Timing of ICAM-I Expression in a Canine Model of Post-Haemorrhagic Cerebral Vasospasm

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Summary

Temporal alterations in endothelial intercellular adhesion molecule I (ICAM-I) expression during the course of post-haemorrhagic cerebral vasospasm (PHCV) are correlated with angiographic and histologic changes in the canine basilar artery.

Angiography was performed in six dogs to obtain baseline measurements of basilar artery diameter. In three dogs subarachnoid haemorrhage (SAH) was created by performing percutaneous puncture of the cisterna magna, and replacing 7 ml of cerebrospinal fluid with 7 ml of arterial blood. The remaining three dogs were used as controls. Daily angiography was performed on all dogs to determine the percent reduction in basilar artery diameter (%RBAD). One dog from each group was sacrificed after 24 hours. The remaining two dogs in each group were sacrificed after 48 hours. Each basilar artery was perfusion fixed and subjected to histologic, and immunohistochemical analysis.

In the SAH group, the average %RBAD was 4 (+/- 3) at 24 hours, and 36 (+/- 1) at 48 hours. In the control group, the average %RBAD was -1 (+/- 1) at 24 hours, and 0 (+/- 2) at 48 hours. Endothelial edema and endothelial expression of ICAM-I were found at 24 hours. At 48 hours post-SAH there was widespread endothelial

desquamation, but no evidence of ICAM-I expression. In the control group, histology was normal and no ICAM-I expression was found at 24 or 48 hours.

The results suggest that a brief window of therapeutic efficacy exists during the first post-ictal 24 hours where ICAM-I antagonists may be useful in suppressing the pathogenesis of PHCV.

Introduction

The syndrome of delayed ischemic deficit develops in 30-50% of patients who survive aneurysmal subarachnoid haemorrhage^{1,2}. It is the principle cause of morbidity and mortality in patients who survive the initial rupture of an intracranial aneurysm. Delayed ischaemic deficits are the clinical manifestation of cerebral vasospasm. Post-haemorrhagic cerebral vasospasm is a reaction to injury that encompasses both acute changes in vasomotor function and chronic changes in vessel structure. The acute phase of the reaction primarily involves functional constriction of cerebrovascular smooth muscle cells³.

The chronic phase involves a combination of inflammation, vascular smooth muscle cell proliferation and fibrosis which collectively result in luminal narrowing, diminished elasticity and

loss of the ability to undergo functional relaxation^{4,7}. These changes may be associated with a transition in vascular smooth muscle cell phenotype from the contractile to the synthetic form. *In vitro* studies have suggested that the loss of alpha actin expression may be a molecular marker of this phenotypic transition⁸.

Recent investigations have suggested that intercellular adhesion molecule I (ICAM-I) may be involved in initiating pathogenesis of the chronic phase⁹⁻¹². Although studies in rats have revealed that haemorrhage induced ICAM-I expression occurs early in the course of cerebral and femoral artery vasospasm, there is no direct experimental evidence indicating that such changes occur in other species^{9,10}. One study in rabbits demonstrated that a marked reduction in the development of cerebral vasospasm could be effected by the intracisternal administration of monoclonal antibodies directed against ICAM-I, suggesting that ICAM-I has a functional role in post-haemorrhagic cerebral vasospasm¹¹. Indirect evidence that subarachnoid haemorrhage (SAH) induced changes in ICAM-I expression may also occur in humans has also been reported¹².

Endothelial expression of ICAM-I may represent a critical intermediate step in the evolution of chronic vasospasm. An understanding of temporal changes in ICAM-I expression is critical to deciphering the pathobiology of cerebral vasospasm, and developing new therapies which prevent the progression of cerebral vasospasm by blocking the function of ICAM-I. We studied the time course of ICAM-I expression and correlated the results with angiographic and histologic findings in a canine model to further elucidate the molecular pathogenesis of post-haemorrhagic cerebral vasospasm. Factor VIII immunohistochemistry was studied to specifically characterize endothelial alterations, and alpha actin immunohistochemistry was studied to delineate changes in vascular smooth muscle phenotype.

