Evolution of tissue damage in compressive spinal cord injury in rats

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The evolution of tissue damage in compressive spinal cord injuries in rats was studied using an immuno-histochemical technique and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The rupture of small vessels accompanied by intense tissue permeation of serum components in and around the hemorrhagic foci appeared to be immediate consequences of the mechanical insult. The loss of cell membrane integrity in neural elements became evident within 1 hour after injury as shown by the diffuse albumin-immunoreactivity of the cytoplasm. At the site of mechanical insult, approximately 30% of the neurofilament proteins were degraded within 1 hour, and 70% of them were lost within 4 hours after injury. A large number of cells positive for glial fibrillary acidic protein were found to demarcate the injured tissue within 1 hour after injury. The progression of tissue damage largely subsided within 48 hours. One week after injury, severe degeneration of the ascending tracts in the posterior funiculus was shown clearly by axon staining and less convincingly by myelin staining. Secondary degeneration of the corticospinal tract in distal segments remained inconspicuous for up to 3 months.

KEY WORDS - experimental spinal cord injury • neurofilament protein • glial fibrillary acidic protein • lysozyme • rat

AN experimental model employing small rodents is obviously advantageous when the assessment of therapeutic intervention requires a large number of tests. As an experimental model for spinal cord injury, the weight-drop method developed by Allen has gained popularity and has been widely used with various modifications in a variety of animals. However, when applied to the rodent, reproducibility of the lesions induced by this technique has been questioned, and use of a compressive cord injury produced with an epidural balloon or spring clip has been suggested as an alternative.

Recently, we developed a compression injury model in rats, and reported on the use of this model for the quantitative assessment of drug effects in acute spinal cord injury. Epidural application of a vascular clip with a relatively weak closing force allowed us to control the severity of injury by alternating the duration of compression. The simplicity of the technique allowed for the use of a large number of experimental animals with relative ease, and the results were reasonably reproducible. The mode of mechanical insult and the anatomy in this animal model, however, are obviously different from those involved in spinal cord injuries in humans.

This communication describes the temporal sequence of tissue damage developing at the site and remote from the site of the mechanical insult in this model. The extent of blood-brain barrier (BBB) breakdown and tissue damage was determined by immuno-histochemical staining of autologous serum albumin. Antibodies against neurofilament proteins (NFP), glial fibrillary acidic protein (GFAP), and lysozymes were utilized to demonstrate the severity of tissue destruction and the extent of reparative reactions. In addition, the degradation of cytoskeletal proteins in the acute stage of injury was sequentially analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the structural proteins extracted from the site of the mechanical insult.

Materials and Methods

Surgical Procedure

Adult male Wistar rats, each weighing 200 to 240 gm, were used within the standards set by the United
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Histopathology and Immunohistochemistry

For histology and immunohistochemistry, the rats were randomly sacrificed at 10 or 30 minutes, 1, 2, 4, 8, 24, 48, or 72 hours, 1, 2, or 3 weeks, or 1, 2, or 3 months after injury. They were transcardially perfused with 4% paraformaldehyde in a phosphate buffer solution, and the entire spinal cord and lower brain stem, complete with the forebrain in some instances, were removed immediately after perfusion and immersed overnight in the same fixative. Longitudinal and transverse semiserial paraffin sections 5 μm thick were prepared. Deparaffinized sections were stained by the method of Hsu, et al., with an avidin-biotin peroxidase complex kit using antibodies against autologous albumin, NP, GFAP, and lysozyme (muramidase) as primary antibodies at dilutions of 1:1000 or 1:2000.* The sections adjacent to those used for immunohistochemistry were stained with hematoxylin and eosin (H & E) or by the methods of Bodian and Klüver-Barrera. Sudan III stain preparations were made from sections cut 20 μm thick on a cryostat.

