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Prevention of vasospasm following subarachnoid hemorrhage in rabbits by anti-CD11/CD18 monoclonal antibody therapy

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Object. Adhesion of leukocytes and their migration into the periadventitial space may be critical in the pathophysiology of vasospasm following subarachnoid hemorrhage (SAH). The cell adhesion molecules involved in this process are lymphocyte function–associated antigen–1 (CD11a/CD18) and macrophage antigen–1 (CD11b/CD18), which are present on neutrophils/macrophages, and intercellular adhesion molecule–1 (CD54), which is present in endothelial cells. A humanized monoclonal antibody (mAb), Hu23F2G, targets CD11/CD18 and prevents leukocyte adhesion to endothelial cells. In this study, systemic administration of Hu23F2G prevented vasospasm in the rabbit model of SAH.

Methods. Twenty-six New Zealand White rabbits were injected with autologous blood into the cisterna magna to induce SAH, after which they were randomized to receive injections of either Hu23F2G (10 animals) or a placebo at 30 minutes and 24 and 48 hours after SAH (six animals). Control animals underwent sham operations (four animals) or SAH alone (six animals). The animals were killed 72 hours after SAH, their bodies perfused and fixed, and their basilar arteries processed for morphometric analysis. Peripheral white blood cells (WBCs) were counted at 72 hours. The percentages of lumen patency were compared using the Student t-test. The presence of neutrophils and macrophages was confirmed by immunohistochemical analysis in which a rat anti–rabbit anti-CD18 mAb and cresyl violet were used.

Treatment with Hu23F2G resulted in the significant prevention of vasospasm. Animals treated with Hu23F2G had 90 ± 7% lumen patency compared with 65 ± 7% in the placebo group ($p = 0.025$). The percentage of lumen patency in the SAH-only group was 59 ± 10%. The mean WBC count was 16,300 ± 2710/μl in the treatment group, compared with 7000 ± 386/μl in the control group ($p = 0.02$). Administration of Hu23F2G produced increased numbers of WBCs in 70% of the animals treated.

Conclusions. This study supports the concept that leukocyte–endothelial cell interactions play an important role in the pathophysiology of chronic vasospasm after SAH. Systemic therapy with an anti-CD11/CD18 mAb prevents vasospasm after SAH by inhibiting adhesion of neutrophils and macrophages and their migration into the periadventitial space.

Key Words • vasospasm • subarachnoid hemorrhage • leukocyte–endothelial cell interaction • anti-CD11/CD18 monoclonal antibody • rabbit

Leukocyte–endothelial cell interactions, as mediated by CAMs, appear to play a major role in the development of posthemorrhagic cerebral vasospasm after aneurysmal SAH.[14,15,23,36,40,42,44] Specific CAMs involved in leukocyte migration into the periadventitial space after hemorrhage include ICAM-1 (CD54),[4,23,29,32,38,40,44,45] and the β-2 integrins LFA-1 (CD11a/CD18)[15] and Mac-1 (CD11b/CD18).[4,15] Intercellular adhesion molecule–1, a member of the Ig superfamily, is expressed by endothelial cells and is one of the main ligands for β-2 integrins.[16,18,30,49] Intercellular adhesion molecule–1 is upregulated after periadventitial blood deposition in animal models of vasospasm,[1,23,44] and increased levels of the molecule are present in the serum and CSF of patients who experience angiographic vasospasm after SAH.[29,32,40] Lymphocyte function–associated antigen–1 is located in the plasma membrane of neutrophils and macrophages, and constitutes the main integrin for adhesion and transendothelial migration of these cells after interacting with endothelial ICAM-1.[17,25,48] Similarly, Mac-1 is present on the surface of macrophages and neutrophils,[9] and facilitates ICAM-1–mediated adhesion of these cells to the endothelium.[46] Other CAMs that may be involved in posthemorrhagic vasospasm include VCAM-1 and E-selectin.[40]

An anti-CD11/CD18 humanized mAb, Hu23F2G has been shown to disrupt leukocyte–endothelial cell interactions.[3,5,8,27,41,52] To "humanize" the antibody, a murine mAb against CD11/CD18 (m23F2G) was developed initially. The complementarity-determining region of the murine antibody that is responsible for antigen binding and is located in the variable fragment of the antibody was selected and inserted into the variable fragment of human IgG4, which was chosen because it does not fix complement and has decreased binding of Fc receptors.[8,52] The Hu23F2G antibody binds to the β subunit of the integrins Mac-1, LFA-1, p150, 95(CD11c/CD18), and αd/β2.[51] It prevents the interaction of Mac-1 and LFA-1 with ICAM-1 and, thus, blocks the mi-

