Cerebral microvascular architecture following experimental cold injury

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The sequential changes in microvascular architecture following local cold injury in rat brains were studied post mortem by scanning electron microscopy and the vascular casting method. The findings were compared with the results of immunohistochemical studies of injured endothelial cells using the bromodeoxyuridine (BUDr) and anti-BUDr monoclonal antibody technique.

Repair of the microvascular architecture had begun by the 3rd day after injury, with hematogenous cells and reactive astrocytes present in the edematous brain participating in the regenerative process. The normal microvascular architecture was reconstructed starting from the edge of the lesion nearest to the brain surface. On the other hand, in the most severely injured part of the brain surface, newly formed microvascular architecture appeared, resembling that of the developing fetal and newborn rat cortex. Seven days after injury, the entire microvascular architecture in the region of the lesion had been reconstructed.

Key Words □ microvasculature □ brain edema □ immunohistochemistry □ bromodeoxyuridine □ scanning electron microscopy □ rat

Brain edema is a frequent and serious problem in man. Many investigators have performed morphological studies on various aspects of brain edema (including blood-brain barrier damage and vascular permeability) for which they have often employed the cold-lesion model of vasogenic brain edema. In this cold-injury model, several methods by which edema fluid can be removed have been clearly established.8,10,13 Spontaneous reconstruction of the microvascular architecture and regeneration of the endothelium following clearance of the edema fluid occur after cold injury,2 but these events have not been studied systematically. In particular, it seems that little attention has been paid to the posttraumatic changes in the microvascular architecture.

The purpose of the present study was to clarify the sequential changes in microvascular architecture following cold injury. The findings obtained were compared with those of previous related studies. An immunohistochemical study of injured endothelial cells was also carried out.

Materials and Methods

A total of 42 Wistar albino rats, each weighing between 200 and 250 gm, were used in this investigation (18 rats for the vascular cast study, and 24 rats for the immunohistochemical study). The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), and a craniectomy was performed in the right parietal region, forming a burr hole approximately 3 mm in diameter. The dura was left intact. A cold lesion was then made in the cortex by the application for 15 seconds of a metal plate cooled to -78°C with a mixture of dry ice and acetone. The contralateral hemisphere served as a control for this study.

Vascular Cast Method

The rats were sacrificed at 1, 2, 3, 4, 5, and 7 days after injury. For macroscopic visualization of the area of injury and of the maximal extension of edema, the rats were injected intravenously with 2% Evans blue dye (2 ml/kg) 1 hour prior to sacrifice. Artificial respiration was performed through a tracheostomy. After thoracolaparotomy, the aorta was tied off between the aortic arch and the diaphragm. A No. 18 cannula was then inserted into the left ventricle, and the right auricle was cut. At the same time, blood was removed by perfusion with 0.9% saline solution at 37°C. After the cannula had been advanced up to the origin of the aorta and fixed with a silk suture, reperfusion was carried out, first with a fixative solution of 10% for-
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Malin in phosphate buffer, 1 ml/gm body weight (pH 7.3) at 37°C, and then with a cold fixative solution of identical composition in order to cool the rats to 4°C. The perfusion pressure was within the range of 100 to 120 mm Hg. Meanwhile, 20 ml of a base resin* at 4°C, mixed with a polymerizer in a ratio of 40:1, was injected manually via the cannula into the origin of the aorta at a pressure of 100 to 120 mm Hg, which was checked with a pressure transducer (flow rate approximately 4 ml/min).

Two hours after injection of the resin, the brain was removed carefully from the cranial cavity and slices 5 to 10 mm thick were cut in the center of the Evans blue-stained area. Slices for study were immersed in 20% NaOH solution for about 12 hours and then transferred to 0.5% NaClO solution for about 14 days. Once the nervous tissue had completely dissolved, the vascular casts remaining were washed thoroughly in distilled water, frozen in liquid nitrogen, and then dried by means of a critical-point drying system to avoid collapse. The dried specimens were coated with gold in a vacuum evaporator and observed with a JSM-T300 scanning electron microscope (SEM) using an accelerating voltage of 15 kV.

**Immunohistochemical Method**

At 1, 2, 3, 4, 5, and 7 days after cold injury, bromodeoxyuridine (BUDr), 10 mg/kg, was administered intravenously. One hour after BUDr administration, the rats were killed by *in vivo* perfusion fixation. Perfusion was carried out with a 0.9% saline solution, followed by 70% ethanol. The brains were removed, immersed in 70% ethanol, and embedded in paraffin.