SDS-PAGE of Structural Proteins

For chemical analysis of structural proteins, the injured and uninjured control rats were transcardially perfused with phosphate-buffered saline containing 2.5 mM ethylenediaminetetra-acetic acid (EDTA) and 2.5 mM ethyleneglycol tetra-acetic acid (EGTA) to cleanse the tissue of blood and to minimize protein degradation by endogenous proteases during the sample preparation. Rats were randomly sacrificed at 0.5, 1, 2, 4, 8, 16, or 72 hours after the injury, and the injured tissue from three rats, two segments from each rat, was pooled at each sampling and homogenized at 0°C in 4 ml of 20 mM sodium phosphate buffer containing 1% Triton X-100, 1 mM sucrose, 100 mM sodium chloride, 2.5 mM EDTA, and 2.5 mM EGTA. The Triton insoluble fraction was pelleted by centrifugation at 100,000 G for 1 hour. The pellets were dissolved in 1 ml of phosphate buffer containing 4% 2-mercaptoethanol (2-ME), 1% SDS, 2.5 mM EDTA, and 2.5 mM EGTA. Sixty microliters of phosphorylase b was added to each sample as an internal standard. The solution was subjected to heat treatment at 100°C for 5 minutes and insoluble materials were removed, after which 70% trichloroacetic acid (TCA) was added. Proteins insoluble by TCA were collected by centrifugation at 10,000 G for 5 minutes, washed twice with ethanol, and dried. The samples were then dissolved in 1 ml of phosphate buffer containing 1% SDS, 4% 2-ME, and 20% glycerol, and prepared for electrophoresis using 7.5% polyacrylamide gels by the method of Laemmli. The gels were stained with Coomassie brilliant blue R-250 and scanned with a densitometer.†

Results

Evolution of Tissue Damage at Injury Site

In the specimens obtained 10 and 30 minutes after injury, massive hemorrhage in the gray matter and petechial hemorrhage radiating into the surrounding white matter constituted prominent histopathological features (Fig. 1A). In most instances, the subarachnoid space was free of blood. The majority of small vessels in the lesion were emptied after transcardial perfusion of a fixative. Extravasation of serum components, mostly from capillaries, and fibrin deposits in the tissue were often encountered at the margin of the hemorrhagic lesions (Fig. 1B). Except for a few pyknotic neurons, neural cells appeared to be well preserved, and normal-appearing neurons were often found in fields of extravasated blood. The white matter in histological sections also appeared normal. When stained with antibodies against autologous albumin, however, the extent of tissue damage was much wider than that disclosed by H & E staining (Fig. 1C). Albumin-immunoreactivity had already developed not only in the hemorrhagic foci but also in the adjacent gray and white matter. Despite the extensive permeation of the serum into neural tissue, most of the cellular components remained free of albumin-reactivity at this stage of injury (Fig. 1D).

In the specimens obtained at 1 hour after injury,
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**FIG. 1.** A: Photomicrograph of a hemorrhagic necrotic lesion that developed less than 10 minutes after a 30-second compression with a vascular clip. H & E, × 25. B: A high-power view of the area indicated by the *arrow* in A. A fibrin deposit (*arrow*) is seen at the edge of a ruptured capillary which has been emptied after perfusion with a fixative solution. H & E, × 250. C: Photomicrograph showing extensive permeation of serum albumin in and around the hemorrhagic tissue, revealed by the avidin-biotin complex (ABC) staining method with anti-rat-albumin serum in a section adjacent to that in A. × 25. D: A high-power view of the area indicated by the *arrow* in C. The majority of vessels are emptied after transcardial perfusion. Note the lack of albumin-immunoreactivity in neurons (*arrows*). v = vein. ABC, × 125.

Albumin-immunoreactivity in the adjacent tissue became intense, and many neurons and glial cells in the vicinity of the hemorrhagic foci now showed strong albumin-immunoreactivity indicating the impairment of cell membrane integrity (Fig. 2). At 4 hours after compression injury, edematous changes had become evident in both the gray and the white matter adjacent to the hemorrhagic foci, and petechial hemorrhage (possibly secondary in nature) was often seen, mostly along the central canal and at the junction of the white and gray matter in longitudinal sections. The distribution of edematous lesions correlated well with that of albumin-immunoreactivity. At the margin of edematous lesions, however, neurons were spared and albumin-immunoreactivity was confined to the perivenous spaces or to the neuropil.

