Ultrastructural Studies of Pial Vascular Endothelium Following Damage Resulting in Loss of Endothelium-Dependent Relaxation

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The changes in pial arterioles of 7 cats were examined by electron microscopy after injury that eliminates endothelium-dependent relaxation to acetylcholine or bradykinin. The injury was produced by exposing the vessels to mercury light in situ in the presence of intravascular sodium fluorescein dye. Previous studies showed that, at the time of initial injury and loss of endothelium-dependant responses, the endothelial cells displayed minimal ultrastructural evidence of injury. Because these changes might indicate the beginning of a sequence of irreversible alterations representing or leading to cell death, the present study was carried out 3½–4 hours later, when ultrastructural evidence of progressive cell degeneration should readily be recognized. No such changes were observed. Instead, most vessels showed only the minimal alterations observed initially (endothelial vacuolation, blebs, and lucencies). Four of 19 vessels were completely normal. The findings fail to support the hypothesis that irreversible cell damage or death caused by the light + dye injury has caused the associated loss of endothelium-dependent relaxation. Rather, the findings support the concept that much lesser degrees of trauma are sufficient to impair the dilating responses of cerebral microvessels. This greatly expands the potential spectrum of pathologic states that might result in loss of endothelium-dependent relaxation. (Stroke 1987;18:927–931)

It is now known that many vasodilators do not directly relax vascular smooth muscle. Rather, they reach and interact with receptors on endothelial cells, and these cells in turn synthesize and release endothelium-dependent relaxing factors (EDRFs). It is EDRF that in turn relaxes vascular smooth muscles.1,2 We have previously demonstrated that endothelial injury in the microvessels of the brain can eliminate the ability of either acetylcholine (ACh) or bradykinin (BK) to relax the injured arterioles.3 This consequence of endothelial damage was equated with loss of 1 or more EDRFs. In analogous fashion, endothelial injury had already been used to establish the existence of endothelium-dependent relaxant (EDR) in larger arteries in vitro.1,2 However, our data provided 3 relatively novel extensions of the original studies with large vessels: 1) we demonstrated EDR in vivo rather than in vitro, 2) we demonstrated EDR in small arterioles rather than large arteries or veins, and 3) in contrast with reports of data from large vessels, we found that denudation of endothelial cells was not essential for elimination of EDR.3–5 To eliminate EDR in vivo from arterioles, we damaged the endothelium with light from a mercury lamp in the presence of intravascular sodium fluorescein dye.3 Not only was EDR eliminated without denuding the endothelium, but the elimination of EDR appeared when endothelial cells revealed only subtle endothelial abnormalities such as cytoplasmic vacuolation or increased lucency.4,5

While these findings established that removal of the endothelium was unnecessary for loss of EDR in the microcirculation, they left open the possibility that the slight degree of ultrastructural change was misleading. Perhaps the endothelial cells were dying, and morphologic evidence of irreversible damage would have appeared had the vessels been examined after a longer time following injury. The studies described below address this issue by injuring the pial vessels in one of the species, cats, used in the original morphologic study and by performing ultrastructural studies 3½–4 hours rather than immediately after injury.4 A failure of morphologic changes to progress beyond those originally reported would suggest that the injury that eliminated EDR was not lethal to the endothelial cells. This in turn would expand the possible pathologic significance of EDR by showing that the injury to endothelium that at least temporarily eliminates EDR need not be severe enough to irreversibly injure the vessel.

Materials and Methods

Seven adult cats of either sex were used in the present investigation. After initial anesthetization with 30 mg/kg i.v. pentobarbital, the femoral artery and vein were cannulated, tracheostomy was performed, and the cats were artificially ventilated with room air and a positive-pressure respirator. During the experiment, expiratory CO₂ concentrations were continuously

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monitored and maintained at 30 mm Hg. A cat was placed in a stereotactic frame, and a 11-mm diam. craniectomy was performed over the left middle suprasylvian and ectosylvian gyri. On removal of the bone flap, the underlying intact dura was carefully resected. With removal of the dura mater, the underlying pial vasculature could be directly observed within the subarachnoid space. A closed pial window was inserted at this site and filled with mock cerebrospinal fluid (CSF). A Wild dissecting microscope (Farmingdale, N.Y.) was used for initial observation of the pial vessels. The microscope was fitted with an image splitter to measure arteriole diameter using TV microscopy as described by Baez. A halogen lamp illuminated the field circumferentially from above.