Methods

Experimental Design

Six adult female dogs, weighing between 25 and 30 Kg, were used for these studies. The study protocol was approved by the Institutional Animal Care and Use Committee of Emory University, in accordance with National Insti-

tute of Health guidelines. All procedures were conducted under general endotracheal anesthesia with respiratory support. In three dogs, an artificial subarachnoid haemorrhage (SAH) was produced after performing baseline vertebral angiography for measurement of basilar artery diameter on day zero. In three control dogs, without SAH, only baseline vertebral angiography was performed on day zero. Animals from each group were sacrificed at selected intervals to obtain basilar artery specimens for histologic and immunohistochemical analysis. Daily selective vertebral angiography was performed on all dogs until the time of sacrifice. In the control group, two dogs were sacrificed 48 hours after baseline angiography, and one dog was sacrificed 24 hours after baseline angiography. In the SAH group, sacrifice followed SAH and baseline angiography by 48 hours in two dogs, and by 24 hours in one dog.

Production of Subarachnoid Haemorrhage and Cerebral Angiography

Animals were sedated by the subcutaneous injection of morphine sulfate (2 mg/Kg). After placement of a peripheral intravenous catheter, anesthetic induction was accomplished by the intravenous injection of diazepam (0.7 mg/Kg), ketamine (10 mg/Kg) and atropine (0.016 mg/Kg). Following oral endotracheal intubation, animals were mechanically ventilated. Ventilation and FiO₂ were adjusted according to the results of arterial blood gases, to maintain a pH of 7.36 to 7.40, a pO₂ of 80 - 100 torr and a pCO₂ of 36 to 40 torr. Anesthetic maintenance was accomplished by the administration of inhaled isoflurane. Rectal temperature was monitored, and normothermia maintained with the use of an adjustable electric heating blanket.

A two dimensional measuring grid, consisting of radioopaque lines spaced at one centimeter intervals, was positioned beneath each dog's head to serve as an internal standard for measuring basilar artery dimensions. Using sterile Seldinger technique, a 6 french introducer sheath was percutaneously inserted into the right common femoral artery. Under fluoroscopic control, a 5.0 french catheter was advanced over a guidewire, into the proximal vertebral artery (to the level of the C3-C4 intervertebral disc space). A selective vertebral an-

Figure 1 Percent reduction in basilar artery diameter over time. The percent reduction in basilar artery diameter (%RBAD) for the control and SAH groups is plotted as a function of time in a 3 dimensional bar graph. The data for individual experimental subjects is presented in the accompanying table. The table also displays the mean value for each experimental group with its standard deviation in parentheses.

giogram was obtained by rapid hand injection of 6 ml of 76% hypaque. In all six dogs magnified digital images were simultaneously acquired in the anteroposterior projection with an OSEC Dasonics C-arm at a frame rate of 30/second. The single image in each series showing the greatest filling of the vertebrobasilar system was used for subsequent basilar artery measurement.

Immediately after baseline angiography, animals were placed in the prone position with head flexed 30 degrees from the horizontal plane. Using sterile technique, percutaneous puncture of the cisterna magna was performed

under fluoroscopic control with a 19 gauge spinal needle. Seven milliliters of cerebrospinal fluid (CSF) was replaced with 7 ml of autologous non-heparinized arterial blood. The rate of CSF removal, and blood injection were strictly controlled at 3.5 ml per minute. Upon completion of the cisternal injection, each animal was placed in a 30 degree trendelenberg position for 30 minutes to prevent diffusion of blood into the spinal subarachnoid space.

At the conclusion of each procedure, the femoral introducer sheath was removed, and the animal was permitted to recover from anesthesia. All animals were provided with a bal-

Figure 2 Temporal progression of angiographic vasospasm. Serial magnified digital subtraction angiograms obtained by hand injection of contrast into the proximal vertebral artery show the basilar artery projected over a measuring grid, with lines spaced at one centimeter intervals. The basilar artery is indicated by white arrows. A) Baseline digital subtraction obtained on day 0, prior to subarachnoid haemorrhage. B) 24 hours after subarachnoid haemorrhage. C) 48 hours after subarachnoid haemorrhage.

anced diet and were given free access to water. If fluid consumption fell below one liter per day, supplementary hydration was accomplished by the intravenous administration of 0.45% saline.

