Ultrastructure of cerebral arteries following experimental subarachnoid haemorrhage

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SUMMARY Scanning and transmission electron microscopy have been used to examine the ultrastructure of cerebral arteries taken from dogs up to nine days following the injection of autologous blood into the suprachiasmatic cistern and using meticulous perfusion-fixation technique. No ultrastructural changes in the cerebral arteries were noted either at sites of radiologically demonstrated arterial constriction or elsewhere. The only abnormality noted was the presence of some leucocytes and macrophages in the subarachnoid space surrounding the arteries. These results are discussed in relation to changes in cerebrovascular reactivity that occur at this stage following subarachnoid haemorrhage.

Post-mortem studies in man have revealed that where death occurs at least three weeks after subarachnoid haemorrhage, there may be subendothelial infiltration with lymphocytes and macrophages followed by fibrosis localised to the segment of artery shown previously to be in spasm by angiography.†‡§ Where death occurs earlier there may be some inflammatory changes within the adventitia and possibly some necrosis within the tunica media. Controversy has arisen over whether or not such structural changes are reproducible in laboratory animals. Three types of experimental subarachnoid haemorrhage have been used: the cisternal injection of autologous blood; the rupture of an intracranial artery and the application of high concentrations of spasmogenic agents directly onto a cerebral artery. Within days, at least in the subhuman primate and the dog, the changes described included swelling and separation of endothelial cells from the internal elastic lamina and degeneration of some smooth muscle cells. However, more recent work using the technique of cisternal injection of autologous blood has failed to replicate these findings in the cat, dog and baboon (see discussion for references). This method, however, is known to induce changes in the responsiveness of the cerebral circulation to hypercapnia and drug induced hypotension, when studied one week later both in the baboon and dog and is associated with abnormal CSF prostanoid levels.†‡§

The aim of our study was to determine whether such "physiological" changes are associated with ultrastructural changes in the cerebral arteries. A possible explanation for the contradictory results in the literature might be poor fixation and hence we paid particular attention to the technique of perfusion fixation in order to correlate the scanning and transmission electron microscopic appearances with the angiographic findings.

Material and methods

General preparation
Twenty-three adult mongrel dogs of either sex were used weighing between nine and 25 kg. There were three categories of dogs, namely three controls, seven acute and thirteen chronic preparations. The animals were starved overnight and on the day of the experiment anaesthesia was induced with intravenous sodium thiopentone (10–30 mg/kg iv). The animals were intubated and allowed to breathe spontaneously on a mixture of 70% nitrous oxide and 30% oxygen. Under sterile conditions a femoral artery was exposed and a headhunting catheter inserted. Using an image intensifier this was guided into a common carotid artery and angiography performed by injecting 5 ml of contrast (300 mgI/ml) when taking lateral subtraction views.
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Induction of subarachnoid haemorrhage and angiography

The same catheter was used for the withdrawal of blood for estimation of arterial blood gases and blood for the subarachnoid haemorrhage. Subarachnoid haemorrhage was produced by the injection of 0.75 ml/kg of autologous arterial blood through a 20 gauge lumbar puncture needle inserted percutaneously through the optic foramen into the suprachiasmatic cistern. The injection was performed after prior withdrawal of CSF and the animal's head was kept dependent in order to keep the blood within the head. Angiography had been performed on the dependant side and was repeated 10 minutes after the injection of blood into the subarachnoid space and in the chronic preparation, one week (two cases—nine days) later. Following the injection of blood the animals usually became apnoeic and were artificially ventilated for a few minutes until satisfactory spontaneous respiration recurred. In total, carotid angiography was performed in ten of the chronic animals and four of the acute animals. Angiography was not performed in all animals to exclude the possibility that some of the previously reported morphological changes might be due to this procedure. After definitive angiography, anaesthesia was maintained under controlled ventilation and the animals were killed by in vivo perfusion fixation. Arterial blood gases and arterial blood pressure were monitored during this period to ensure that hypoxic brain damage was not induced. In the acute preparations intracranial pressure was monitored via a subdural catheter inserted through a burr hole to ensure that intracranial hypertension had subsided following the subarachnoid injection. This ensured there would be adequate perfusion fixation.