Before any experimental interventions were initiated, all vessels in the field were selected, and a single arteriole and venule were selected for continued detailed monitoring. Then, to document an initially intact vasculature that gave EDRs, the response to 10^−7 M ACh (ACh chloride, Sigma Chemical Co, St. Louis, Mo.) was monitored. The cranial window was filled with mock CSF containing ACh and was left in place until steady-state dilation was achieved. The fluid under the window was then washed out with mock CSF. The Wild microscope was then replaced by a Leitz Ultrapak microscope (Rockleigh, N.J.) fitted with a filtered mercury lamp. When the lamp was not in use, illumination came from a halogen lamp and fiberoptic probe. The mercury lamp was filtered with Leitz KG-1 and BG-12 filters providing peak transmission at 400 Å and removing harmful ultraviolet wavelengths from the light emitted by the mercury lamp. This explains why no harmful effects were seen even after 40 minutes of exposure unless the fluorescein was present and excited by the light passing through the BG-12 filter. It should be noted that this light was focused by the Ultrapak microscope on only 1 microscopic field within the craniectomy site. Within seconds of changing to illumination from the mercury lamp, we initiated the infusion of 2% sodium fluorescein at a rate of 1.2 ml/min via the femoral vein, and within approximately 1 minute the pial vessels in the illuminated field fluoresced brightly as previously described. A maximum of 20 ml of fluorescein was infused. Within 10 minutes of the onset of the light + dye insult, platelet aggregates were seen. If one interrupts the mercury light immediately after aggregates appear in the vessels, no aggregates will ever appear in the arterioles. We used this maneuver to investigate the effects of endothelial injury without the complicating factor of platelet aggregation in the arterioles. To prevent endothelial injury from progressing and aggregates from developing in the arterioles, at this point in each experiment we switched back to the Wild dissecting microscope and halogen lamp. In 6 of the 7 cats, the response to ACh was again assessed to show success.

Once again identified on the sketch of the illuminated microscopic field. The cat was then prepared for transcardial perfusion. A rapid thoracotomy and pericardotomy were performed, and the left ventricle was pierced with a cannula through which normal saline was perfused. After 2 minutes of saline perfusion, the craniectomy site was reopened, and the site was flushed with topically applied fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer). Concomitant with this topical lavage of fixative, the saline perfusion was discontinued, and 1,000 ml of fixative was perfused. On termination of fixation, the brain with its meningeal investments was removed from the cranial vault and placed in the fixative. After 2 hours, the brain was transferred to 0.1 M sodium phosphate buffer, and the site under the cranial window was identified. Using a protocol described in detail elsewhere, we stripped the pia mater and its related vasculature from this site and placed it in buffered osmic acid. After 2 hours, this tissue sample was placed under a dissecting microscope; on the basis of the previously drawn sketch, we identified those individual arteriole segments whose response to ACh was tested in vivo. These were dissected free of the pia mater.

A total of 19 arterioles were examined from the 6 cats monitored in vivo before and 15 minutes after injury and the 1 additional cat whose vessels were monitored only before injury. Control arterioles were sampled from nonilluminated microscopic fields. In addition, 3 cats had a second window placed over the contralateral hemisphere, which was never exposed to mercury light. Two arterioles from each of these 3 windows were also used as controls.

The chosen microvascular segments were dehydrated in chilled ethanol and propylene oxide, embedded in Medcast resin (Ted Pella Inc, Tustin, Calif.), and transferred to the stage of an ultramicrotome, where the vessels were cut in a plane parallel to their long axis. Alternate thick sections, followed by multiple semiserial thin sections, were cut through the vessels' entire extent. In this fashion, we examined 75 sections from each of the preselected vessels as well as 20–30 sections from all other harvested vessels. The semiserial thin sections were collected in order on membrane-coated slotted grids and were then stained with uranyl acetate followed by lead citrate. All thin sections were viewed and photographed on a JEOL 1200 EX electron microscope (Peabody, Mass.). The arterioles ranged in diameter from 80 to 225 μm.

Results

As previously reported, the injury produced by mercury light + fluorescein dye eliminated the relaxation normally produced by ACh. Before injury, ACh dilated the arterioles to 121 ± 11% (mean ± SD) of baseline diameter. Fifteen minutes after injury, the average response was a slight constriction, the diameter following ACh being 98 ± 7% of baseline. A paired t test showed these postinjury responses to be significantly smaller than control responses (p <
Figure 1. Top: Typical, subtle pial arteriolar change observed 3½-4 hours following mercury light + fluorescein dye injury. Note limited endothelial vacuolization (arrowhead) without change in underlying smooth muscle cells (SM). Bottom left: More dramatic endothelial vacuolization (arrowheads) in pial arteriole 4 hours following injury. Again note endothelial change without concomitant change in medial smooth muscle. Bottom right: Rarely, 4 hours after injury, isolated focus of denudation. Note endothelial (End) detachment, basal lamina exposure (curved arrow), and inclusion-containing (arrow) smooth muscle cell. This area of denudation, approximately 2 endothelial cells long, was the longest seen in any specimen. Few damaged arterioles displayed such a finding, and no muscle damage was found immediately after injury when endothelial-dependent relaxation was already lost.3,4

0.01). Four hours after injury, there was only partial recovery of the response to ACh, the vessels relaxing to 107 ± 9% of baseline. A paired t test showed this response to be significantly larger than that immediately after injury (p < 0.01).