At 8 hours after injury, the spread of the hemorrhagic and edematous lesions appeared to be completed on transverse sections. The maximum distribution of albumin-immunoreactivity on longitudinal sections was not seen until 48 hours after injury, when it extended rostrally and caudally for almost two segments from the site of direct impact. At the margin of the lesions, albumin-immunoreactivity tended to converge toward the posterior column. Strong albumin-immunoreactivity remained in the injured tissue for at least 1 week, and then gradually disappeared.

Alteration of NFP-immunoreactivity in the injured tissue appeared to be biphasic. The initial change occurred instantaneously after mechanical impact and the other was apparently associated with the development of tissue edema. As early as 10 minutes after injury, immunoreactivity of neural elements against anti-NFP sera was markedly reduced in the tissue to which pressure had been directly applied (Fig. 3A). The area of poor immunoreactivity was sharply demarcated from the adjacent tissue and did not correspond to the extent of the hemorrhagic lesion or to the extent of BBB breakdown. In the later stage of the injury, the reduced NFP-immunoreactivity was associated with the devel-
opment of edematous changes in the tissue (Fig. 3B). Another early change disclosed by NFP staining was ballooning of neurites (possibly axon terminals) in the gray matter, often adjacent to the edematous lesion, 1 hour after injury (Fig. 3C, arrows). Two hours after injury, granular disintegration of axons in the white matter and dusty staining of neurites in the edematous gray matter became conspicuous; end-bulb formation in the adjacent white matter became apparent by 4 to 8 hours after injury. No differences were detected in the distribution and intensity of the NFP pathology when the results of staining with anti-NFP antibodies against the NFP triplet (68k, 150k, and 200k) were compared.

Lysozyme-immunoreactive cells were first encountered 8 hours after injury and were sparsely dispersed in the vicinity of the hemorrhagic foci. Numerous lysozyme-positive cells tending to form clusters were seen in the edematous tissue at 48 hours after injury. One week after injury, the majority of cells in the necrotic foci were lysozyme-positive.

In the uninjured control tissues, GFAP-positive cells were localized solely in the subpial region and root entry zones. Within 1 hour after injury, a group of cells surrounding the hemorrhagic foci became strongly GFAP-positive in both the gray and the white matter (Fig. 4A). The number of GFAP-positive cells steadily increased in the surrounding tissue. Their GFAP-immunoreactivity was rather weak in the early stage, but increased over the next 24 hours and, between 48 to 72 hours after injury, the surrounding tissue was

Fig. 2. Photomicrograph showing strongly albumin-immunoreactive neuronal cells in the vicinity of a hemorrhagic lesion 1 hour after injury. Avidin-biotin complex method, × 55.

Fig. 3. Photomicrographs of the pathology revealed by the avidin-biotin complex method with anti-neurofilament protein (NFP) sera. A: Specimen obtained 10 minutes after injury showing immediate loss of NFP-immunoreactivity from the tissue to which pressure was directly applied. Anti-200k stain, × 25. B: Specimen obtained 8 hours after compression. Secondary loss of NFP-immunoreactivity is apparently associated with tissue edema and it is less sharply demarcated than the lesion in A. Anti-200k stain, × 25. C: Swelling of axon terminals in the adjacent gray matter is seen in this specimen obtained 1 hour after injury. Strongly immunoreactive materials (arrows) are scattered within the neuropil. Anti-150k stain, × 125.
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thoroughly paved with strongly GFAP-positive cells (Fig. 4B).

Between Days 3 and 7, hemorrhagic and necrotic tissue insidiously began to liquefy, and microcystic lesions developed. Numerous foamy cells, mostly lysozyme-immunoreactive, were seen either perivascularly or within the cysts. Proliferation of fibrous connective tissue became conspicuous at the margin of cystic lesions by Day 7. Microcysts tended to fuse with each other, and a large cyst, demarcated by gliomesenchymal tissue, formed in the center of the injured tissue between 1 and 3 weeks after injury.