Perfusion fixation

Briefly, after heparinisation (1000 IU/kg iv) a thoracotomy was performed and a cannula inserted via the left ventricle into the proximal aorta where it was secured by a ligature. After clamping the thoracic aorta and incising the right atrium, the upper part of the animal was perfused with 500 ml of physiological saline at a mean pressure of 120 mm Hg. The perfusion was then changed to 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 (a volume of 1:5:1 was used per animal delivered at a pressure of 120 mm Hg). At the end of the procedure the brain and carotid arteries were removed and immersed in the same fixative at 4°C for a minimum period of 12 hours.

Neuropathology

Electron microscopy Using a dissecting microscope, segments of the extra- and intracranial arteries corresponding to those assessed for vasospasm (vide infra) and including the verteobasilar arterial system, were taken and prepared for scanning (SEM) and transmission electron microscopy. The specimens were post-fixed in osmium tetroxide, dehydrated in graded ethanols and those for scanning electron microscopy were taken through amyl acetate and critical point dried in a Polaron CO2 bomb, mounted on SEM stubs using silver Dag, sputter coated with gold in an argon atmosphere at 0-15 mm Hg for three minutes at 1:2 kV and 40 mA using a Polaron SEM Corning Unit E5000 (this gives a gold coat of approximately 500Γ in thickness) and observed in a Cambridge Stereoscann 600 microscope in the University Department of Anatomy. Specimens for transmission microscopy were taken through propylene oxide to araldite and ultra-thin sections cut from representative segments of all the major arteries stained by uranyl and lead were examined in a Philips 201 microscope in the University Department of Neuropathology.

Light microscopy Sections 1 μm thick were cut from all the blocks and stained with toluidine blue. The cerebral hemispheres of each brain were cut in the coronal plane into slices 0-8 cm thick. After recording any abnormalities, multiple representative blocks of the cerebral and cerebellar hemispheres, and the brain stem were embedded in paraffin wax and sections were stained by haematoxylin and eosin, a method combining luxol fast blue and cresyl violet, the Prussian blue reaction for iron, Van Gieson for collagen and Miller's stain for elastica. The sections were examined by conventional light microscopy.

Analysis of angiograms

The subtraction angiograms were assessed for vasospasm. Where the reduction in calibre was less than one-third it was classified as grade 1, between one-third and two-thirds grade 2, greater than two-thirds grade 3. The distribution and grading of spasm was recorded at six points:—the proximal part of the internal carotid artery, the distal part of the internal carotid artery, the stem of the anterior cerebral artery, the stem of the middle cerebral artery, the proximal portions of the first bifurcation of the middle cerebral artery.

Results

Angiography

In the four acute preparations, the intracranial vessels became reduced in calibre subsequent to the injection of blood into the subarachnoid space (fig 1a and b). Most of the spasm was classified as Grade 2. In the ten chronic preparations, when examined at ten minutes after the injection of blood, spasm occlusion of the internal carotid artery had occurred in one case and the intracranial vessels had become reduced in calibre in five. Again the spasm was mainly Grade 2. When re-examined a week later, the animal which had shown spasm occlusion now had grade 2 to 3 spasm. Some spasm persisted in four of the five which had had reduced calibre though most vessels now showed grade 1 narrowing. In the fifth animal and in the four that had been normal after injection of subarachnoid blood, no abnormality was detected (table).

Neuropathology

There was a moderate amount of subarachnoid haemorrhage in the basal cisterns of all the acute animals; a small amount of blood was present in the chronic animals. As judged by the uniform hardening of the specimens and by the absence of blood in the vessels, perfusion fixation appeared to be good in all dogs. There was no evidence of brain swelling and internal herniation was not seen. The ventricles
of four acute animals were dilated and contained casts of blood clot. In contrast the ventricles of the control and chronic preparations were similar in size.

**Light microscopy**
In none of the animals were the histological artefacts, the “dark cell” and “hydropic cell”, seen. In eight, however, there were foci of haemorrhagic necrosis in the inferomedial quadrant of either the left or right frontal lobe: this could be attributed to the technique of suprachiasmatic injection of blood. In none of the animals was there histological evidence of ischaemic damage within the distribution of any of the cerebral arteries or their branches.

The subarachnoid space in acute animals was filled with unaltered blood. Very much less blood was seen in the meninges of the chronic animals and in some there was a moderate number of lymphocytes and macrophages, some of the latter containing haemosiderin pigment.

