A randomized placebo-controlled double-blind trial of nimodipine after SAH in monkeys

Part 2: Pathological findings

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Chronic cerebral vasospasm was induced in monkeys by placement of an autologous blood clot after the basal cisterns had been opened over the arteries of the circle of Willis on one side. The experimental protocol was detailed in Part 1 of this paper. Twenty of the 30 monkeys studied from both groups (one receiving placebo and the other nimodipine) underwent cerebral fixation (Day 14) at controlled pressure by intra-arterial perfusion. The arteries at the base of the brain were studied by light microscopy and scanning (SEM) and transmission electron microscopy (TEM). Cerebral angiography on Day 7 showed that vasospasm was significantly more common (p < 0.0001) and more severe (p < 0.01) on the clot side compared to the control or non-clot side. Vasospasm was less severe on Day 14, just before sacrifice. On SEM, 80% of the 20 middle cerebral artery (MCA) specimens that had been in spasm (Day 7) showed marked corrugation, and in some the endothelium had a fish-scale appearance. All of the 10 MCA’s on the clot side examined by TEM that had been in spasm (Day 7) showed marked changes such as endothelial swelling, subendothelial proliferation, corrugation of the elastic lamina, and myonecrosis. With few exceptions, none of the basilar arteries or MCA’s on the non-clot (control) side showed any abnormalities. The pathological findings of vessels in spasm were considered to be slightly less severe in the nimodipine group; however, the trial drug (1 mg/kg/8 hrs) did not prevent such abnormalities from occurring. The ultrastructural changes in the arterial walls of specimens from both placebo and nimodipine groups in vasospasm are described. Since dramatic changes are present in the vessel walls even after radiologically visible vasospasm has almost completely abated, we believe that vasospasm is due to long-lasting smooth-muscle constriction and not to vessel wall thickening caused by a cellular or subcellular infiltrate.

Key Words • cerebral vasospasm • electron microscopy • nimodipine • subarachnoid hemorrhage • vascular pathology

Cerebral arterial spasm induced by blood within the subarachnoid space has been associated with structural and morphological changes in the vessel wall. These findings may provide valuable information related to the pathogenesis, pathophysiology, and possibly treatment of vasospasm. A recent report, however, failed to document any pathological changes in the brain arteries in dogs subjected to subarachnoid hemorrhage (SAH) and in humans suffering from ruptured aneurysm. Eldevik, et al., suggested that findings in previous investigations could be artifacts from inadequate fixation techniques. In vivo perfusion fixation in humans dying from delayed ischemia due to vasospasm is clearly impossible. Potential postmortem artifacts limit the interpretation of microscopic studies. While subendothelial proliferation and myonecrosis have been attributed to vasospasm, the same changes may be nonspecific and atherosclerotic in nature, and not necessarily due to spasm.

The present studies in monkeys were designed to determine and compare the presence of any anatomical changes among arteries exposed to a subarachnoid hematoma and arteries not directly exposed to blood clot.
Nimodipine and cerebral vasospasm, Part 2

(c)ntrol). We also attempted to determine whether nimodipine could prevent any ultrastructural changes in the cerebral arterial wall.

Materials and Methods

The detailed description of methods of inducing the SAH and clinical and radiological techniques of documenting vasospasm are given in Part 1 of this study.

Cerebral Fixation by Intra-Arterial Perfusion

The monkey brains were fixed by intra-arterial perfusion after the last angiogram was obtained on Day 14 post-SAH. While the animal was mechanically ventilated with a mixture of O2 (66%) and N2O, a wide thoracotomy was performed at the level of the fifth intercostal space. The ascending aorta was cannulated via the left ventricle, the right atrium widely opened, and the descending aorta ligated. Heparin (3000 IU) was given intravenously 5 minutes before normal saline (150 ml at 22°C) was infused for 30 seconds at a pressure of 110 mm Hg. Immediately after the blood was washed out, a mixture of 2% glutaraldehyde and 2% formaldehyde in 0.12 M Millonig’s buffer (pH 7.4) was infused for about 5 to 10 minutes, also at 110-mm Hg pressure. The brain was removed within 15 to 30 minutes of fixation and placed whole in the same mixture at 4°C for a minimum of 24 hours and for a maximum of 4 weeks. Under an operating microscope, the basilar artery and the middle cerebral arteries (MCA’s) of each animal were very carefully dissected, removed, and cut into three segments; one of the sections of each vessel was processed for light microscopy, one for scanning electron microscopy (SEM), and one for transmission electron microscopy (TEM).