Anatomic, Histologic and Immunohistochemical Techniques

Upon completion of all angiographic studies, animals were sacrificed on the angiography table by pentobarbital overdose (324 mg/Kg).

Figure 3 Anatomic distribution of subarachnoid haemorrhage. Ventral surface of excised brainstem specimen obtained 48 hours after subarachnoid haemorrhage (original magnification $\times 2.5$). The brainstem and basilar artery adventitia are encased in clotted blood.

The common carotid arteries, and the intracranial vessels in all six dogs were perfusion-fixed under physiologic pressure with 10% phosphate buffered formalin. Immediately after perfusion fixation, posterior fossa craniectomy with bilateral laminectomies of the C1 and C2 vertebrae was performed to conduct a gross examination of the brainstem and basilar artery. The basilar artery and sections of common carotid artery were sharply excised, and fixed overnight in 10% phosphate buffered formalin. On the following morning, the specimens were embedded in paraffin blocks. Five micron thick cross-sections, of each basilar artery, were then stained separately with haematoxylin/eosin and elastica van Gieson. Additional sections from each block were separately immunostained for canine alpha-actin smooth muscle cell antigen (SMCA), canine factor VIII-Von Willebrand Factor (VWF), and canine Intercellular Adhesion Molecule-1 (ICAM-I). Five micron thick sections of common carotid artery from each

subject were immunostained for SMCA, VWF, and ICAM-I to quality check the reagents, and exclude tissue fixation technique as a source or error. Tissue sections were prepared for immunohistochemistry by dewaxing in a series of graded ethanol/xylene solutions.

Non-specific protein binding was blocked with 1% gelatin-phosphate buffered saline. All tissue sections were rehydrated in phosphate buffered saline, then stained with primary antibody during a one hour incubation. Primary antibodies were prepared in 1.0% crystalline grade bovine serum albumin (BSA) in phosphate buffered saline (PBS). Primary anti-ICAM-I antibodies (CD18/1D8) were kindly provided by C. Wayne Smith, Ph.D., Speros P. Martel Laboratory of Leukocyte Biology, Baylor College of Medicine, Houston, TX. Primary anti-VWF antibodies were obtained from the DAKO Corporation, 6392 Via Real, Carpinteria, CA. Primary anti-SMCA antibodies were obtained from Sigma, 3050 Spruce street, St.

Louis, MO, 63103. After staining with primary antibody, each tissue section was washed twice in PBS, then stained with secondary antibody during a 30 minute incubation. Secondary antibodies were prepared in 1.0% crystalline grade BSA in PBS. After staining with secondary antibody, tissue sections were washed twice in PBS and incubated for 45 minutes in a solution of Vector ABC Alkaline Phosphatase (Vector Laboratories, 30 Ingold road, Burlingame, CA, 94010). Tissue sections were then treated with a substrate solution provided by the manufacturer, then rinsed and counterstained with haematoxylin. All tissue preparations from the SAH and control groups were then matched with each other according to the time of tissue harvesting. A vascular pathologist performed detailed histologic examinations of matched tissue sections side by side.

Quantitative Assessment of Cerebral Vasospasm

The degree of cerebral vasospasm present on each angiogram was quantitatively estimated as the average percent reduction in basilar artery diameter. Selected views from each angiogram were independently evaluated by two neuroradiologists, blinded to the source of the images. The basilar artery diameter was measured to the nearest tenth of a millimeter at the midpoint of its proximal (10 mm distal to the confluence of the vertebral arteries), middle (2 mm distal to the anterior inferior cerebellar artery origin), and distal (5 mm proximal to the basilar bifurcation) segments. The percent reduction in basilar artery diameter was calculated for each segment (segmental %RBAD), at each timepoint *t*, by dividing the difference between the baseline diameter and the diameter at time *t*, by the baseline diameter. For each animal, the average % RBAD at a given timepoint was determined by averaging the three segmental %RBADs (proximal, middle and distal) at that timepoint.