Abbreviations used in this paper: BA = basilar artery; CAM = cell adhesion molecule; CSF = cerebrospinal fluid; ET = endothelin; ICAM-1 = intercellular adhesion molecule–1; Ig = immunoglobulin; LFA-1 = lymphocyte function–associated antigen–1; mAb = monoclonal antibody; Mac-1 = macrophage antigen–1; NO = nitric oxide; SAH = subarachnoid hemorrhage; VCAM-1 = vascular CAM–1; WBC = white blood cell.

gration of leukocytes into the periadventitial space. Preliminary studies in a nonhuman primate model of SAH indicate that Hu23F2G might be of benefit in the treatment of vasospasm.[14]

We and others have previously shown that blocking leukocyte–endothelial cell interactions with mAbs directed against CAMs prevents posthemorrhagic vasospasm in rats, rabbits, and monkeys.[4,14,15,38] Systemic therapy with an anti–ICAM-1 mAb prevented chronic vasospasm and decreased the number of periadventitial macrophages and neutrophils[38] in the rat femoral artery model of vasospasm. Treatment with anti–ICAM-1 and anti–LFA-1 mAbs also prevented chronic vasospasm and decreased the number of periadventitial inflammatory cells in rats.[15] Antibodies against ICAM-1 and CD18 administered intrathecally prevented vasospasm in a rabbit model as well.[4] Similarly, ibuprofen, which inhibits the endothelial expression of ICAM-1 and VCAM-1,[26,35] prevented vasospasm after SAH in dogs[12,13] and in the femoral artery model of vasospasm in rats.[50]

We hypothesized that the systemic administration of an anti–CD11/CD18 mAb, which could block the adhesion of neutrophils and macrophages and their migration into the periadventitial space, prevents vasospasm after SAH. In this study we describe the effect of systemic therapy with Hu23F2G on the lumen patency of the BA and on the number of peripheral WBCs in the rabbit model of vasospasm after SAH.

Materials and Methods

Experimental Design

Animals were randomized to four experimental groups. Animals in the first group (sham-operated group, four animals) underwent surgery with exposure of the dura mater only and without CSF extraction or a cisternal injection of blood. Animals in the second group (SAH-only group, six animals) underwent injection of 1.5 to 2 ml of autologous arterial blood into the cisterna magna to create an SAH. Animals in the third (SAH + Hu23F2G group, 10 animals) and fourth (SAH + placebo group, six animals) groups underwent intraperitoneal injections of either Hu23F2G or vehicle at 30 minutes and 24 and 48 hours after blood injection. The rabbits were killed at 72 hours post-SAH, when peak vasospasm occurs in rabbits as previously determined by our group and others,[10,19,22] and underwent perfusion fixation for histological and morphometric analysis of the BA. Peripheral WBCs were counted 72 hours after SAH.

Animal Selection

Twenty-six New Zealand White rabbits (*Oryctolagus cuniculus* obtained from Robinson Services, Inc., Winston-Salem, NC), each weighing 1.5 to 2.5 kg, were used in this experiment. The animals were housed in standard animal facilities with free access to Baltimore city water and rodent chow. The Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all experimental protocols.

Monoclonal Antibody Treatment

Both Hu23F2G and vehicle placebo were provided by ICOS Corporation (Bothell, WA). The Hu23F2G antibody was formulated as 10 mg/ml in 120 mM sodium chloride, 45.2 mM sodium acetate, and 0.02% Tween-20, which was adjusted to pH 5.6 with acetic acid. Placebo was formulated as water for injection (US Pharmacopeia) with 120 mM sodium chloride, 45.2 mM sodium acetate, and 0.02% Tween-20, with the pH adjusted to 5.6 with acetic acid. The Hu23F2G antibody was synthesized as previously described.[8] Briefly, m23F2G, a murine anti-CD11/CD18 mAb, was humanized by inserting the complementarity-determining region of m23F2G into the variable region of human IgG4. The DNA of the new construct was transfected into Chinese hamster ovarian cells, which produced the humanized antibody. Either Hu23F2G or placebo was administered by intraperitoneal injection at a dose of 4 mg/kg.