Pathology of Lesions Remote from Injury Site

In the rostral cord segments, no pathological changes were detected by routine histological techniques on Day 3. By Day 7, however, severe degeneration of the white matter had become evident. Discrete wedge-shaped lesions were readily identified in the most dorsal portion of the posterior funiculus in the cervicothoracic segments by Bodian staining (Fig. 5a). The myelin destruction, however, was much less conspicuous than that of axons (Fig. 5b). At this stage and thereafter, no axons were demonstrable in the ascending tracts in the posterior funiculus but the tracts were filled with Luxol fast blue-positive material. Lipid droplets were only sparsely seen on Sudan III staining. In the lateral funiculi, a marginal zone became symmetrically pale but the change was indistinct. Degenerating tissue was slowly cleared by macrophages. Two months after injury, the lesions in the posterior funiculus had been thoroughly replaced by glial scars and had shrunk, and

![Fig. 4. Photomicrographs showing astroglial reaction at the site of injury. Avidin-biotin complex with anti-GFAP (glial fibrillary acidic protein) serum. c = central canal; H = hemorrhagic lesion. A: The lesion is demarcated with GFAP-positive cells within 1 hour after injury. × 25. B: At 48 hours after injury, the surrounding tissue is paved with GFAP-positive cells. × 25.](image1)

![Fig. 5. Photomicrographs of the posterior funiculus in the distal segments 1 week after injury. A distinct wedge-shaped lesion is revealed by the Bodian stain (a) in the most dorsal portion of the cervical cord, but it is less conspicuous with Klüver-Barrera staining (b). Only loosening of nerve bundles and slight hypercellularity are suspected in the lumbar cord, as shown by a Bodian stain (c) and Klüver-Barrera stain (d). a and b: × 50; c and d: × 100.](image2)
the lesions in the lateral funiculi had become inconspicuous. Pathological changes in the caudal segments were much less remarkable than those in the rostral segments throughout the observation period up to 3 months after injury. Even 1 week after injury, white matter degeneration was hardly discernible in the anterolateral and posterior funiculi on Bodian or Klüver-Barrera staining, and only loosening of nerve bundles and increased cellularity in the most ventral portion of the posterior funiculus were suspected (Fig. 5c and d). Although Bodian and Klüver-Barrera stains revealed no apparent pathology in the gray matter, GFAP staining disclosed diffuse isomorphic gliosis in the posterior and anterior horns in the lumbar and sacral segments (Fig. 6). Glial reaction in the gray matter of the caudal segments was most conspicuous 1 week after injury and was not detectable 1 month later.

SDS-PAGE of Structural Proteins

Degradation of NFP at the site of impact was clearly revealed by SDS-PAGE analysis. In uninjured tissue, the NFP triplet (200k, 150k, 68k) and GFAP (51k) were identified as discrete major bands in the SDS-PAGE analysis of the Triton-insoluble fraction (Fig. 7). No band corresponding to the NFP triplet was seen in the SDS-PAGE of the Triton-soluble fraction (not shown), although obvious reduction in the total amount of the Triton-insoluble fraction from injured tissues suggested that a substantial amount of structural proteins might have become Triton-soluble. The density of NFP bands rapidly decreased after injury, and in most instances it concurred with an increase in the amount of lower-molecular-weight materials below 55k. Semi-quantitation of SDS-PAGE profiles with a gel scanner is shown in Fig. 8. Approximately 40% of 200k and 150k proteins was degraded within 30 minutes after injury while the 68k component was largely preserved. At 4 hours after injury, however, almost 70% of all NFP triplets was degraded. Afterward, degradation of the 68k component was apparently retarded and 10% to 20% of 68k was still retained 72 hours after injury. On the other hand, the 200k and 150k components of NFP continued to degrade rapidly and only traces of them were found 16 hours after injury. Glial fibrillary acidic protein was also degraded but at a much slower rate and less extensively than NFP, and approximately 40% of the control levels was still recovered 72 hours after injury.

