. All cell population gates were based on FMO negative controls. The numbers inside or near each gate refer to the percentage of cells on the entire plot that fall within the respective gate.
**Supplemental Figure II.** Inflammatory monocytes represent the largest population of CD45$^{hi}$ blood-derived leukocytes in the brain 2 days after ICH. Bars indicate mean ± SD. N=4.