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Depletion of Ly6G/C+ cells ameliorates delayed cerebral vasospasm in subarachnoid hemorrhage

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Abstract

Background—The etiology of delayed cerebral vasospasm (DCV) after aneurismal subarachnoid hemorrhage (SAH) has remained elusive. Growing evidence supports a role for inflammation in the pathogenesis of DCV. We showed that CSF neutrophils predict which patients will develop DCV.

Methods—We evaluated a murine model of SAH to test the hypothesis that myeloid cells are required for the cerebral damage associated with DCV.

Results—SAH was associated with decreased middle cerebral artery caliber on day 1 which normalized at day 3 and recurred at day 6. In addition, behavioral testing with a Barnes maze showed executive dysfunction that progressively worsened after the seventh day post hemorrhage. To test the role of innate immune responses, we administered a myeloid cell-depleting monoclonal antibody against Ly6G/C prior to experimental SAH. Myeloid cell depletion ameliorated angiographic vasospasm measured by MCA vessel caliber and normalized behavioral testing.

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Competing Interests

The authors have no conflicting interests for this work.

Conclusion—Our findings support the role of Ly6G/C⁺ cells in the development of DCV after SAH and suggest that immune modulation of neutrophils or other Ly6G/C⁺ cells may be a strategy for the prevention of DCV.

Introduction

Delayed cerebral vasospasm (DCV) is a late complication of aneurysmal subarachnoid hemorrhage. Patients who experience DCV show both focal neurological signs and global acute cognitive decline during periods of DCV. The cerebral arteries in DCV patients are narrowed 4–14 days after subarachnoid hemorrhage (SAH) (Provencio and Vora, 2005). The precise mechanism by which bleeding from cerebral aneurysms causes this delayed reaction remains unclear but several lines of evidence implicate inflammation in the pathogenesis of the vasculopathy (Takizawa et al., 2001, Gallia et al., 2006, Manno et al., 1997, Lin et al., 2005).

In our laboratory, we have found that neutrophils in the cerebral ventricular fluid (CSF) predict which patients with SAH will later develop DCV (Provencio et al., 2010). A number of studies in animal models have also suggested a role for innate immune inflammation in its pathogenesis. Blockade of E-selectin, a trafficking molecule for neutrophils and other inflammatory cells across the vascular endothelium, has been shown in a murine model to ameliorate vasospasm (Lin et al., 2005). Fasudil, a protein kinase inhibitor that is known to decrease chemotaxis of granulocytes also decreased vasospasm in a dog model (Shibuya et al., 2001). Administration of a humanized anti-CD11/CD18 antibody decreased vasospasm in a rabbit model (Pradilla et al., 2004).

Direct evidence of innate inflammatory cell action is lacking in the development of DCV. Ly6G/C is a cell surface marker found on cells of the myeloid lineage, predominantly neutrophils and monocytes (but also reported in small quantities on activated CD8⁺ T cells) (Daley et al., 2008). Antibodies against Ly6G/C have been exploited as markers of innate immune cells (Tazawa et al., 2003, Kurizaki et al., 2004). Because the innate inflammatory pathway may be a reasonable target for treatment or prevention of DCV, we investigated the role of Ly6G/C⁺ cells in DCV.

Methods

All experiments were conducted under the supervision of the Cleveland Clinic Institutional Animal Care and Use committee (IACUC).

SAH and the evaluation of vasospasm

We studied male C57BL/6 mice (Jackson Labs, Maine) weighing 20–32 g, 10–12 weeks of age (Table 1). Our murine model of SAH has been described (Altay et al., 2009). Briefly, mice were anesthetized and placed in a prone position. An incision was made in the midline of the neck, the alanto-occipital membrane was punctured and a subarachnoid vein was transected. The bleeding was allowed to stop spontaneously after which the incision was closed. Saline injection sham surgery involved the same procedure except that the alanto-occipital membrane was entered with a 30g needle and 50µl of saline was instilled.