Twenty sets of arteries (11 from the animal group that received placebo and nine from the nimodipine group) were studied by SEM. Of these arteries, 10 (six from the placebo group and four from the nimodipine group) were examined and photographed using a Zeiss microscope.*

Procedure for Light Microscopy

After fixation of the tissues, the samples were dehydrated through a graded ethyl alcohol series and cleared in xylene. Tissues were then transferred to an incubator (58°C) and moved through two changes of paraffin (Tissueprep, 56.5°C) for 30 minutes each. Following paraffin embedding, 8-μm sections were cut on a steel knife using a rotary microtome, floated on an albumin-ized slide, and allowed to dry overnight at 40°C. Routine staining was performed using Harris’ hematoxylin counterstained with alcoholic eosin Y. The samples were examined and photographed using a Zeiss microscope.*

Procedure for Electron Microscopy

After primary fixation of the tissues (see above), they were then washed for 45 minutes (three changes) with 0.12 M Millonig’s buffer and postfixed for 1 hour in 1% osmium tetroxide in 0.07 M Millonig’s buffer. After a 30-minute wash (three changes) with double-distilled water, the tissues were dehydrated through a graded series of ethanol solutions.

The samples for SEM were transferred in absolute ethanol to a Seevac carbon dioxide critical point dryer.† The dried tissues were mounted on aluminum stubs, sputter-coated with gold, and examined in a Philips 505 scanning electron microscope at 25 kV.*

Samples for TEM were moved from absolute ethanol into propylene oxide for 30 minutes (three changes) and then into a 1:1 mixture of propylene oxide and Araldite (CY212) epoxy resin for 3 to 4 hours. They were then placed into pure resin in embedding blocks and left overnight at room temperature. Polymerization was carried out over 48 hours at 60°C. Thin sections were cut on a Reichert-Jung Ultracut ultramicrotome, mounted on 300-mesh copper grids, counterstained with uranyl acetate and lead citrate, and examined in a Philips 410 transmission electron microscope at 80 kV.§

Pathology

Gross pathology of the brain was noted in detail, and coronal or horizontal sections were made and photographed. Two observers, unaware of the monkeys’ angiographic status, assessed all arterial specimens for the presence or absence of morphological spasm and any structural abnormalities in the intima, media, and/or adventitia of the cerebral arterial wall.

Results

A detailed description on neurological status, incidence and severity of vasospasm, presence of collateral circulation, and cerebral blood flows is given in Part 1 of this study. Gross examination in all animals showed a hematoma surrounding the narrowed arteries on the side of the craniotomy. The contralateral vessels looked normal. In only one brain (from a monkey with delayed ischemic deficit due to severe vasospasm)§ was there a wedge-shaped infarction in the territory of the MCA. The clinical and radiological manifestations in this monkey (see Part 1)§ correlated with this pathological finding (Fig. 1).

* Standard 14 laboratory light microscope manufactured by Carl Zeiss, D-7082, Oberkochen, West Germany.
† Critical point dryer, CPD-100, manufactured by SeeVac, Inc., 683 Regency Drive, Pittsburgh, Pennsylvania.
Light Microscopy

Light microscopy of the cerebral arteries that had been in spasm showed few changes compared to control arteries. In general, the adventitia looked thicker and had more abundant collagen. The media also appeared thicker, but no other abnormalities were seen. The internal elastic lamina together with the intima were corrugated and the endothelial cells in some cases looked rounded at the crests of the corrugations. In all of the MCA's from the non-clot side and all of the basilar arteries (controls), the endothelium was single-layered and flat, the internal elastic lamina had no corrugations and looked intact, and the media and adventitia appeared normal.

