Axonal Caliber and Neurofilaments are Proportionately Decreased in Galactose Neuropathy

Hitoshi Nukada, M.D.,* Peter J. Dyck, M.D., Phillip A. Low, M.D., Alfred C. LaIs, and Margaret F. Sparks

Abstract. Feeding galactose to rats induces nerve conduction abnormalities, increased levels of nerve galactitol, endoneurial edema, elevated pressure and hypoxia of endoneurial fluid, and pathological abnormalities of nerve fibers. To investigate the cellular mechanisms of the fiber lesions and their possible relationship to alterations in the nerve microenvironment, rat peroneal nerves were morphometrically evaluated eight months after the commencement of galactose feeding. Whereas the density of neurofilaments (NF/μm²) in the transverse axonal area of myelinated fibers was not significantly different between the nerves of galactose-fed and control rats, axonal areas and the number of NF/axon, when related to myelin spiral length, were significantly less in nerves of galactose-fed rats. Myelin alterations, characteristic of axonal atrophy, were also significantly increased. The present data provide evidence of a proportionate decrease in axonal caliber and the number of NF/axon in myelinated fibers in experimental galactose neuropathy, suggesting that galactose induces either decreased NF synthesis, assembly or transport. The possible role of microenvironmental alterations, including endoneurial hypoxia and hyperosmolarity, in the production of this axonal atrophy is discussed.

Key Words: Atrophy, axonal; Axonal caliber; Axons; Hypoxia, endoneurial; Myelin spiral length; Neurofilaments; Neuropathy, galactose.

INTRODUCTION

Experimental galactosemia, induced in the rat by excess galactose feeding, has been used as a model to study the clinical and pathological consequences of sugar alcohol accumulation. Galactose, an isomer of glucose, is metabolized into galactitol by the polyl pathway, reproducing many of the effects of experimental diabetes on the peripheral nerves, lens and retina (1, 2). The cataracts which form in rats with galactose feeding are associated with galactitol accumulation in the lens and an osmotic effect has been postulated (3).

Gabbay and Snider (4) were the first to demonstrate edematous nerve and impaired nerve conduction in experimental galactose neuropathy (EGN). In subsequent investigations, endoneurial edema was most prominent in the subperineurial region (5, 6). The edema is thought to be related to sugar alcohol accumulation (4, 7) rather than a breakdown of the blood nerve barrier (8, 9). The endoneurial edema results in a significant increase in fascicular area, elevated endoneurial fluid pressure (EFP) (5, 6, 10–13), and prominent subperineurial edema due to the increased compliance of this area (6, 12, 13). Low et al (14) recently demonstrated endoneurial hypoxia in EGN due to increased intercapillary distance secondary to endoneurial edema.

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The hypoxia is sufficient to lead to a reduction in the in vivo local oxygen consumption.

During the first few months of galactose feeding, no nerve fiber lesions, apart from a slight reduction in both the maximal and average diameter of myelinated fibers (MF), were observed (10). A higher percentage of axonal degeneration was demonstrated in teased fiber preparations (12). Powell and Myers (15) reported segmental demyelination and remyelination, minor onion-bulb formation, glycogen-filled Schwann cells and focal axonal swelling with accumulation of axonal organelles in rat sciatic nerves after two years of galactose feeding.

Because EGN and diabetic neuropathy may be caused by similar mechanisms, it is important to examine the pathogenesis of EGN. In both EGN and experimental diabetic neuropathy, the polyol pathway is active and the concentration of nerve-free myoinositol is reduced (2, 7, 16). Common alterations in nerve physiology and the microenvironment include: slowing of nerve conduction; resistance to ischemic block; endoneurial hypoxia/ischemia; and sugar alcohol accumulation, and associated endoneurial edema and increased EFP. The latter is even more pronounced in EGN than in experimental diabetic neuropathy (5, 6, 9, 11–13, 17–20).

To investigate the cellular mechanism of the nerve fiber lesions in EGN, we performed quantitative ultrastructural studies of MF including the measurements of transverse axonal area, myelin spiral length and the number of neurofilaments (NF) and microtubules, and analyzed teased fibers from rat peroneal nerves after eight months of galactose feeding. We found a proportionate decrease of axonal...

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TABLE 1
Densities of Neurofilaments and Microtubules from Peroneal Nerves of Galactose-fed and Control Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Densities (μm², mean ± SD)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galactose-fed (n = 5)</td>
<td>Control (n = 6)</td>
</tr>
<tr>
<td>Neurofilaments</td>
<td>126.5 ± 22.0</td>
<td>123.2 ± 10.0</td>
</tr>
<tr>
<td>Microtubules</td>
<td>14.7 ± 2.1</td>
<td>14.5 ± 1.5</td>
</tr>
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caliber and in the number of NF/axon relative to myelin spiral length (the best indicator of the previous size of the MF).

MATERIALS AND METHODS

Twelve adult Sprague-Dawley rats, each weighing approximately 250 g, were divided into two groups and housed in similar cages with plastic floors which were covered with sawdust. Six rats were placed on a diet containing 40% galactose for eight months. The other six were fed the same diet but without galactose.