Myeloid cell depletion

animals were pretreated with the myeloid cell-depleting anti-ly6G/C antibody (RB6-8C5) at a dose of 13mg/ kg i.p. 24 hours prior to surgery and immediately before surgery, or 50 µl of saline (saline control). The dose of RB6-8C5 was chosen based on previous reports at 10mg/ kg i.p (Tazawa et al., 2003, Kurizaki et al., 2004). A slightly higher dose was chosen due to

the larger size of the animals than in previous studies which may not be reflected completely in weight algorithms. Pilot experiments with control animals given pretreatment with the irrelevant isotype-matched antibody, anti- β -Gal (GL113) prior to SAH yielded similar responses to saline controls and were therefore not included in this analysis.

India ink assessment of vessel caliber

Animals were anesthetized with pentobarbital (6 mg/100 g i.p.); trans-cardiac perfusion was performed with 20 ml 4% paraformaldehyde followed by 10 ml of warmed 5% India ink in gelatin. Animals were decapitated, and their brains were removed carefully preserving the vasculature. The circle of Willis vasculature was examined under the surgical microscope and relevant pictures captured (Leica, Wetzler, Germany) and analyzed with Adobe Photoshop CS2 (San Jose CA). The diameter of the middle cerebral artery (MCA) segment was measured 1 mm from the posterior wall of the carotid artery by a member of the research team not involved in the surgery and blinded to the intervention. Measurements were made on the 1st, 3rd, 6th, and 9th day after the hemorrhage. Ten mice were used for each of the three conditions (Sham, SAH, and myeloid cell depleted SAH) for 1st, 3rd, and 6th day analysis (30 mice per day total); five were used for the 9th day analysis.

Flow cytometry

We tested the neutrophil populations in whole blood by flow cytometry. Whole blood samples were obtained with SAH and myeloid cell depletion in a heparinized syringe using cardiac puncture just prior to perfusion. The samples were stained using APC-conjugated Anti-Mouse CD45, FITC-conjugated Anti-Mouse neutrophil specific Ly-6G mAb, and PE-conjugated anti-mouse CXCR2 mAb (BD Pharmingen). Flow cytometric analysis was performed on a LSRII flow cytometers (Becton Dickinson). CD45^{hi} cells that were bright for Ly6G and CXCR2 were defined as neutrophils.

Behavioral testing

Animals in the behavioral study underwent three batteries of tests in groups of 12 mice (4 mice from the three experimental conditions were tested concurrently and the experiment was repeated 3 times (total 36 mice)). All tests were done at the same time of the day by a single handler (SKM) and scored by a different examiner (SS). On day 1 and 13 post-hemorrhage, the mice underwent rotorod testing to evaluate motor function and coordination. The second and 11th day post-hemorrhage, the mice underwent Y-maze testing to evaluate spatial working (immediate) memory. On days three through 10, the mice were tested in a Barnes maze to test spatial learning, short-term and long-term memory (Figure 1).

In the rotorod test, time on the rod and maximum speed of rotation (RPM) were measured. Animals were placed in one of 4 separated chambers with the spinning rod and a landing base. After a habituation for 30 seconds at low speed, the mice were tested with a continuously increasing rate of speed for 5 minutes or until the subject fell off the rod. In the Y-maze, animals were placed in a random orientation in the maze which has 3 open arms of the same length in a Y configuration. The mice were recorded during 5 minutes of exploration and scored later. Percent alterations was calculated as the ratio of the number of times the animals chose to enter the three arms of the maze in succession to the total number of unique arms the animal chose to enter.

In the Barnes maze, the mice are placed on a table with multiple open holes one of which has a box that represents a safe haven. There are visual cues on the wall around the table for the animal to orient itself. Each animal had 2 days of training where it was gently guided to the goal box. On subsequent days, animals were placed in the center of the table in a covered enclosure until ready for the trial. The cover was lifted and mice were allowed to find the

goal box. The movements were recorded and scored later. The time it took to find the goal box (latency) was recorded.