Scanning Electron Microscopy

Most of the control arteries (MCA on the non-clot side and basilar arteries) were normal, except in two instances; one MCA and one basilar artery had small endothelial convolutions but otherwise they were normal. The other control specimens studied under SEM showed clearly defined flat and uniform endothelium with fusiform cells oriented along the longitudinal axis (Fig. 2).

In specimens in which cerebral angiography demonstrated unequivocal narrowing or vasospasm, SEM showed endothelial convolutions that were longitudinally oriented. In some cases, the endothelial cells appeared as if they were dragged in the direction of blood flow (fish-scale appearance), and the crests of the convolutions abutted one another (Figs. 2 and 3). Balloon-like protrusions, crater-like deficits, and areas with detached endothelium with adherent platelets or fibrin thrombi were occasionally seen in vessels in spasm (Fig. 2). There were no differences in pathological findings of the endothelial surface between the placebo or nimodipine groups. Table 1 summarizes some of the radiological and pathological features. In general, there was good agreement between the radiological appearances (Day 14) and pathological findings on SEM.

Transmission Electron Microscopy

Pathological changes were not seen among control arteries except for the basilar artery of the monkey with delayed ischemia, which had subendothelial edema, disruption of tight junctions of the endothelium, and fibrosis of the muscularis. The MCA's on the clot side always demonstrated pathological findings in all three layers. These changes were more apparent in specimens obtained from the placebo group. Pathological findings in the intima included swelling and vacuolization of endothelial cells and morphological changes resulting in a round or plump appearance and disruption of tight junctions. In some cases there was subendothelial swell-

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**TABLE 1**

Degree and time course of vasospasm and pathological findings

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>Vasospasm*</th>
<th>Pathological Findings†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
</tr>
<tr>
<td>placebo group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>mild</td>
<td>mild</td>
</tr>
<tr>
<td>50</td>
<td>moderate</td>
<td>mild</td>
</tr>
<tr>
<td>52</td>
<td>mild</td>
<td>mild</td>
</tr>
<tr>
<td>53</td>
<td>severe</td>
<td>mild</td>
</tr>
<tr>
<td>55</td>
<td>moderate</td>
<td>mild</td>
</tr>
<tr>
<td>58</td>
<td>severe</td>
<td>mild</td>
</tr>
<tr>
<td>61</td>
<td>moderate</td>
<td>mild</td>
</tr>
<tr>
<td>64</td>
<td>moderate</td>
<td>moderate</td>
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<td>69</td>
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<tr>
<td>nimodipine group</td>
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<td></td>
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<td>45</td>
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</tr>
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</tr>
<tr>
<td>68</td>
<td>moderate</td>
<td>mild</td>
</tr>
</tbody>
</table>

*Degree of spasm of the middle cerebral artery (clot side) as assessed angiographically: mild = -11% to -30% reduction in vessel caliber (VC); moderate = -31% to -50% reduction in VC; severe = -51% to -100% reduction in VC.

†Pathological findings on scanning electron microscopy: 0 = normal; + = small endothelial convolutions; ++ = medium endothelial convolutions; FS = fish-scale appearance of endothelial cells.
FIG. 2. Scanning electron micrographs of the luminal surface of cerebral arteries from the non-clot (control) and clot sides. Magnification factors and scales are at the bottom of each picture. Upper Left and Center Left: Normal-appearing endothelium of control arteries (basilar and left middle cerebral artery (MCA), respectively). Lower Left: Balloon-like protrusions and areas in the detached endothelium with adherent platelets and leukocytes on a right MCA (clot side) which had been in spasm on Day 7. Right: Marked longitudinal convolutions covered by abnormal endothelium (upper and lower part of pictures) with fish-scale appearance of the MCA's (clot side) from three monkeys.
F. Espinosa, et al.

Flc. 3. Scanning electron micrographs of the luminal and adventitial surfaces of middle cerebral arteries (MCA's) from the non-clot side and clot side 14 days after subarachnoid hemorrhage. Magnification factors and scales are shown at the bottom of each picture. Left: Normal adventitial and luminal surface of control (non-clot side) MCA's from three different monkeys. Note the presence of nerves and vasa vasorum on the adventitia. Right: Markedly convoluted endothelial surface of three MCA's (clot side). Note the absence of nerves and vasa vasorum on the adventitia.

