Intimal proliferation of cerebral arteries after subarachnoid blood injection in pigs

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A model of experimental subarachnoid hemorrhage in young pigs has been created using two subarachnoid blood injections. Cerebral arteries of the pig demonstrate intimal proliferation and medial necrosis 10 days after experimental blood injection; this appears to be a reaction to arterial injury. The similarity between the arterial reaction to subarachnoid blood and the general process of atherosclerosis is noted, and steps have been taken to insure that the vasculopathy described is truly a response to the injected blood. The authors conclude that the intimal proliferation observed between 1 and 2 weeks after experimental subarachnoid blood injection is an indicator of arterial injury and is, therefore, a good end point for further studies.

KEY WORDS □9 vasospasm □9 experimental subarachnoid hemorrhage □9 pig □9 intimal proliferation

IN 1976, we2.3,6 reported that the intimal proliferation of cerebral arteries seen after subarachnoid blood injection is similar morphologically to the generalized arterial reaction to injury which has been proposed by Ross and Glomset.34,35 as a theory for the pathogenesis of atherosclerosis. Recently, Kassell, et al.,24,32 suggested that post-subarachnoid hemorrhage (SAH) vasospasm is an acute proliferative vasculopathy, which supports this similarity to atherosclerosis. Since pigs are considered one of the best animals for induction of experimental atherosclerosis and are well known for developing spontaneous atherosclerosis,15,16,37 it seemed appropriate to determine if a pig model would develop intimal proliferation in cerebral arteries after subarachnoid blood injection and, therefore, be useful for future studies. Because some investigators have questioned the significance of morphological changes, we have taken considerable precautions to insure that our observations are not caused by artifact or spontaneously occurring abnormalities.

Materials and Methods

Animal Preparation

Seventeen young female pigs, each weighing 16 to 24 kg, were used in the experiments. The pigs ranged in age from 10 to 24 weeks and they were fed regular commercial chow. The experimental design is shown in Table 1. The surgical procedures and angiograms were performed under halothane anesthesia with controlled ventilation. The concentration of halothane was maintained at 1.0% with 50% nitrous oxide. Several agents such as ketamine hydrochloride, atropine sulfate, and sodium thiamylal were used for restraint and preanesthesia tranquilization.

The 14 experimental pigs had catheters inserted into the prepontine cisterns via a C-2 laminectomy using sterile precautions and microsurgical technique. After the dura mater was opened, the arachnoid membrane was incised immediately beneath the C-2 nerve root on the left side, and two 6-cm Silastic catheters, 1 mm in diameter, were passed ventrally to the spinal cord cephalad into the prepontine cistern. After it was confirmed that cerebrospinal fluid (CSF) was flowing through the catheters, the proximal ends of the catheters were occluded and left in the subcutaneous tissue for subsequent blood injection. The dura mater was closed in a watertight fashion by continuous suture, and the wound was closed in layers.

Between 5 and 8 days after laminectomy, the skin of the neck was opened and 12 cc of nonheparinized autologous arterial blood was injected slowly through one of the catheters without removal of CSF. A second injection of 12 cc of blood was given through the other catheter 2 days after the first injection in all except two pigs (Table 1). The second injection was delayed in Pigs 1 and 2 due to technical difficulties. The pigs were kept in a 30° head-down position for 15 minutes after each injection to secure a cephalad distribution of the subarachnoid blood. One animal (Pig 13) was used for
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TABLE 1

Experimental design and results of subarachnoid hemorrhage in pigs

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Procedure</th>
<th>Day of Study*</th>
<th>Arterial Intimal</th>
<th>Medial Necrosis‡</th>
<th>Clot†</th>
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* O = C-2 laminectomy and placement of catheters in the subarachnoid space; A = angiography; B = subarachnoid blood injection; S = sacrifice.
† Amount of clot on gross observation: ++ = dense clot; + = patch clot; - = no clot.
‡ + = feature present; - = feature absent.

Acute angiographic and pathological studies 1 hour after the first injection.

One animal (Pig 14) was used for pathological study to determine whether the operation and catheter insertion had any effect on the arteries. This animal received no blood injection. In addition to the experimental pigs, three pigs of similar age and weight in which no catheters were placed and no subarachnoid blood was injected were used as controls for the histological study.

Angiography

Angiographic evaluation of arterial caliber was not done routinely on these animals because ultrastructural artifacts can be produced by the contrast medium. Angiography was performed in six of the pigs, including Pig 13, to document the arterial diameter, which was then classified according to our definition. If arterial narrowing lasted less than 1 hour, it was considered vasoconstriction; if it lasted from 1 to 24 hours, it was considered acute vasospasm; and if it lasted more than 24 hours, it was considered chronic vasospasm.