Immunohistochemistry

The monoclonal anti-microglial cell antibody (IBa1) (gift from Dr. Bruce Trapp) and the monoclonal anti-neutrophil antibody (7/4) (Serotec, Raleigh, NC) were used. Thirty micron brain slices were incubated in primary antibody for 24 hours using a free floating technique. Briefly, 30 μ m slices were microwaved for 3 minutes in citrate buffer followed by incubation in 500 μ l of 3% goat serum and primary antibody at 20°C overnight in a 24 well plate. Secondary antibodies were resolved with either 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich, Saint Louis, MO) or by direct conjugation to fluorescent signals (Jackson Immunoresearch, West Grove, PA). Neutrophils in the brain were determined using a semi-quantification method by matching coronal brain sections at the level of the third ventricle and counting neutrophils in a 40 $\times$ grid over the inferior aspect of the left parietal lobe (adjacent to the basal cistern) (Figure 3B).

Quantitation of microglial activation was accomplished by selecting 10 fields using the same grid shown in Figure 3D and calculating the total cell area and percent of field covered by stained microglia for each experimental group. The images were generated with a Leica 4000B microscope at 40 $\times$ magnification. Images were stored in a standard format (TIFF file: 4.64 $\times$ 3.47in. at 300 dpi). Measurements were made using Image J software (NIH, Bethesda, MD) from the images and are reported in pixels.

Statistics

Statistical analysis was performed with the aid of Graphpad Prism 5.0 (Graphpad Software Inc. San Diego, CA). Continuous variables were compared using one-way and two-way ANOVA for tests done once and those done sequentially over time, respectively. Where there was significant interactions, individual groups were compared using Student's t-test. Fisher's exact test was used for non-continuous variables. A p-value of <0.05 was considered significant in all comparisons.

Results

DCV occurs at day 6 in our SAH model

There was no operative or perioperative mortality in this study; all animals survived until the end of testing. After surgery, all animals returned to feeding and ambulating normally within 3 hours. Mice subjected to experimental SAH were found to have significant narrowing of their MCA compared to controls on the first day post hemorrhage ($0.080 \text{ mm} \pm 0.004$ vs. $0.111 \text{ mm} \pm 0.005$ [mean $\pm$ SEM], $p < 0.0001$) that resolved by the 3rd day (0.107 ± 0.009 vs. 0.111 ± 0.007 , $p = 0.712$). On the 6th day post hemorrhage, DCV was noted with significant narrowing of the MCA (0.086 ± 0.003 vs. 0.114 ± 0.006 , $p = 0.004$) (Figure 2A and B). On Histology, vasospastic arteries showed corrugation of the arterial wall not seen in controls (Figure 2A and B). By the 9th day post hemorrhage, there was no vessel narrowing (0.115 ± 0.003 vs. 0.115 , $p = 0.979$) (Figure 2D). This suggests a period of acute vasospasm on the first day post hemorrhage that resolved by day 3 and DCV developing by day 6 before dissipating by day 9.

Myeloid cell depletion prevents delayed cerebral vasospasm

To test whether myeloid cells play a role in the development of vasospasm, we administered the anti-Ly6G/C antibody (RB6-8C5) prior to experimental SAH. The frequency of total neutrophils (CD45^{bright}/Ly6G⁺/CXCR2⁺ cells) in the blood of mice treated with the Anti-Ly6G/C antibody decreased 60% (15% to 6%) compared to control animals 24 hours after

SAH and remained low until day 6. The neutrophil percentage in the SAH group increased from 20% to 40% between day 3 and day 6 (Figure 3A).

The diameter of the MCA was significantly larger in myeloid cell depleted animals with SAH than in SAH animals alone one day after hemorrhage ($0.119 \text{ mm} \pm 0.005$ vs. 0.080 ± 0.004 [mean $\pm$ SEM], $p < 0.0001$). Because non-depleted SAH animals had normalization of MCA diameters on the 3rd day, the diameters were not significantly different from depleted animals with SAH. By the 6th day, delayed vasospasm was seen in the non-depleted animals but not the depleted animals (0.086 ± 0.003 vs. 0.106 ± 0.003 , $p = 0.003$) (Figure 2D). The MCA diameter of the depleted group with SAH was not significantly different from sham treated animals on the 1st through the 9th day post procedure ($p = 0.615$).