Examination of the paraffin and araldite sections of the cerebral arteries failed to reveal any significant abnormalities and in particular there was no evidence of myonecrosis. Some lymphocytes and macrophages were seen in the adventitia of the cerebral arteries after subarachnoid haemorrhage.

**Electron microscopy**

**Scanning electron microscopy** The scanning electron microscopic appearance of the control vessels revealed well organised endothelium that was consistent with previous descriptions of normal endothelial cell morphology. The luminal surface was either convoluted or flattened except for ovoid protrusions indicating the position of underlying endothelial nuclei. Endothelial cells varied from fusiform to rhomboid in shape. Cell borders, marginal folds and surface striations were easily seen. The endothelium of both the acute and chronic vessels was indistinguishable from controls (figs 2a and b). There was no evidence of “balloon” formation, the adherence of platelets or fibrinous thrombi. The occasional rare “crater” was seen (fig 3). There were no areas devoid of endothelium.

### The incidence and grading of spasm after induced subarachnoid haemorrhage in the acute and chronic preparations

<table>
<thead>
<tr>
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<th>Internal carotid artery grading</th>
<th>Anterior cerebral artery grading</th>
<th>Middle cerebral artery grading</th>
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<tr>
<td></td>
<td>0 1 2 3 SO</td>
<td>0 1 2 3 SO</td>
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<tr>
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<td>0 1 3 0 0</td>
<td>0 1 3 0 0</td>
<td>0 0 3 1 0</td>
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<tr>
<td>Chronic (10)</td>
<td>{Immediately post SAH 1 week}</td>
<td>4 0 5 0 1</td>
<td>4 3 2 0 1</td>
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<td></td>
<td>7 1 2 0 0</td>
<td>5 3 1 1 0</td>
<td>6 2 1 1 0</td>
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SO—Spasm occlusion case
SAH—Subarachnoid haemorrhage
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Transmission electron microscopy In both the acute and chronic preparations no abnormalities of note were seen in either the intima or the media. The endothelium retained its normal morphology and the tight junctions remained tight, with both overlapping and interdigitation. The internal elastic lamina consisted of amorphous granular substance which was denser on the luminal side. The amount of corrugation was similar in control, acute and chronic animals. The media consisted of smooth muscle cells between which there was scanty intercellular collagen and elastic fibres. Each smooth muscle was surrounded by a layer of basement membrane and was essentially spindle shaped with a centrally disposed nucleus of similar shape. The cytoplasm contained abundant myofibrils: the other cellular organelles were normal. The cell membrane had well developed caveolae and in some there were a few dense bodies. The number of dense bodies, however, was similar to those seen in controls, an appearance that was interpreted as physiological degeneration and regeneration of smooth muscle fibres. There was no evidence of myonecrosis (fig 4). A few leucocytes were seen in the adventitia associated with the subarachnoid haemorrhage. In the chronic animals there were some lymphocytes and macrophages and an increase in the number of fibroblasts was seen in the adventitia in some.

Discussion

Following Crompton's original description in 1964 of histological changes in the cerebral arteries taken from patients who died at least three weeks after subarachnoid haemorrhage, controversy has arisen over whether such changes can be distinguished from pre-existing arterial pathology and whether there is any histological change in arteries taken from patients who succumbed within three weeks of bleed. This latter point is of considerable interest clinically since it is usually safe to operate on patients three weeks after their initial haemorrhage and it is obviously important to know whether the high risk period from three to ten days approximately following subarachnoid haemorrhage can be ascribed to or associated with ultrastructural changes within the cerebral arteries.\textsuperscript{2,3,10-13} Part of the discrepancy with the results of other workers who have reported ultrastructural changes in cerebral arteries within a week of the cisternal injection of blood may be due to problems with perfusion fixation.\textsuperscript{14-17} In addition, the site of injection of blood (suprachiasmatic or cisterna magna) might produce different distributions of blood in the basal cisterns and fourth ventricle and theoretically predispose to hydrocephalus and intracranial hypertension. However, hydrocephalus was not seen in our series nor reported elsewhere.