The blood supply to the brain of pigs has some special characteristics that deserve mention. The major contribution to the circle of Willis is derived from the ascending pharyngeal arteries through the carotid rete, so there are no true intracranial vertebral arteries. The basilar artery is formed by the union of the caudal divisions of both internal carotid arteries and tapers from cephalic to caudal.

In animals subjected to angiography, the first angiogram was performed at the time of the C-2 laminectomy and this was used as the control. A second angiogram was taken 2 days after the second subarachnoid blood injection, except in Pig 13 (Table 1). Angiography was performed by way of the common carotid artery, which was exposed in the neck and punctured with No. 20 Angiocath.* The tip of the catheter was placed in the ascending pharyngeal artery. Three to 5 cc of Conray (isothalamate meglumine) was injected for each study. The pig's head was placed at a standard distance from the x-ray tube and the film so as to reproduce the same amount of magnification in all animals. All angiograms were reviewed independently by two of us (T.T. and J.F.A.) and graded for the presence or absence of arterial narrowing.

Fixation and Tissue Examination

All 17 animals were sacrificed by an overdose of sodium thioumal. We decided to fix the tissues by immersion instead of by perfusion because of our interest in preserving intravascular platelets or other debris adherent to the endothelium. The brain was removed within 10 minutes. We intended to sacrifice Pigs 1 to 12 between 10 to 14 days after the first subarachnoid blood injection, but two of them (Pigs 4 and 7) were sacrificed earlier than the scheduled time because of neurological deterioration after the second SAH. Pig 13 was sacrificed at 1 hour after subarachnoid blood injection and Pig 14 was sacrificed at 24 days after C-2 laminectomy and placement of the prepontine catheters (Table 1).

At the time of brain removal, the presence or absence of clotted blood in the basal cisterns was noted on gross observation and recorded as: dense clot, patch clot, and no clot. After removal, the brain was immediately immersed in 2% phosphate-buffered glutaraldehyde where it remained for at least 12 hours. The circle of

* No. 20 Angiocath manufactured by The Deseret Co., Sandy, Utah.
Willis and attached major arteries were dissected free and cut into 25 pieces. These were further fixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Each of the 25 specimens was sectioned transversely with a microtome. Thick sections stained with toluidine blue were examined with a light microscope. Thin sections stained with uranyl acetate and lead citrate were examined with a Zeiss EM 9 transmission electron microscope.

Sections from each of the 25 specimens of cerebral arteries removed from each pig were examined microscopically with attention directed mainly to the intima and media of the arterial wall. If abnormalities consisting of at least two layers of intimal proliferation were found in at least two specimens of vessel, the animal was regarded as positive for pathological findings. If intimal proliferation was not found in two specimens the animal was regarded as negative for pathological findings.

**Results**

**Gross Observations**

Pig 13, which was autopsied 1 hour after injection of 12 cc of blood into the subarachnoid space, had a wide distribution of blood clots at the base of the brain and much blood in the cisterna magna (Fig. 1 left). The basilar artery and intracranial carotid arteries were found to be buried within an accumulation of clotted blood (dense clot). The animals that were sacrificed later showed patchy clots (Fig. 1 right) or no clots, accompanied by yellow pigmentation of the arachnoid membrane, which was presumed to represent recently absorbed blood. The tips of the Silastic catheters were found in the subarachnoid space ventral to the medulla oblongata in all pigs. No sign of infection was observed.

**Microscopic Findings**

**Normal Artery.** There were no recognizable histological differences between Pig 14 (catheter control) and the three nonsurgical pigs. The fine structure of the arterial walls was the same throughout the various segments of the circle of Willis, except for the normal intimal thickening at sites of branching.

The tunica intima consisted of an endothelial cell layer, subendothelial layer, and an internal elastic lamina (Fig. 2). There was no cellular component in the subendothelial layer, but extracellular fibrils and collagen were occasionally observed. Endothelial cells were joined by tight junctions, with overlapping or interdigitation of cell processes. Their cytoplasm was relatively clear, showing no difference between adjacent cells. The internal elastic lamina formed a continuous band between the intima and the media. The tunica media consisted of four to 10 layers of smooth-muscle cells with interspersed collagen and elastic fibers. The cytoplasm of the smooth-muscle cells was filled with the usual complement of myofilaments and cell organelles localized near the nucleus. There was no finding of medial necrosis.