Anesthesia and Surgical Technique

For all procedures, the animals were anesthetized by an intramuscular injection of a mixture of ketamine (50 mg/kg [100 mg/ml ketamine HCl; Abbott Laboratories, Chicago, IL]) and xylazine (10 mg/kg [100 mg/ml XYLA-JECT; Phoenix Pharmaceutical, Inc., St. Joseph, MO]).

We have previously described the surgical technique used for the rabbit SAH model.[22] Briefly, after induction of anesthesia, an intramuscular injection of ceftriaxone (20 mg/kg) was administered and, through a midline incision, the atlantooccipital membrane was exposed. In animals in the sham-operated group the operative field was irrigated with saline solution, and the incision was closed. In the other three experimental groups 1 ml of CSF was aspirated by cisternal puncture, followed by aspiration of 1.5 to 2 ml of nonheparinized blood from the central ear artery, which was then injected into the cisterna magna. Animals in Groups 3 and 4 received either Hu23F2G (4 mg/kg) or placebo by intraperitoneal injection 30 minutes and 24 and 48 hours after SAH.

Histological Assessment

Angiographic evidence of peak vasospasm in the rabbit model of SAH is present 72 hours after blood injection into the cisterna magna.[10,19,22] Animals were therefore killed 72 hours after induction of experimental SAH by an intraperitoneal injection of sodium pentobarbital (200 mg/kg), after which in situ perfusion fixation was performed. The animals were anesthetized as described earlier, a midsternal thoracotomy was performed, the right atrium was pierced for exsanguination, and the left ventricle was cannulated. Transcardiac perfusion was performed with 300 ml of a 0.1-M phosphate-buffered saline solution followed by 500 ml of ice-cold 4% paraformaldehyde, which was delivered by a peristaltic pump at 100 rpm (25 ml/minute) (Watson-Maulden-Bredel, Falmouth, UK).

The BA and the brainstem were harvested en bloc and immersed in a 0.1-M phosphate buffer–20% sucrose solution at 4°C for 3 days for cryoprotection. Specimens were snap-frozen in −60°C methylbutane and stored at −80°C. Transverse sections (20-μm) were obtained with a microtome cryostat at 200-μm intervals beginning at the termination of the BA. Tissue slices were mounted on Superfrost Plus slides (Fisher Scientific Co., Pittsburgh, PA) for H & E staining. Additional staining of BA cross-sections (four per group) was performed using a rat anti–rabbit CD18 antibody and cresyl violet to detect the periadventitial localization of CD18-positive cells.

Morphometric Analysis

Luminal cross-sectional areas were outlined and the circumference of the BA was measured by performing computerized analysis (MCID; Imaging Research, St. Catharines, ON, Canada). Six sections of each BA (each section 20 μm thick and obtained 200 μm apart) were evaluated and averaged to control for vessel deformation and off-transverse sections. The vessel perimeter was obtained by interactive measurements of vessel sections. Estimated cross-sectional areas were converted to lumen-patency percentages and absolute values were defined by the average of cross-sectional areas from sham-operated animals.

Statistical Analysis

Vessel perimeters and WBC counts are expressed as mean values ± standard errors of the means. Mean perimeters of the BAs are expressed as percentages of lumen patency obtained by dividing the mean perimeter of each group by the mean perimeter of the sham-operated group. Mean vessel perimeters (in millimeters) were compared using the Student t-test. A probability value less than 0.05 was considered significant. Statistical analysis was performed using SPSS Version 8.0 for Windows (SPSS, Inc., Chicago, IL).

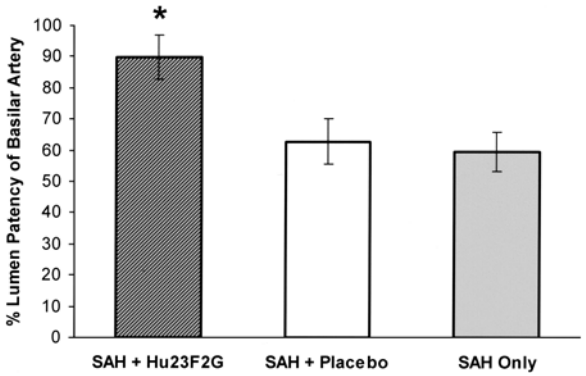

FIG. 1. Bar graph showing that treatment with Hu23F2G resulted in a significant increase in the percentage of lumen patency of the BA compared with treatment with placebo or no treatment. *Asterisk* indicates significance.