...ing and, in others, subendothelial proliferation or, more likely, inward migration of smooth-muscle cells (Fig. 4). The internal elastic lamina was frequently corrugated, thinned in some areas, and disrupted in others (Figs. 4 and 5). In the media, some smooth-muscle cells appeared in spasm, and others had intracytoplasmic vacuoles of various sizes either containing fine amorphous material or apparently empty. Some smooth-muscle cells contained many lysosome-like dense bodies. In general, there was swelling and abundant fibrous
Fig. 4. Transmission electron micrographs of endothelial swelling and migration of smooth-muscle cells to the subendothelium of vessels that had been in spasm. The vessel lumen is at the top of each picture. Upper Left: Migration of smooth-muscle cells to the subendothelium. × 6265. Upper Right: Plumped endothelial cells. × 13,290. Center Left: Proliferation and swelling of the subendothelium. × 6265. Center Right: Swelling of the endothelium, detachment of the basement membrane, and swelling of the subendothelium. × 10,175. Lower Left: Migration of smooth-muscle cells to the subendothelium, thinning of the elastic lamina, and fibrosis and early signs of necrosis (pale smooth-muscle cell with intracytoplasmic vacuole) of the media. × 5105. Lower Right: Swelling and vacuolization of the endothelium and disruption of the tight junctions. × 16,710.
tissues in the intracellular space of the media, and in some sections frankly pyknotic muscle cells were encoun tered (Fig. 6). The adventitia was generally thick ened by fibrous material, and in some vessels the hematoma was still attached to it (Fig. 7). In control vessels normal nerve fiber bundles, both myelinated and unmyelinated, with distinct Schwann cells were frequently seen. Despite the search for nerve fibers in arteries that were exposed to a hematoma and were proved to be in spasm, none was found.

Discussion

Cerebral vasospasm is the radiological appearance of sustained arterial narrowing that develops as a complication of SAH due to ruptured aneurysm.\(^4,5,12\) The degree of narrowing is dependent on the volume of blood contained in the subarachnoid space.\(^5,7\) It is not entirely clear, however, why vasospasm lasts for several days to several weeks.

Conway and McDonald,\(^4\) Mizukami, \textit{et al.},\(^16\) and Peerless, \textit{et al.},\(^18\) suggested that the luminal narrowing seen angiographically in SAH patients is due not merely to contraction of smooth-muscle cells but also to various histological changes such as cellulosfibrous thickening of the intima, subendothelial proliferation, and organization of luminal thrombus. Other investigators\(^5,12\) have also observed such findings. Hughes and Schianchi\(^12\) and Peerless, \textit{et al.},\(^18\) found that the magnitude of structural and morphological changes in the arterial wall was related to lapse of time after SAH. Early changes (3 weeks or less after SAH) were the most prominent manifestations. These consisted of swelling of the endothelium with areas of displacement from basement membrane, migration of smooth-muscle cells...
FIG. 7. Electron micrographs of hematoma 14 days after placement within the subarachnoid space. Upper: Scanning electron micrograph showing deformed erythrocytes and abundant collagenous tissue. • 448. Lower: Transmission electron micrograph showing three macrophages with intracytoplasmic vacuoles and lysosomes and a plasma cell (lower left of picture). • 2150.

to the subendothelium, fragmentation and corrugation of the internal elastica, swelling and necrosis of smooth-muscle cells, and an inflammatory reaction of the adventitia containing lymphocytes, plasma cells, and macrophages. Faleiro, et al., found that arteries close to ruptured aneurysms had an increase in mast cells in the lamina muscularis. Patients who had more mast cells in the media of their arteries had less spasm and survived for a longer period of time after SAH. No firm conclusions were drawn due to the small number of patients studied.