**Subarachnoid Blood Injection Group.** Nine (75%) of the 12 pigs examined after subarachnoid blood injection showed intimal proliferation (Fig. 3). In these animals, the intima was thickened and the endothelial cells had increased endoplasmic reticulum and a long pedicel extending into the subendothelial space. In the subendothelial space, which is normally cell-free, there were numerous irregularly shaped cells that looked like proliferating smooth-muscle cells capped by elastin and
collagen fibers. The internal elastic lamina was split and fragmented. What appeared to be the remnant of the normal internal elastic lamina was thinned, but new layers of elastic fibers were seen, which suggested the formation of new elastic material (Fig. 3). No foam cells were observed in the subendothelial space.

Eleven (92%) of the 12 pigs showed necrosis in the media (Table 1). This included all nine of the animals that had intimal proliferation. The severity of the necrosis was highly variable, however, and the pathological smooth-muscle cells were seen randomly distributed throughout the media. Some cells were intact but had abnormal vacuoles containing fine granular substances and some were actually necrotic as evidenced by their high electron density (Fig. 3). In addition, areas of extracellular debris were seen, indicating lysis of necrotic smooth-muscle cells. All of these changes were considered evidence of medial necrosis.

One animal (Pig 11) had severe damage with denudation of the endothelial cells and the adherence of platelets to the subendothelial structures (Fig. 4). This animal also showed marked necrosis of smooth-muscle cells under the internal elastic lamina. Neither the intima nor the media of the pig sacrificed 1 hour after SAH (Pig 13) showed any abnormality.

**Angiographic Findings.** In Pig 13, angiograms were performed before and 15 minutes after the subarachnoid blood injection. The basilar artery became straightened and narrowed. According to our definition this was considered vasoconstriction. Of the five pigs that underwent angiography 2 days after the second blood injection, three showed chronic vasospasm and two appeared normal (Fig. 5).

**Discussion**

There have been numerous clinical and experimental studies concerning the morphological changes in the wall of cerebral arteries after SAH with or without vasospasm. Crompton observed necrosis, edema, and subendothelial polymorphonuclear cell permeation of the vessel wall in a postmortem study of 119 patients with cerebral infarction following the rupture of cerebral aneurysms. Conway and McDonald reported a series of autopsied SAH cases, and showed that the lumina of the intracranial arteries were narrowed by intimal thickening and that this structural change became prominent 4 weeks after the hemorrhage. They thought that the initial vasospasm caused either mechanical or anoxic damage to the intima with subsequent intimal proliferation in the most severely affected arteries. Hughes and Schianchi and Sakaki observed marked concentric intimal thickening by subendothe-
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FIG. 5. Angiogram of Pig 11 obtained at the time of laminectomy (left) and 2 days after the second blood injection (right). Chronic vasospasm of the internal carotid artery is seen.

Familial fibrosis and necrosis in the tunica media in autopsy studies of patients who died 3 weeks after SAH with vasospasm. Mizukami, et al., described medial necrosis, medial thinning, and endothelial proliferation in patients with longer survival after vasospasm. A few authors have reported no changes in the arteries, even of those patients with vasospasm antemortem, but their patients were studied within 3 weeks after SAH, which makes the identification of pathological changes very difficult.

Weir, et al., reported no morphological abnormalities in monkey vessels after experimental SAH; however, in electron microscopy studies in 1974, Alksne and Greenhoot observed post-SAH changes in the tunica media in monkeys. Tanabe, et al., first described intimal thickening of arteries after experimental SAH in dogs. In that study, intimal thickening was mostly observed between 3 days and 1 month after SAH. Nevertheless, other investigators have been unable to confirm these findings and have suggested that they are not significant.

Recently, two groups of investigators have reported the use of a double blood injection model in dogs. In both instances, the authors reported pathological changes in the vessel wall but neither showed the marked intimal proliferation found in our animals. We have used dogs in the past but discontinued use of these animals because the arterial changes were much less than those seen in humans or monkeys. In order to progress to reliable experiments on prevention, it is essential to have an unequivocal pathological abnormality.

Our experiments clearly demonstrate that intimal proliferation of cerebral arteries occurs in response to experimental SAH in pigs. This supports earlier reports that what has been considered vasospasm in the past may actually be associated with a pathological process. Since others have failed to detect these changes, we have taken considerable precautions to insure that our observations are not caused by artifact or spontaneously occurring abnormalities unrelated to SAH.

We have already pointed out the similarity of the arterial reaction to subarachnoid blood to the general process of atherosclerosis. According to the injury hypothesis of atherogenesis proposed by Ross and Glomset, the initiating insult is injury to the endothelium which results in a sequence of events that progress ultimately to plaque formation. Ross and Glomset and Jellinek reported that the fundamental arterial reaction to injury is the appearance and/or increase in number of intimal smooth-muscle cells and accumulation of connective tissue fibers and matrix in subendothelial layers. These changes are called "intimal proliferation," "thickening of intima," "subintimal thickening," "myointimal thickening," and "myointimal proliferation." We use the term "intimal proliferation" in this article.