Microglial activation early after SAH is myeloid cell-dependent

Immunohistological evaluation of the brains and meninges of mice one day after SAH showed neutrophils (7/4⁺) in the brain parenchyma. Occasional neutrophils were seen in the meninges of sham treated and myeloid cell depleted animals but parenchymal neutrophils were uncommon. Quantitative analysis of neutrophils in the brain parenchyma showed significantly more neutrophils per high powered field after SAH without myeloid cell depletion than sham or SAH with myeloid cell depletion ($4.8 \text{ neutrophils/HPF} \pm 0.73$ [mean $\pm$ SEM] vs. 0.2 ± 0.13 vs. 0.4 ± 0.16 , $p < 0.0001$, respectively) (Figure 3B). By day six, there were no neutrophils seen in the tissue for any of the groups (data not shown).

Microglia in SAH showed significant activation defined by thickened processes and enlarged cell bodies (ameboid morphology) (Figure 4B) not seen in the sham animals (Figure 4A) one day after SAH. Although in the SAH mice, the majority of microglia were activated, the highest density of activated microglia were near the basal cistern where the blood clot was concentrated. Analysis of the total visible area of cell/field showed that microglia after SAH had significantly increased cellular area compared to Sham ($1001 \text{ pixels} \pm 98$ vs. 546 ± 66 , $p = 0.0016$). After myeloid cell depletion, the microglia showed markedly less cell area than SAH ($310 \text{ pixels} \pm 39$ vs. 1001 ± 98 , $p < 0.001$) (Figure 4E). Immunofluorescent double staining for microglia and neutrophils showed that parenchymal neutrophils were directly apposed to the cell bodies of activated microglia (Figure 4D). On day 6 after SAH, there was no change of Sham mice microglia but the SAH mice with and without myeloid cell depletion appeared less activated than on day 1 (Figure 4A,B,C).

Myeloid cell depletion reverses behavioral deficits in delayed cerebral vasospasm

Behavioral testing was done in 12 mice per group and included rotarod testing to determine if there are physical deficits that could confound Y-maze and Barnes maze cognitive tests. Rotarod testing of mice after experimental SAH showed no motor deficits or incoordination on day one post hemorrhage compared to sham or neutrophil depletion in either time on the rod ($114 \text{ seconds} \pm 11$ vs. 133 ± 14 vs. 114 ± 11 [mean $\pm$ SEM] respectively, $p = 0.409$) or maximum RPM (15.8 ± 1.22 vs. 13.8 ± 1.00 vs. 13.7 ± 0.95 , respectively, $p = 0.337$). There was similarly no difference at day 13 post hemorrhage. Myeloid cell depleted mice with SAH showed no difference from control or non-depleted mice with SAH in rotarod testing.

Y-maze testing in SAH and sham revealed significantly decreased percent alternations in SAH animals on the second day post hemorrhage ($47.7\% \pm 2.51$ vs. $58.2\% \pm 1.65$, $p = 0.002$) that was not apparent on retesting at day 13. Myeloid cell depleted mice with SAH showed no difference from sham in Y-maze testing.

Barnes maze testing done every day after a two-day education period showed that the three groups were significantly different ($p < 0.0001$). SAH animals initially took significantly longer to find the goal box (latency) than controls and myeloid cell depleted mice (266.9

seconds $\pm$ 23.6 vs. 47.33 ± 13.5 vs. 96.5 ± 26.54 respectively, $p < 0.0001$). By the 7th day post hemorrhage, there were no differences between the groups (89.3 ± 20.5 vs. 64.11 ± 17.7 vs. 52.58 ± 13.2 , $p = 0.326$). After the 7th day, the SAH group progressively declined. From Day 8 to 10, SAH significantly altered behavioral performance compared to sham and myeloid cell depletion ($p < 0.0001$). The myeloid cell depleted mice were similar to sham. After the 7th day, the SAH mice had worsening latency not seen in the myeloid cell depleted SAH group (Figure 5). These data support the hypothesis that Ly6G/C⁺ cell depletion protects from delayed behavioral deficits after SAH.