Discussion

As noted previously in both human and experimental compressive spinal cord injuries, a primary event in this experimental cord injury appeared to be vascular damage preferentially involving the central gray matter. While fibrinoid necrosis of vessel walls has been suggested as an immediate consequence of impact, initial hemorrhage in the present experiments seemed more likely to be due to tears in thin-walled vessels. Immunohistochemical studies of autologous albumin clearly demonstrated the evolution of tissue damage, including the distribution of edematous lesions and

![Fig. 6. Photomicrograph showing diffuse gliosis in the otherwise normal gray matter of the lumbar cord 1 week after injury. D = posterior funiculus. GFAP stain, × 30.](image)

![Fig. 7. Sequential 7.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) profiles of the cytoskeletal proteins extracted from the injured tissue at various times after injury. Note the rapid degradation of neurofilament protein (NFP) with relative sparing of glial fibrillary acidic protein (GFAP).](image)
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the extent of cellular damage. The compression injury employed in the present study was severe enough to render the majority of animals paraplegic and to induce hemorrhagic necrotic lesions preferentially involving the central gray matter, leading to cavitation of several cord segments. Autologous albumin staining clearly showed immediate and extensive tissue permeation of serum components with or without extravasation of blood cells. The rather diffuse albumin-immunoreactivity of the tissue in the absence of blood cells might be attributable to the breakdown of the BBB and subsequent extravasation of serum components. Albumin-immunoreactivity was already evident 10 minutes after injury in the gray and white matter adjacent to the hemorrhagic foci (Fig. 1C), and apparently preceded or occurred simultaneously with the development of tissue edema.

The albumin-immunoreactive area steadily expanded in the next 48 hours in both rostral and caudal directions. The progression of albumin-immunoreactivity, however, was largely restricted to the gray matter. Consequently, 48 hours after injury the albumin-immunoreactivity in the gray matter spanned almost three entire cord segments, whereas that in the white matter was usually confined to a single segment. Thus, the present study involving albumin-immunoreactivity also supports the previous notion that preferential vulnerability of the gray matter and relative durability of the white matter are characteristics of compressive spinal cord injury.\textsuperscript{2,8,17} As noted previously,\textsuperscript{8} the temporal sequence of the events in this model indicated that the first 48 hours is the window for treatment of acute spinal cord injury, since by then the progression of tissue damage has largely subsided.

Diffuse albumin staining of the cytoplasm has been considered to represent accumulation of extracellular components as a consequence of the impaired integrity of cell membranes.\textsuperscript{6} We had frequently observed the phenomenon in ischemic lesions of the central nervous system (CNS), and it appears to be analogous to the dye exclusion test commonly used to assess the viability of tissue culture cells. In the present experiment, diffuse cytoplasmic albumin-immunoreactivity was first seen 1 hour after injury (Figs. 1D and 2). Ten minutes after injury, the majority of cells in hemorrhagic foci and their vicinity were albumin-negative and stood out in the albumin-positive background of the neuropil. One hour after injury, strong albumin-immunoreactivity developed in both neuronal perikarya and glial cells in and around the hemorrhagic foci and also in some distant neurites in the neuropil. Therefore, cellular damage in this model seemed to be largely secondary to vascular damage rather than the direct consequence of mechanical insult, although neural functions might have been impaired instantaneously.\textsuperscript{19}

Astrocytic reactions as revealed by GFAP staining

\begin{figure}
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\includegraphics[width=\textwidth]{figure8}
\caption{Results of gel scanning of two time-course experiments on cytoskeletal protein degradation at the site of injury. Each experiment is indicated by closed and open symbols: stars = GFAP; circles = 68k; triangles = 150k; and squares = 200k.}
\end{figure}
deserve a few comments. In the present study, an increase in the number of GFAP-positive cells was clearly seen as early as 1 hour after injury at the margin of the injured tissue. Astrocytic reaction seems to be one of the earliest reparative processes in CNS tissue injury and has been reported to occur within 40 minutes after reperfusion in ischemic rat brains.