Serial coronal sections, 3 µm thick, were obtained from each brain at the level of the cold-injured area. Deparaffinized sections were incubated for 15 minutes in methanol with 0.3% H2O2 to avoid nonspecific reaction of peroxidase in the tissue. The sections were then incubated for 20 minutes in 4 N HCl in order to denature the DNA (deoxyribonucleic acid). Sections were rinsed five times with phosphate-buffered saline (PBS). A 1:10 dilution of purified anti-BUDr monoclonal antibody† containing 1% bovine serum albumin was used to cover the tissue sections for 1 hour at room temperature in a 100%-humidified atmosphere. Biotinylated anti-mouse immunoglobulin G (IgG) and peroxidase-labeled Streptavidin‡ were then applied to the sections for 20 minutes each, respectively. Finally, the slides were rinsed three times with PBS and reacted for 10 minutes with 20 µg of dianaminoazobenzene tetrahydrochloride and 100 µl of 0.05 M Tris buffer containing 0.005% H2O2. Other mirror sections were subjected to immunohistochemical staining with anti-Factor VIII-related antigen polyclonal antibody§ using the peroxidase-antiperoxidase (PAP kit system) method. The slides were incubated for 2 minutes at 37°C in 0.05% actinase E for the purpose of enzymatic digestion before 1 hour's incubation of the primary antibody. Control sections for each antibody were incubated with normal serum obtained from animals of corresponding species after adjustment of the IgG concentration.

**Results**

The extent of each edematous lesion was estimated by the intensity and extent of the Evans blue-stained area in the coronal brain slice. The lesion was maximal in extent at 1 day, and showed a gradual decrease, disappearing entirely at 7 days.

**Vascular Cast Study**

At 1 and 2 days after placement of the cold injury, the vascular network was very sparse within the lesion, and no capillary network could be seen. The residual vascular casts had irregular walls. Outside the Evans blue-stained area, the blood vessels had identical diameters (6 to 8 µm), ran smoothly, and showed three-dimensionally constant distances between them.

At 3 days after cold injury, reconstruction of the normal microvascular architecture had begun from the edge of the lesion toward the center (Fig. 1). The vascular network was less sparse than at 2 days. Most of the vessels still had irregular walls, and a few localized protrusions or blind ends of the casts were observed within the lesion (Fig. 2). There were between 10 and 20 of these protrusions, which decreased by approxi-

* Mercox CL-2R base resin supplied by Dianippon Ink and Chemical Inc., Tokyo, Japan.
† Monoclonal antibodies supplied by Becton-Dickinson Monoclonal Center Inc., Mountain View, California.
‡ Streptavidin, supplied in a Stravigen B-SA kit, manufactured by Biogenex Laboratories, Dublin, California.
§ Polyclonal antibodies supplied by DAKO Corp., Santa Barbara, California.
FIG. 2. Scanning electron micrographs of a vascular cast on the 3rd day showing sprouting branches (arrows) arising from a small venule (left) and a capillary (right) at the brain surface. Bar = 10 μm.

FIG. 3. Scanning electron micrograph of a vascular cast on the 5th day showing dilated and tortuous vessels at the brain surface with many nodular protrusions and a septum formation (arrows). Bar = 100 μm.

FIG. 4. Scanning electron micrograph of a vascular cast on the 5th day. Many capillaries at the edge of the lesion show kinking (arrows) and irregular walls. Bar = 10 μm.

At 4 and 5 days after cold injury, some elongated and dilated casts were seen within the lesion. No regular nuclear notches were seen on their surfaces. In most specimens, some nodular protrusions, septal formation, and irregular surfaces were revealed (Fig. 3), and the vascular network was generally sparse. At the edge of the lesion, the abnormal microvascular casts were continuous with the developing normal microvascular architecture, which took shape gradually. Capillaries with irregular walls became reduced in number (Fig. 4).

At 7 days after cold injury, the entire microvascular architecture of the lesion had regenerated, and no areas of poor vascularization were observed. On a small part of the brain surface, there were some elongated and dilated casts similar to those seen at 5 days. Sinusoidal dilated vessels were also seen (Fig. 5), but, apart from these, no vessels with irregular walls were present. Normal capillary architecture (Fig. 6) was observed in all specimens except in the severely injured part of the brain surface.

Immunohistochemical Study

Immunohistochemical sections prepared by means of BUdR demonstrated immunoreaction products exclusively in the cellular nuclei. Bromodeoxyuridine-positive nuclei were observed in reactive astrocytes, macrophages, or endothelial cells from 1 to 7 days after injury. In the present study, most of the BUdR-positive endothelial cells were seen within the area of the lesion. The earliest evidence of BUdR-positive endothelial cells was observed at 2 days after injury, the largest number was recorded at about 3 days, and at 7 days none could be detected (Fig. 7 and Table 1).