Discussion

The pathogenesis of DCV after SAH remains elusive despite extensive investigation. In SAH patients, elevated neutrophil levels in the CSF predict which patients will develop DCV (Provencio et al., 2010). In addition, other inflammatory markers have been associated with DCV. Recently, the systemic inflammatory response syndrome (SIRS) on admission to the hospital has been associated with DCV (Dhar and Diringer, 2008).

The present study demonstrates a role for Ly6G/C⁺ cells in a murine model of DCV after SAH suggesting strongly that myeloid cells are involved in the pathology of DCV. Importantly, myeloid cell depletion prevents both the vascular sequelae and the behavioral manifestations seen in DCV. It is unclear if partial doses of the antibody have any effect on DCV or the behavioral abnormalities. Microglial activation, evidence of a cerebral inflammatory response is seen one day after the hemorrhage in normal mice but not in myeloid cell depleted animals. In fact, some activated microglia appear to be physically associated with neutrophils suggesting possible communication.

Other studies support the role of inflammatory cells in the pathogenesis of DCV. Administration of an E-selectin (a mediator of leukocyte entry into the central nervous system) blocker (Lin et al., 2005) and administration of antibodies against the neutrophil/macrophage adhesion molecule CD11/CD18 decrease the severity of angiographic vasospasm (Pradilla et al., 2004). Vasospasm in these studies was present at less than 48 hours not the 6 days seen in our study. The delayed nature of vasospasm in our study is more closely temporally associated with the human disease. A recent study shows that a blocker of TLR4 receptor prevents neurological deterioration early after experimental SAH but did not investigate DCV (Ma et al., 2009). The present study is the first work to our knowledge that suggests the specific role of myeloid cells in the pathogenesis of DCV.

Additionally, the finding that myeloid cell depletion ameliorates the vasospasm seen on day one suggests that the process of inflammation that leads to DCV likely begins very early in the course of SAH. Human studies have linked early vasospasm with poor outcome (Baldwin et al., 2004). Zhang and colleagues have published a number of studies focused on early inflammation in SAH leading to more cell death (Ostrowski et al., 2006, Zubkov et al., 2000). It is tempting to link the process of acute vasospasm and early inflammation with the later development of DCV. The finding of normalization of blood vessel diameter on day 3 and recurrence of vasospasm at day 6 combined with the worsening in executive function testing suggests that the process of DCV is a distinct syndrome that may be tied to early inflammation but is not a continuation of the earlier event.

The pattern of behavioral deficits is interesting. The lack of deficits on Y-maze and rotarod testing suggests that physical disability and immediate recall are not affected. This may be due in part to the mild nature of the DCV in our model compared to other models (Mesis et al., 2006). The major finding of deficits in executive function is supported by a study of psychosocial outcome in patients after SAH in which Powell and colleagues found that

executive function was impaired up to 18 months after the ictus (Powell et al., 2004). The authors also noted an unusually low level of employment which may suggest executive difficulties.

Based on our studies in patients with SAH where neutrophil increase was positively associated with DCV and where no evidence of significant monocyte or CD8 T-cell accumulation was present (unpublished observation) we feel that the most likely culprit cell is the neutrophil (Provencio et al., 2010). A report by Oruckaptan and colleagues found specifically that myeloperoxidase (MPO) activity was not implicated in angiographic vasospasm in a rabbit model of SAH (Oruckaptan et al., 2000). This raises the question of whether other components of the myeloid response that are affected by Ly6G/C antibody administration are important in DCV. The techniques used in this work were substantially different than our evaluation which may contribute to the differences in results. Additionally, MPO activity is one of many mechanisms by which neutrophils affect damage in the local environment, but it is possible that mechanisms other than MPO in neutrophils may have a role.