Results

Treatment with Hu23F2G resulted in a significant increase in BA lumen patency as well as in the peripheral WBC counts. Whereas the lumen patency of the SAH + Hu23F2G group was 90 ± 7%, that of the SAH + placebo group was 65 ± 7% ($p = 0.025$) (Fig. 1). By comparison the lumen patency of the SAH-only group was 59 ± 10%. Similarly, although the peripheral WBC count of the SAH + Hu23F2G group was 16,300 ± 2710/μl, that of the SAH + placebo group was 7000 ± 386/μl ($p = 0.02$; Fig. 2). It was noted that within the SAH + Hu23F2G group seven of 10 animals had elevated peripheral WBC counts, but three did not. The mean peripheral WBC counts in animals whose response was an elevated count was 20,517 ± 2371/μl, compared with 6310 ± 505/μl in animals without elevated WBC counts ($p = 0.005$). Qualitative observations of immunohistochemical tests for CD18 showed a decrease in CD18-positive cells in the periadventitial space of BAs from rabbits treated with Hu23F2G when compared with the periadventitial space of BAs from animals treated with placebo (Fig. 3).

Discussion

In this study we describe the systemic administration of a humanized anti-CD11/CD18 mAb for the treatment of vasospasm in a rabbit model of SAH. We found that treatment with Hu23F2G resulted in increased lumen patencies, prevention of morphometric vasospasm, and increased numbers of peripheral WBCs. The Hu23F2G antibody binds to β-2 integrins including LFA-1 and Mac-1, which are expressed on the surface of macrophages and neutrophils. Such binding prevents their interaction with ICAM-1 and, thus, their adhesion to the endothelial surface. The biological effect of Hu23F2G was confirmed by an increase in peripheral WBCs at 72 hours after SAH, which was observed in 70% of the treated animals.

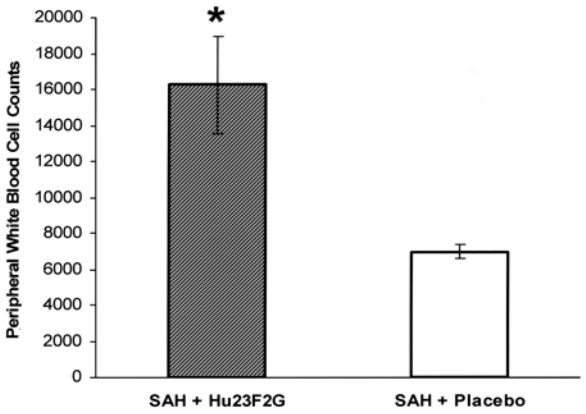

FIG. 2. Bar graph demonstrating that systemic treatment of rabbits with Hu23F2G caused a significant elevation in the number of peripheral WBCs, whereas treatment with placebo did not.

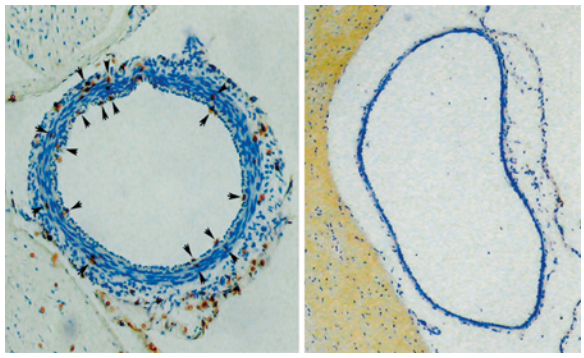

FIG. 3. Photomicrographs showing frozen cross-sections of the BA. *Left:* Tissue obtained from a rabbit treated with placebo after SAH. *Arrowheads* indicate CD18-positive cells (macrophages and neutrophils). *Right:* Tissue obtained from a rabbit treated with Hu23F2G after SAH. Cresyl violet and anti-CD18 mAb, original magnifications × 15 *(left)* and × 10 *(right)*.