Discussion

There has been no previous description of detailed sequential changes occurring in microvascular archite-
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FIG. 5. Scanning electron micrograph of a vascular cast on the 7th day showing blood vessels with irregular walls at the brain surface. Sinusoidal dilated vessels are also present. Bar = 10 μm.

FIG. 6. Scanning electron micrograph of a vascular cast on the 7th day. The vascular architecture shows smooth-running microvessels with a uniform diameter and three-dimensionally even distribution. Bar = 10 μm.

FIG. 7. Photomicrograph, with anti-bromodeoxyuridine (B UdR) preparation, of a specimen obtained 3 days after cold injury. Most B UdR-positive endothelial cells (arrow) and hematogenous cells (arrowhead) were seen within the lesion on the 3rd day. × 480.

TABLE 1

<table>
<thead>
<tr>
<th>Day Posttrauma</th>
<th>No. of Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>13.5 ± 2.4</td>
</tr>
<tr>
<td>4</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means for four animals. Significance of difference by Student’s t-test: Day 2 vs. Day 3: p < 0.05; Day 2 vs. Days 4 and 5, and Day 3 vs. Days 4 and 5: p < 0.01.

Process of reconstruction. The form of vascular repair observed was of two types. In one, the normal microvascular architecture was reconstructed from the edge of the lesion toward the brain surface, starting 3 days after injury. In the other type, an abnormal form of microvascular architecture, which was different from that of the normal adult rat brain, was seen in a small part of the brain surface. Nodular protrusions, elongated and dilated casts, and septum formation were

ture following local cold injury, although the vascular system of the normal and developing brain has been well studied. There is no record of when and in which area newly formed vessels appear or how normal vessels are reestablished. We therefore studied the microvascular system by SEM using the cerebrovascular casting method.

It was revealed that repair of the microvascular architecture was initiated on the 3rd day after cold injury, and that at 7 days the microvascular architecture at the lesion site had been entirely regenerated. It is considered that similar changes in the microvascular architecture would have continued after 7 days, and that the number of newly formed vessels would diminish. Clarification of this mechanism would have been very interesting, but in the present study we focused specifically on the
features regarded to be evidence of structural alteration. These architectural features strikingly resembled the microvascular structure of the developing fetal and newborn rat brain reported by Yoshida, et al. \(^1\) Previous ultrastructural studies of endothelial cells have indicated that the production of new blood vessels in the developing brain arises from terminal sprouting of the original vessel, with various proliferation patterns, such as septum formation in the lumina and development of small ring-like vessels. From this viewpoint, we consider that the microvessels seen in small areas of the brain surface were newly formed vessels.

Bromodeoxyuridine is one of the halopyrimidines and, like thymidine, is incorporated into cellular nuclei at the time of mitotic DNA synthesis. It is rapidly degraded by the liver (more than 90% of a single bolus of BUDr is debrominated within 20 minutes). Although the new immunohistochemical method using BUDr and anti-BUDr monoclonal antibodies has recently been employed for the study of tumor cell kinetics, there has been no report of any study using this method for elucidating the mechanism of tissue repair. In the present immunohistochemical study with BUDr, regeneration in most of the endothelial cells was initiated within the lesion at 3 days, and these cells had disappeared by 7 days. We also noted newly formed vessels within the lesion.

We believe that the difference observed between the two types of repair is dependent on the severity of the injury. In a slightly injured area, the basement membrane is preserved and reconstruction of the microvascular architecture may occur along this membrane. In a more severely injured area, part of the basement membrane may be disrupted and discontinuous, so that the microvascular architecture must be formed de novo. Hauw, et al., have reported that in the early stage of vessel formation some immature vessels exist within the discontinuous basement membrane.

The reconstructed vessels had irregular walls soon after injury which disappeared gradually. We consider that this gradual disappearance of the wall irregularities is due to a decrease of intraluminal thrombi and thinning of the regenerated endothelium. Ikuta, et al., have reported that, at 3 or 4 days after cold injury, hematogenous cells and reactive astrocytes in an edematous lesion participate in its repair, and that morphological alterations seen in brain edema, such as a massively expanded extracellular space and freely floating cells within the fluid, are also observed in the developing normal fetal brain. Interestingly, these findings match our present results: that is, repair of the microvascular architecture was started on Day 3, and the newly formed vascular architecture resembled that of the developing fetal brain.

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Reference


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