Results

Morbidity and Mortality

There were no unplanned animal deaths, and no complications from angiography during the course of this study. Two of three dogs in the SAH group made complete recoveries within 24 hours. Lethargy, ataxia and paresis of the

hind limbs were invariably present in the first 12 hours after SAH. Of three dogs in the SAH group, only one demonstrated significant neurologic disability after SAH. This dog (dog 4) developed a dense left sided haemiplegia that was apparent immediately after recovery from anesthesia on day zero. He remained lethargic throughout his survival period, until he was sacrificed after angiography on day 2. At autopsy, a large subarachnoid clot was found extending into the sylvian cistern on the right side.

Angiographic Vasospasm

For each angiogram, the imaging parameters were identical. Core temperature, heart rate, mean arterial blood pressure, and arterial blood gases were not significantly different between procedures.

In the SAH group, cerebral vasospasm, as measured by the percent reduction in basilar artery diameter (%RBAD), was only very mild in the first 24 hours after haemorrhage (figure 1). By day 2, marked vasospasm was present in the SAH group (figure 2).

None of the subjects in the control group displayed a significant reduction in basilar artery diameter on any angiographic study.

Anatomic Pathology

In two of three dogs from the SAH group, subarachnoid clot was restricted to the infratentorial compartment. In each case, a thick, poorly organized clot, circumferentially encased the brain stem along its entire length (figure 3). Mechanical distortion of the brainstem, suggesting mass effect, was not present in any specimen. No evidence of vascular or parenchymal trauma, as a result of cisternal puncture, was found in any of the specimens. In one dog (dog 4), there was significant extension of subarachnoid clot into the right sylvian cistern. Although no mass effect was evident, the clot appeared to be in contact with the right middle cerebral artery. This finding suggests middle cerebral artery vasospasm as a possible mechanism for the animal's left hemiparesis.

Histopathology

Histopathologic analysis of the basilar artery was performed 24 hours after SAH in one dog

Figure 4 Basilar artery histology 24 hours after subarachnoid haemorrhage. A) Longitudinal section of basilar artery (haematoxylin and eosin; original magnification x20). Clusters of leukocytes (black arrowheads) are adherent to the abluminal surface of the vessel. Erythrocytes are present within the lumen (straight black arrow) and adventitia (curved black arrow). B) Transverse section of basilar artery (elastica van Geison; original magnification x 10). The internal elastic lamina is corrugated, but no evidence of elastolysis is seen.

Figure 5 Endothelial changes in the basilar artery after subarachnoid haemorrhage. Transverse section of basilar artery (original magnification $\times 40$) stained for Factor VIII, 24 hours after subarachnoid haemorrhage. Factor VIII positive endothelial cells (black arrowheads) appear swollen, and rounded up. Small foci of endothelial desquamation are evident as gaps in the endothelium (black arrows).

(figure 4). The basilar artery adventitia was extensively infiltrated by erythrocytes and leukocytes, and the intima was mildly corrugated. Platelets and leukocytes were found in sparsely distributed clusters on the abluminal surface of the artery. Although the internal elastic lamina was corrugated, no evidence of elastolysis was found. Factor VIII immunohistochemistry revealed that the endothelium was intact, with occasional small focal patches of desquamation (figure 5). Numerous endothelial cells appeared swollen, or rounded up. Immunohistochemistry disclosed that smooth muscle alpha actin expression was identical in control and SAH subjects. Sections of basilar artery obtained from the matched control dog were histologically normal without evidence of adventitial infiltrate, intimal corrugation, or endothelial injury.

Histopathologic analysis of the basilar artery was performed 48 hours after SAH in two dogs (figure 6). Numerous erythrocytes remained

scattered throughout the basilar artery adventitia, and there was extensive adventitial infiltration by neutrophils, lymphocytes and macrophages. The entire thickness of the intima was deeply folded into regularly spaced corrugations. Neutrophils, macrophages and platelets were attached to the luminal surface of the artery in large numbers. Focal deposits of leukocytes and platelets were clustered in the pits between consecutive corrugations of intima. In some areas, leukocytes were seen infiltrating into the intima and media. There was definite fragmentation of the internal elastic lamina, with numerous focal defects indicating elastolysis. Basilar artery cross-sections immunostained for Factor VIII revealed widespread endothelial cell desquamation. In two matched control subjects, the basilar arteries were histologically normal without evidence of adventitial infiltration or endothelial injury. There was no difference in alpha actin staining between the SAH and control basilar arteries.