The Hu23F2G antibody has been used previously in New Zealand White rabbits, nonhuman primates, and humans. A reduced likelihood of immunogenic reactions has been achieved by humanizing the antibody, which markedly decreases the likelihood of a human anti–mouse antibody type reaction and maintains the binding efficacy of the murine precursors. Whereas a human anti–mouse antibody response has been identified in 20 to 40% of patients treated with murine antibodies, such a response has been identified in only 7% of patients treated with humanized antibodies.[6] The Hu23F2G antibody uses the human IgG4 heavy chain, which has a decreased fixation of complement and low binding of Fc receptors.[27,52]

Upregulation of ICAM-1 and other CAMs on the endothelial surface following aneurysmal SAH promotes binding of macrophages and neutrophils through LFA-1 and Mac-1, thus creating a determinant event in the pathogenesis of posthemorrhagic vasospasm.[15] After aneurysmal SAH, erythrocytes in the subarachnoid space cluster around the vessel wall as a thrombus. Acute-phase reactants such as interleukin-1β,[39] tumor necrosis factor–α,[1] and interferon-γ,[24] among others, are produced in the thrombus and induce upregulation of selectins and ICAM-1 in the endothelial layer.[42] Upregulation of ICAM-1 causes the arrest of rolling leukocytes, adhesion, and diapedesis into the subarachnoid space.[47] Macrophages and neutrophils in the subarachnoid space are then attracted into the periadventitial thrombus by released chemoattractants. These leukocytes proceed to phagocytose erythrocytes and debris. After phagocytosis, however, the leukocytes die in the subarachnoid space and release ETs,[11,20] O_2 free radicals,[43] chemo-

kines,[21] and other products,[7,15,20,42] which damage the endothelium, decrease the synthesis of endothelium-derived NO, and cause chronic vasospasm.

The rabbit model of SAH was first described by Offerhaus and van Gool[37] in 1969 to analyze electrocardiographic changes after SAH. Nevertheless, the most common type of rabbit model used to induce vasospasm after SAH is the one described by Chan and associates[10] in which SAH is created by injection of arterial blood into the cisterna magna.[28] This method has achieved excellent correlation between angiographic vasospasm and morphometric measurements of perfusion-fixed cross-sections of the BA.[31] Injection of blood into the cisterna magna results in histopathological[10] as well as chemical changes[34] in the BA with peak vasospasm occurring 72 hours after SAH.[19,22] In our series we had a mortality rate of less than 10%. Disadvantages of this model include the development of subacute vasospasm (on Day 3 in comparison with the human disease in which vasospasm occurs 7–10 days after SAH) and the absence of neurological deficits after vasospasm; however, the latter is true for all animal models of posthemorrhagic vasospasm.[28,31]

The inflammatory CAM hypothesis of vasospasm after SAH can be reconciled with the extensive evidence that shows the important role of ETs and NO depletion in vasospasm. Macrophages and neutrophils in the subarachnoid space release vasospastic molecules, particularly ET-1.[7,20,42] Endothelin-1, the most common isoform of ET, is a potent vasospastic molecule involved in posthemorrhagic vasospasm. In addition, ET-1 is also secreted by endothelial cells, neurons, and astrocytes.[7,20] Endothelin-1 binds primarily to the ET_A receptor located mainly in smooth-muscle cells and activates a Gαs protein that controls voltage-dependent Ca channels, resulting in vasoconstriction.[11] Simultaneously, ET-1 contributes to the decreased synthesis of NO, impairing NO-dependent vasodilation.[2] After neutrophils and macrophages degranulate, free radicals such as OH^- and O_2^- are released into the periadventitial space and eliminate endothelium-dependent relaxation[2] through destruction of endogenous NO. These free radicals also cause lipid peroxidation, enzymatic inhibition, and elevation of Ca^{++}, among other events,[33] which damage the endothelium and contribute to the development of vasospasm.

Conclusions

Treatment with Hu23F2G, which disrupts interactions between endothelial ICAM-1 and the leukocyte integrins LFA-1 and Mac-1, and thus could prevent the migration of neutrophils and macrophages into the periadventitial space, prevents morphometric vasospasm after SAH in rabbits. These findings support the role of leukocyte–endothelial cell interactions in the pathogenesis of chronic posthemorrhagic vasospasm.

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