Eldevik, et al., could not confirm any morphological changes ascribed to spasm. They studied nine patients who died from SAH and cerebral infarction due to vasospasm, and 10 dogs weighing 11 to 20 kg in which an SAH was created by the injection of 5 ml of blood (once or more) into the cisterna magna. The volume of blood used for induction of SAH was probably small for their dogs. We used an average of 7 ml of blood for clot placement in our 3-kg monkeys. The failure to reproduce morphological changes in vessels in spasm may be due to the fact that the posterior circulation is less densely innervated than the anterior one, or that clot did not form in the vessels subsequently studied. It is severe chronic vasospasm that causes brain ischemia and is associated with morphological changes in the arterial wall.

Others have found that vasospasm in experimental SAH is associated with morphological and structural changes of the affected vessels. Fein, et al., created an SAH in monkeys, either by intracisternal injection of 3 ml of blood or by puncture of the intradural internal carotid artery. Chronic vasospasm (for more than 7 days) developed only when the carotid artery was punctured, suggesting that with this method a large SAH was created. In these cases, electron microscopy showed vacuolization and fibrosis of the media and some areas of myonecrosis; the elastic lamina was more dense than normal and the endothelial cells looked rounded. Clower, et al., reported similar experiences. Tanabe, et al., and Tani, et al., also studied dogs subjected to SAH and found necrosis, vacuolization, and widening of the interstitial space of the media. They also described vacuoles and dense bodies in endothelial cells, detached endothelium, and thickening of the intima. More recently, Varsos, et al., and Liszczak, et al., using a model similar to Eldevik, et al., found changes in the arterial wall 30 days post-SAH. They reported subendothelial edema and accumulation of debris in the myointimal region, corrugated intima and elastic lamina, degenerating smooth-muscle cells and accumulation of membrane-bound vesicles and collagen fibers within the media, and adventitial inflammatory reaction associated with vasospasm. The authors suggested that adventitial changes more than intimal or medial changes may be responsible for the pathogenesis of vasospasm.

Ohta, et al., using a rat model of acute spasm, examined the luminal surface of normal and spastic vessels. They observed marked convolutions of the luminal surface in SEM that did correspond to the changes seen on TEM. Mayberg, et al., also studied the cerebral arterial endothelium after SAH was induced by a 2-ml blood injection in cats. Scanning electron microscopy showed mild longitudinal convolutions of the luminal surface of the basilar artery that correlated with angiographically demonstrated vasospasm. These findings, which were not seen in control arteries, persisted after fixation at physiological pressure. As shown by others, intimal folds can be avoided by fixation using intra-arterial perfusion at pressures above 80 mm Hg. Although Greenhill and Stiefens perfused their rabbits at controlled pressure, they observed luminal convolutions in normal carotid arteries. It is likely that these corrugations were post-mortem artifacts, since sacrifice of their animals preceded chest opening and cannulation of the thoracic aorta for the infusion of the fixative.

Our findings agree with most of the previous inves-
tigators in that changes in the arterial wall were associated with vasospasm. However, we did not see mast cells in the media, and inflammatory reaction in the adventitia was minimal. We could not document the presence of intraluminal thrombus in vessels in spasm. We did not see erythrocytes or debris from them in the media or intima. Our findings suggest that structural and morphological changes of the arterial wall may not be a major factor causing vessel narrowing, because vasospasm was maximal on Day 7 and the cerebral arteries were fixed on Day 14 when the last angiogram showed that vessel lumen was almost normal. Cerebral vasospasm is more likely a nonphysiological (sustained) contraction of smooth-muscle cells in response to blood in the subarachnoid space and not vessel narrowing from histological changes, as suggested by others.4,6,18

Media changes may be ischemic in nature from sustained spasm. Endothelial changes were likely induced by blood travelling at high velocity through a narrowed arterial segment; the endothelial cells looked as if they were dragged in the direction of blood flow which, if high, could perhaps detach the damaged endothelial cells from the basement membrane. Nimodipine (1 mg/kg/8 hrs) did not prevent the occurrence of these morphological changes.

The structural and morphological changes in the vessel wall seem to be the result of sustained spasm and not the cause of the vessel narrowing seen angiographically. Therapy of vasospasm should be directed at preventing smooth-muscle contraction.

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References


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