Electron microscopy showed that although 3½-4 hours had elapsed since the insult, evidence of marked endothelial injury was rarely noted. Overall, the observed morphologic abnormalities resembled those reported previously4 in vessels obtained immediately after injury at a time when EDR was lost.

A total of 19 injured arterioles were harvested from 7 cats. Four of the 19 arterioles were totally normal; 11 displayed only scattered endothelial vacuoles, blebs, or intracytoplasmic lucencies (Figure 1, bottom left).
Four of the 19 showed more severe damage. In 3, there was a single focus of denudation, varying from several (Figure 1, bottom right) to only 1 endothelial cell in size. In 1 arteriole, disintegration of the endothelium was observed; however, in this situation the basal lamina was not directly exposed. Occasionally, although not visible with light microscopy, degranulating platelets were found.

In 6 of the 19 arterioles, smooth muscle alteration was also noted, consisting of electron-dense smooth muscle, electron-dense inclusions (Figure 1, bottom right), or degenerating mitochondria scattered throughout a limited portion of the medial smooth muscle. Two of the 6 vessels with muscle damage were vessels that also had a single focus of endothelial denudation.

No alterations of smooth muscle were found in the control arterioles, nor was alteration of endothelium observed.

Discussion

Previous studies demonstrated that EDRF(s) for ACh and BK must exist in pial and mesenteric vessels of mice and cats3,5,12 by showing that, within minutes of endothelial injury, the relaxation produced by ACh and BK was eliminated. Ultrastructural studies showed only slight endothelial damage and normal smooth muscle cells within minutes of injury.4 The present ultrastructural studies were performed to see whether increased evidence of cell damage would appear within 3½–4 hours after injury.

Three-and-a-half to 4 hours after injury, the endothelium in most arterioles looked like that described in our original report.4 Only 3 vessels showed breaks in the endothelial layer, and these rare breaks were very minute. The slight endothelial alteration in most vessels indicates that no irreversible changes were induced by the original injury.

No platelet aggregates were noted in the arterioles at any time during our in vivo observations. However, aggregating platelets were seen in some ultrastructural preparations. Platelets were either too small or too few to be detected in vivo. Since aggregating platelets may release materials toxic to endothelium,13,14 they might have caused the very rare foci of severe damage seen with electron microscopy. If so, even these few foci cannot be interpreted as "ripening" of the original injury, but represent foci of new damage occurring after the original insult.

The reason for focal muscle injury in one third of the vessels is unclear. This injury was not present initially,4 and therefore cannot account for loss of EDR. Indeed, it could not account for such loss in any case since it was ultimately seen in only a minority of vessels.

The ultrastructural changes could not be graded in a manner that correlated with degree of response to ACh 4 hours after injury. The damage may result in a graded response to ACh, depending upon its intensity; however, graded response was not demonstrated here, nor was it an aim of the study. However, it is possible to produce graded loss of EDR in pial arterioles as shown by recent studies in which we used a HeNe laser to injure the endothelium.5,15,16 There again we found only minimal ultrastructural changes at the site of laser impact, but EDR was lost in a graded fashion upstream and downstream from that site.

The ultrastructural data in the present study supports the view that no irreversible sequence of events need be triggered when mercury light + fluorescein dye are used to injure endothelium and eliminate EDR. If irreversible changes had been initiated, we would expect progression of ultrastructural change. Instead, in the overwhelming number of vessels, endothelial damage was as slight as or even slighter than that reported 4 hours earlier,4 when loss of EDR was first initiated.3 Of course, we cannot rule out the possibility of further cell degeneration developing >4 hours after injury. However, the issue addressed here is whether the initial insult triggered ultimate cell death even though only minor ultrastructural perturbations were found immediately after injury. The absence of features of endothelial cell death17–20 such as nuclear pyknosis, membrane discontinuities, disrupted organelles, or loss of cytoplasmic content even 4 hours later makes it unlikely, in our opinion, that irreversible injury was produced. Rather, it appears that lesser damage, which does not result in endothelial cell death, may be all that is required to impair endothelium-dependent dilation.

References

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