Figure 6 Basilar artery histology 48 hours after subarachnoid haemorrhage. A) Longitudinal section of basilar artery (haematoxylin and eosin; original magnification x 40). The abluminal surface of the vessel appears devoid of endothelial cells, and numerous leukocytes (black arrowheads) are adherent to the underlying basement membrane. The basement membrane is irregular, and has a moth eaten appearance. B) Longitudinal section of basilar artery (elastica van Geison; original magnification x 20). The internal elastic lamina is fragmented, and numerous focal lytic defects are present.

Figure 7 ICAM-I expression after subarachnoid haemorrhage. Longitudinal section of basilar artery stained for ICAM-I, 24 hours after subarachnoid haemorrhage (100X). ICAM-I positive endothelial cells (red) appear swollen (black arrows).

Control specimens of common carotid artery from each subject demonstrated expression of immunoreactive Factor VIII by the endothelium, and uniform staining of the tunica media with anti-smooth muscle alpha actin antibodies.

ICAM-I Immunohistochemistry

In control basilar artery sections there was no detectable expression of immunoreactive ICAM-I at 24 hours or 48 hours.

In basilar artery harvested 24 hours after exposure to SAH, there was uniform expression of immunoreactive ICAM-I throughout the endothelium (figure 7). In contrast, there was no detectable expression of ICAM-I in basilar arteries harvested 48 hours after exposure to SAH.

None of the common carotid artery specimens obtained from the six animals stained positive for ICAM-I.

Discussion

In a canine model of post-haemorrhagic cerebral vasospasm we have demonstrated that endothelial expression of ICAM-I is histologically correlated with endothelial cell swelling and adhesion of inflammatory leukocytes to the endothelium at 24 hours post-haemorrhage. At 48 hours post-haemorrhage, the disappearance of immunoreactive ICAM-I is correlated with widespread endothelial desquamation, adhesion of leukocytes to the arterial luminal surface, lytic changes within the internal elastic lamina and mononuclear cell infiltration of the adventitia. Catheter angiography revealed that these changes were associated with mild vasoconstriction at 24 hours, and intense vasospasm at 48 hours. In control subjects undergoing vertebral angiography without demonstrable vasospasm, there was no detectable endothelial expression of ICAM-I, or change in arterial histology.

Although animal models of SAH differs from the human condition in a number of ways, the canine model of SAH has been established as a satisfactory representation by numerous investigators. In canine models of SAH, two distinct forms of cerebral vasospasm are observed: early spasm, which develops within minutes and spontaneously resolves over a period of hours, and delayed spasm, which peaks within two days and persists for several additional days^{3,13-15}.

It is generally agreed that early spasm is a brief, self-limited process that has little relevance to the human clinical syndrome of delayed ischaemic deficit. Delayed cerebral vasospasm, produced experimentally in canine models of SAH, resembles the human clinical condition very closely. Similar to human vasospasm it is a sustained process, lasting several days, that is associated with chronic structural and functional changes in the affected artery^{16,17}. In this study, delayed vasospasm had its onset within the first 24 hours, and progressed to severe levels within 48 hours, consistent with previous experimental studies of cerebral vasospasm, employing a single SAH^{13,18-20}.

According to currently accepted paradigms, delayed vasospasm is a reaction to injury, which

encompasses both acute changes in vasomotor function, and chronic changes in vessel structure^{3,4-7}. The chronic phase represents a proliferative arteriopathy involving a combination of inflammation, vascular smooth muscle cell proliferation and fibrosis, which collectively cause luminal narrowing of the affected artery⁴⁻⁷. The development of structural changes in the spastic cerebral artery marks a critical event in the evolution of the clinical syndrome of delayed ischaemic deficit, since the chronically spastic cerebral artery is less likely to respond to medical and pharmacologic intervention and may require mechanical angioplasty for successful treatment. Both clinical and experimental studies have shown that cerebral vessels in spasm develop chronic changes that influence their elasticity, and their vasomotor reactivity^{16,21-24}.