The development of reactive astrocytes in the gray matter of distant segments has also been observed after cord transection in rats. The pathognomonic significance of the astrocytic reaction in remote sites is not clear but it serves to repair the degeneration of axon terminals in the gray matter, since the distribution of GFAP-positive cells within the gray matter of lumbar-sacral segments correlates well with that of degenerating axon terminals as revealed by the Fink-Heimer method. Also, the appearance of GFAP-positive cells is concomitant with the development of degenerating axons.

The loss of NFP-immunoreactivity was apparently biphasic. Initial loss was confined to the tissue where mechanical compression had been directly applied (Fig. 3A). Reduced NFP-immunoreactivity at a later stage was associated with the development of tissue edema (Fig. 3B). Instantaneous loss of immunoreactivity to antibodies to all NFP triplets after mechanical compression was hardly correlated with the degradation of NFP's, since at least the 68k protein was well preserved even 30 minutes after injury. Alternatively, reduced immunoreactivity might reflect conformational changes in the NFP structure. The reduced immunoreactivity in the edematous lesions seemed to be associated with the degradation of NFP as supported by the results of both histological observations and SDS-PAGE analysis. The underlying mechanism of the altered NFP-immunoreactivity, however, requires further investigation since it has been shown that the degradation products of NFP retain certain immunoreactivity.

Although selective degradation of the 160k component of NFP by calcium-activated neutral proteases has been reported, we were unable to detect the differential loss of immunoreactivity to antibodies against the 68k, 150k, and 200k components.

Analysis by SDS-PAGE clearly showed the rapid degradation of structural proteins in the injured tissue. An appreciable amount of NFP triplets was already degraded 30 minutes after injury when tissue edema was inconspicuous. One hour after injury, NFP levels were reduced by approximately 30% of control levels. The values would be even higher in damaged tissue, since the sample (two cord segments) included apparent normal tissue. These observations largely agree with the report by Banik et al. The vulnerability of 200k and 150k proteins relative to that of 68k may be related to the tertiary structure of neurofilaments: the decoration of the surface of the 68k core protein with 200k and 150k proteins.

Although the tissue degradation in acute CNS injury may be attributable to the activation of lysosomal enzymes, it is clear that lysosomal hydrolase activity was detectable only in the later stage of spinal cord injury in dogs and suggested that lysosomal enzymes played a minor role in the early stage of the CNS tissue degradation. In support of their view, only a small number of lysozyme-positive cells were encountered within 24 hours after the injury in the present experiment.

Alternatively, as a mechanism of tissue degradation in acute CNS injuries, proteolysis by non-lysosomal calcium-activated neutral proteases (CANP) has recently been implicated. Rapid accumulation of Ca++ essential for the activation of CANP has been shown biochemically and histopathologically in the injured tissue, and remarkable elevation of neutral protease activity in the injured tissue has recently been reported.

Albumin-immunohistochemical studies in the present investigation demonstrated that permeation of serum components into normal-appearing tissue took place immediately after the mechanical insult, and the integrity of cell membranes was severely impaired in a large number of cellular elements less than 1 hour after injury. As a consequence, an increased influx of extracellular components, such as extracellular Ca++, can be safely assumed and subsequent activation of CANP might be expected shortly after injury. Furthermore, we have previously observed substantial suppression of axonal damage in acute spinal cord injury by intraperitoneal administration of exogenous protease inhibitors specific for CANP. Thus, although the evidence still remains circumstantial, and its role may be restricted to the initiation of tissue degradation, proteolysis by CANP seems to be an early and important event in acute spinal cord injury. Therefore, the suppression of cytoskeletal protein degradation with appropriate protease inhibitors may alleviate the neurological sequelae of acute CNS tissue injury.

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Manuscript received June 27, 1986.

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