In the present study using a carefully controlled technique of perfusion fixation we have not been able to find any evidence in the dog of ultrastructural changes within the cerebral arteries up to nine days following the injection of autologous arterial blood into the suprachiasmatic cistern. There were no changes in either vessels of normal calibre or in

Fig 2 (a) Scanning electron microscopic appearances of luminal surface of internal carotid artery. The junctions between adjacent endothelial cells are clearly visible. No abnormalities were seen in the control, acute or chronic preparations. N = ovoid nucleus of endothelial cell protruding into the lumen; R = red blood cells. (× 1200.) (b) Scanning electron microscopic appearance of luminal surface of carotid artery. There is a defect in the junction between adjacent endothelial cells. This appearance was seen occasionally in control and SAH-induced preparations and was attributed to either perfusion fixation or the preparative procedures. (× 3400)

Fig 3 Scanning electron micrograph. There is a "crater" on the surface of an endothelial cell at a distance from its nucleus. This uncommon appearance was only seen in one SAH-induced preparation. (× 1800)
arteries shown to have been constricted angiographically. The only abnormality was the presence of some white cells and macrophages in the subarachnoid space and debris left by the subarachnoid haemorrhage. Such leucocytes did not penetrate into the tunica media or intima of the cerebral arteries. These results confirm our earlier findings with the light microscope in the baboon and the electron microscopic findings in the dog, cat and monkey. All these authors reported the effects of the cisternal injection of blood and avoided artefacts of the internal elastic lamina and "crater-like" defects and "ballooning" of endothelial cells on the luminal surface, which are produced by inadequate perfusion pressure during fixation and by ischaemia. As Eldevick et al made clear the meticulous procedure before, during and after the perfusion fixation of the brain is important in order to avoid artefacts when using scanning and transmission electron microscopy. For example, Tanabe et al exsanguinated their animals prior to perfusion fixation and Alksne immersed the excised brain in 4% phosphate-buffered glutaraldehyde having previously exposed the brain prior to opening the chest and excising the heart.

The absence of ultrastructural changes in our animals implies that the alterations in cerebrovascular reactivity both in vivo and in vitro and changes in intracranial prostanoid production cannot be attributed to overt histological damage to the cerebral arteries. In addition, in the baboon one week after cisternal injection of blood there is no evidence for increased intracerebral platelet deposition as might be expected were there any significant endothelial disruption (Lovick, Pickard, Graham and Fitch, unpublished observations).

In the recent study of Smith et al swelling of the intima was only seen in arteries taken one to nine days following subarachnoid haemorrhage and there was some subintimal cellular proliferation with intramural haemorrhage in arteries taken between 4 and 28 days following a bleed at which time the internal elastic lamina and smooth muscle were found to be normal. It was only in arteries taken between 16 and 40 days following subarachnoid haemorrhage that severe subintimal cellular prolif-

Fig 4 Transmission electron micrograph. Proximal portion of first bifurcation of middle cerebral artery in chronic preparation. Angiography had shown Grade 2 vasospasm in this vessel one week after subarachnoid haemorrhage. The endothelial cells (EN) are flat and are joined by a tight junction and there are overlapping cytoplasmic processes (arrows). The internal elastic lamina (EL) is not corrugated and shows higher electron density on the luminal side (L). Smooth muscle cells (S) are round, oval and polygonal in shape, and contain a central core of organelles and a nucleus surrounded by myofilaments. A basement membrane envelopes each cell and the intercellular space contains bundles of collagen fibres. (× 8000)
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Intraarterial injection was noted with rupture and splitting of the internal elastic lamina, intramural haemorrhage and myonecrosis with fibrosis. Various workers have reported that they could reproduce similar histological changes experimentally in cerebral arteries following tearing of a branch or withdrawal of a previously inserted needle through the wall. It is not always possible reading the literature to know how extensive such changes are throughout the intracranial vasculature. In the work reported by Clowert et al all vessels showed more extensive changes close to the site of rupture but the alterations also occurred both proximal and distal to the point of rupture in the middle cerebral artery. By 30 days varying degrees of subintimal change were observed 6–7 mm distal and 2–3 mm proximal to the initial site of vascular rupture. Both Liszczak et al and Clowert et al suggest that these subintimal changes may reflect dissection of blood into the wall of the artery following rupture.

In conclusion, a consensus is emerging that instillation of blood into the subarachnoid space per se does not appear to cause ultrastructural change at least during the first week. The production of prolonged vasospasm in animals is notoriously difficult and this may relate to the simplicity of their subarachnoid space with very efficient evacuation of subarachnoid clots when compared to man. However, the important conclusion from our work is that the changes in cerebrovascular reactivity and intracranial prostanoid production cannot be attributed to overt ultrastructural changes in the walls of the cerebral arteries.

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