Clinical and experimental studies have shown that the histological changes of chronic vasospasm are preceded by adherence of inflammatory leukocytes to the endothelium, endothelial edema, and endothelial desquamation^{4,5,25,26}. Since ICAM-I is the principal mediator of leukocyte-endothelial cell interactions, it is likely that ICAM-I plays a key role in this process. The activity of immune-inflammatory cells within the arterial wall may be important in driving the fibroproliferative changes and architectural remodeling of chronic vasospasm. The loss of arterial elasticity, which is an integral component of chronic spasm, may be at least partly related to the destruction of elastic tissue by leukocyte elastase^{22,23}. Endothelial expression of ICAM-I may therefore be a necessary prerequisite for the evolution of proliferative arteriopathy. In theory, ICAM-I antagonists may suppress the development of chronic vasospasm if administered early in the course of SAH during the therapeutic window of ICAM-I expression. Synchronizing therapy to the timing of endothelial ICAM-I expression is critical for this approach. Previous studies in rabbits have shown that ICAM-I antagonists markedly ameliorate the development of cerebral vasospasm if administered prior to SAH.

Studies in rats have yielded conflicting results about the timing of endothelial ICAM-I expression following haemorrhage^{9,10}. In a rat femoral artery model of post-haemorrhagic vasospasm, Sills et al reported that endothelial expression of ICAM-I was present between three and 24 hours post-haemorrhage, but ab-

sent at 48 hours post-haemorrhage⁹. In a rat SAH model, Handa et al reported that endothelial expression of ICAM-I coincides with the peak of angiographic vasospasm in the basilar artery two to five days post-haemorrhage¹⁰. Differences in the timing of ICAM-I expression reported by these two studies may be related to regional differences in arterial physiology. Species specific differences in cerebral arterial physiology may account for the different temporal course of ICAM-I expression found in our study. In the canine model of post-haemorrhagic cerebral vasospasm, we have demonstrated that endothelial expression of ICAM-I precedes the onset of angiographic vasospasm, and lasts for at least 24 hours. At 48 hours post-haemorrhage, when angiographic vasospasm is maximal and lytic changes begin to appear within the internal elastic lamina, there is no detectable expression of ICAM-I. Absence of ICAM-I expression at 48 hours post-haemorrhage is probably related to endothelial desquamation rather than physiologic down regulation since histologic analysis in this study revealed early changes of cytotoxic endothelial edema, followed by widespread endothelial desquamation at 48 hours post-haemorrhage. This is consistent with histopathologic data reported in the clinical and experimental literature^{4,25,26}.

We were unable to detect any alteration in basilar artery smooth muscle alpha actin expression at 24 or 48 hours post-haemorrhage. This finding suggests that vascular smooth muscle cell phenotype remains constant in the first 48 hours after subarachnoid haemorrhage. If alpha actin immunohistochemistry accurately reflects the smooth muscle cell population phenotype, this implies that some of the deterministic changes marking the onset of the proliferative phase do not occur in the first 48 hours post-haemorrhage. Requisite changes in vascular smooth muscle phenotype may depend on preceding endothelial responses, and the local activity of immune-inflammatory cells.

Conclusions

Assuming that circulating immune-inflammatory cells play an important role in the pathogenesis of cerebral vasospasm, the results of this study suggest that ICAM-I antagonists might be therapeutically efficacious if they are

administered in the first 24 hours post-haemorrhage. Our study indicates that the delivery of cellular mediators of inflammation to the cerebral arterial wall via ICAM-I mediated interactions with the endothelium precedes the development of elastolysis, endothelial desquamation and maximal angiographic vasospasm, yet the relative contribution of this process to the pathogenesis of vasospasm is unknown. Further study will be necessary to establish the kinetics of endothelial ICAM-I expression, and elucidate the functional significance.

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