In pigs, atherosclerotic changes of the intima, characterized by smooth-muscle cell proliferation, accumulation of connective tissue fibers, and lipid deposition, which is very similar to that which occurs in humans, takes place spontaneously with increasing age. Skold and Getty found spontaneously occurring patches of intimal thickening which stained by Sudan IV at the rate of 5% in the abdominal aorta of 1-year old pigs. Atherosclerosis of the cerebral arteries of pigs has been studied by Luginbühl and Jones and Getty. They reported that many plaques develop in...
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the anterior and middle cerebral arteries, as well as in the basilar artery of the aged pig. They also observed that cerebral arteries of the pig are affected later in life than are the aorta, coronary arteries, and other major blood vessels. In this present study, we used young pigs 10 to 12 weeks of age in order to prevent the possibility of mistaking the spontaneous plaque formation produced by age for the intimal proliferation produced by subarachnoid blood injection.

According to Stary and Strong, non-atherosclerotic intimal thickening, which consists of smooth-muscle cells and ground substance between the endothelium and the internal elastic lamina, can be differentiated from atherosclerosis because non-atherosclerotic intimal thickening has no extracellular lipid. They have defined two morphological types of non-atherosclerotic intimal thickening, the "intimal cushion" and diffuse intimal thickening. The "intimal cushion," which has also been called "intimal pad," "musculoendothelial plaque," "mucoid fibromuscular plaque," "focal intimal proliferation," "localized fibrous plaque," and "spindle-cell cushion," refers to a small area of thickening at the site of arterial branching. This occurs spontaneously in many animals, including young pigs. On the other hand, diffuse intimal thickening is uncommon in young animals.

Because it would be sufficiently difficult to distinguish the intimal proliferation that occurs after subarachnoid blood injection from intimal cushions, we decided to select all of our specimens away from sites of arterial branching. Utilizing this sampling method, we found the endothelium to be closely applied to the internal elastic lamina in all the control animals. Extracellular components such as collagen, elastin, and fine fibrillary material were seen infrequently. Therefore, it seems safe to state that the morphological changes described did not occur spontaneously in the pigs used for this study.

Although all of the tissue has been fixed by immersion instead of perfusion, we believe unequivocally that the morphological changes described are true pathology and not artifact. It would be impossible for the intimal proliferation demonstrated to occur as a fixation artifact because such a process would require actual cell duplication. The medial necrosis could conceivably be secondary to poor fixation, even though the changes described were never seen in the control animals, which were prepared in an identical manner. Therefore, all decisions about the presence or absence of pathological change were based on intimal proliferation.

After preliminary experiments using a single injection of blood failed to produce consistent vasoconstriction or morphological change, we developed the double injection mode. We found that the use of two catheters simplified the technical aspects of the second injection. The incidence of the pathological process we desired to study increased markedly with the double injection method, presumably because of the increased quantity of blood remaining in contact with the arteries.

Nine (75%) of the 12 pigs with double subarachnoid blood injection showed unequivocal intimal proliferation. These results indicate that subarachnoid blood injection such as we performed can be regarded as an injury to the cerebral arteries of the pig sufficient to initiate a pathological injury reaction. Although it is not possible to prove that the pathological changes are the cause of the arterial narrowing commonly referred to as vasospasm, it is noteworthy that in those animals in which angiographic narrowing was seen, vasonecrosis was found. It is not surprising that we did not find changes in all our animals as it is well recognized that even in humans with blood clots demonstrated in the basal cisterns by computerized tomography, vasospasm does not always occur.

The etiology of the intimal proliferation seen after subarachnoid blood injection is not known. Based on our observation of denudation of endothelial cells and adhesion of platelets (Fig. 4), we believe that the process begins with endothelial injury as proposed by Ross and Glomset for atherosclerosis and involves platelet adherence as described by Jorgensen.

In addition to intimal proliferation, there are changes of the media after subarachnoid blood injection. These changes consist of cytoplasmic vacuole formation, necrosis of smooth-muscle cells, and the presence of cell debris in the extracellular space. Recently, Joris and Majno reported that vacuoles and cell debris are observed under physiological conditions such as maximum vasoconstriction. Therefore, intimal proliferation observed between 1 and 2 weeks after experimental subarachnoid blood injection remains the best indicator of actual pathological reaction.

In conclusion, the cerebral arteries of the pig demonstrate intimal proliferation and medial necrosis 7 to 17 days after experimental SAH, which appears to be a reaction to injury. This model may be useful for further evaluation of the relationship between vasospasm and vasonecrosis and for study of the pharmacological prevention of these phenomena.

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