The mechanisms by which neutrophils can cause the vascular and behavioral changes in DCV are still unclear. Work by Clark and colleagues suggest that bilirubin oxidation products lead to vascular damage seen in DCV (Clark and Sharp, 2006, Pyne-Geithman et al., 2005). The source of superoxide in the oxidation of bilirubin is likely NADPH oxidase from neutrophils. Additionally, we found in patients with SAH an increase in neutrophil-derived reactive oxidant species suggesting a role for neutrophils (Provencio et al., 2010). Finally, neutrophils make other compounds that, when released inappropriately, could lead to neuronal dysfunction such as elastase and the metalloproteinases (Nguyen et al., 2007, Naruko et al., 2002).

The findings of this study suggest that myeloid cells (and, in conjunction with human studies, neutrophils) are possibly involved in the development of DCV in humans. This study suggests a possible new direction for development of treatments in DCV. If the flow of neutrophils into the brain can be impaired early after SAH, DCV could be averted. Depleting myeloid cells in patients in the intensive care setting has a number of dangers including the risk of overwhelming infection. A better understanding of the mechanism may allow a more targeted approach that is potentially less dangerous.

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Figure 1. Time line of experiments

(A) Time line for SAH/Sham animals. The Orange box signifies the days in which Barnes maze testing was done. (B) Time line for myeloid cell depletion/sham SAH animals. The dark green box signifies the days of administration of the anti-Ly6G/C antibody.

Figure 2.

Middle cerebral artery (MCA) diameter on day 6 after SAH (left) in Sham (A), SAH (B), and SAH after myeloid cell depletion (C). Black arrows in left panels depict the area where artery was measured. H&E sections of the MCA artery (middle-low resolution; right-higher resolution) show corrugation of artery in SAH (B) but not sham (A) or myeloid depleted mice (C). Black arrow in right panel (B) shows an intravascular neutrophil. (D) Diameter of MCA on days 1, 3, 6, and 9. The differences between the sham, myeloid cell depleted mice and SAH were significant ($p=0.004$). On day 1 and 6, SAH mice had more vasoconstriction than myeloid cell depleted SAH mice.

Figure 3.

(A) Flow cytometric analysis of blood from mice after administration of the anti-Ly6G/C antibody. CD45^{bright}/Ly6G⁺/CXCR2⁺ cells are decreased from day 1 through 6 in the depleted mice despite the increase in percentage of CD45^{bright}/Ly6G⁺/CXCR2⁺ cells on day 6 in the control animals. (B) Quantitative analysis of neutrophils in the brain was accomplished by evaluating the areas of the parietal lobe shown in the diagram. Analysis shows higher neutrophil (7/4⁺ cell) concentration in SAH than either sham or myeloid cell depletion ($p < 0.0001$).

Figure 4.

Microglial staining (Iba1) on day 1 and 6 after SAH. Sham (A) mice showed ramified microglia whereas SAH (B) showed amoeboid microglia. Microglia in mice with SAH after myeloid cell depletion (+Ly6G/C) (C) appeared ramified. By day 6, sham and SAH after myeloid cell depletion remained unchanged. SAH became more ramified signaling a transition to a more quiescent state. In SAH animals at day 1(D), activated microglia (Iba1, Red) were often closely associated with neutrophils (7/4, Green). Higher magnification views show close apposition of neutrophils and microglia. (E) 10 sections were photographed and total cellular area/field was calculated. The total cellular area/field was significantly larger in SAH compared to control and myeloid cell depletion (+Ly6G/C).

Figure 5.

Mice with SAH have impaired behavioral tests. Barnes Maze test showed initial poor performance in finding the goal box in animals with SAH. Over the first 6 days, the times between the three groups converged so that on day 7, there was no difference between Sham, SAH, and myeloid depleted mice. After day 7, the SAH animals significantly worsened compared to Sham, SAH after myeloid cell depletion ($p < 0.0001$). The myeloid cell depleted animals are not different from Sham animals over the time period ($p = 0.409$).

Table 1

Animals in experiments

	India Ink	Behavioral Testing	Immunohistology/Flow cytometry
Sham (day 1,3,6,9)	35 (10,10,10,5)	12	3
SAH (day 1,3,6,9)	35 (10,10,10,5)	12	3
SAH+Ly6G/C (day 1,3,6,9)	35 (10,10,10,5)	12	3