Histological processing has been described previously (21). After perfusing the rats with 4% glutaraldehyde in 0.025 M cacodylate buffer at pH 7.39 for approximately 15 minutes, the nerves were removed and immersed in 2.5% glutaraldehyde in 0.025 M cacodylate buffer overnight and postfixed in 1% osmium tetroxide. After washing, the nerves were cut, dehydrated, infiltrated, and embedded in epoxy. Transverse 0.75 μm semi-thin sections were cut and stained with 1% phenylenediamine or 1% methylene blue. The following tissues were examined: L6 ventral root, L6 dorsal root, sciatic nerve at the upper thigh level, peroneal nerve at midcalf level, proximal and distal sural nerves and the first branch of the tibial nerve to muscle.

Morphometric measurements of myelinated fibers (MF) were performed on electron micrographs of thin transverse sections of peroneal nerves stained with uranyl acetate and lead citrate using previously described methods (22, 23). The peroneal nerve was chosen for morphometric evaluation since endoneurial edema is marked in this nerve. Myelin spiral length and axonal area were calculated using programmed digitization and the number of neurofilaments (NF) and microtubules counted in at least 30% of the axonal area, including peripheral (subaxolemmal) and central regions, and expressed as density (μm²) and as total number/axon. Myelinated alterations in electron micrographs were graded by criteria described previously (24). In teased fiber studies, a minimum of 100 single fibers were prepared and graded (21). Distributed data were compared using the two-tailed Student’s t-test.

RESULTS

Galactose-fed rats developed dense cataracts, but otherwise appeared to be healthy and exhibited no evidence of neuromuscular dysfunction. In galactose-intoxicated nerves, however, evidence of endoneurial edema, especially in the subperineurial region, was seen in the sciatic nerve and its branches (Fig. 1). Intramyelinic edema was found in only a few ventral and dorsal root fibers. The results of quantitative light microscopic studies of peroneal nerves from this group of galactose-fed rats have been reported briefly and showed smaller median diameter of MF without loss of fibers (25).

The mean of median densities of NF/μm² of axonal area was not significantly different between galactose and control nerves (Table 1). The regression lines of the number of NF/axon on axonal area were also not significantly different between galactose and control nerves.

Regression analysis of axonal area on myelin spiral length showed that the trans-
verse axonal area was significantly less, relative to myelin spiral length, in EGN than in control nerves (Fig. 2). When the number of NF/axon was regressed on myelin spiral length, the common regression line was significantly lower for intermediate and large MF from galactose nerves (Fig. 3).

The mean of median densities of microtubules/μm² of axonal area was not significantly different between galactose and control nerves (Table 1). The regression lines of the number of microtubules/axon to axonal area and to myelin spiral length were not significantly different between experimental and control nerves.

Pathological alterations of myelin, of the types characteristic of axonal atrophy (infolded loops, clefts, focal separation and reduplication), were significantly more frequent in galactose than in control nerves (Table 2, Fig. 4). Glycogen accumulation, which was not found in the cytoplasm of Schwann cells, was significantly more common in galactose than in control MF axons (mean ± SD, 4.0% ± 1.2 and 1.4% ± 1.7, respectively, 0.02 < p < 0.025) (Fig. 5). Neither axonal swelling nor an accumulation of axonal organelles was seen. Although not measured, there was no obvious thickening of the capillary basement membrane.

Teased fiber studies of galactose nerves showed a significant increase in the number of fibers with excessive myelin irregularity (condition B, Table 3). The frequency of demyelination or remyelination and axonal degeneration did not reach statistical
Fig. 3. Regression lines relating the number of neurofilaments/axon to myelin spiral length for control (open circles) and galactose-fed (closed circles) rat peroneal nerves, as obtained from electron micrographs (described in text).

significance. Typical pathological abnormalities observed in EGN are shown in Figure 6.

DISCUSSION

Demyelination, secondary to axonal atrophy, has been described in uremic neuropathy (26) and Friedrich's ataxia (27). The sequential cellular changes of secondary demyelination in chronic neuronal injury were confirmed using the permanent axotomy model (28). In each case, axonal atrophy appears to be an early morphological alteration, although different cellular mechanisms may be involved (26–30). The atrophy leads sequentially to secondary segmental demyelination, remyelination and axonal degeneration. Excessive myelin wrinkling also precedes demyelination. This appears to be the consequence of a relatively stable myelin sheath which buckles, splits and forms in-pouchings in response to a reduced axonal caliber.

The present data provide evidence of axonal atrophy of MF in EGN. Axonal area and the number of NF/axon are significantly less relative to myelin spiral length in galactose compared to control nerves. The density of NF in galactose nerves is normal because the decrease in their number is proportionate to the decrease in axonal caliber. Myelin wrinkling was more frequently seen in galactose than in control nerves, at statistically significant levels and using quantitative ultrastructural and

Fig. 4. Electron micrographs illustrate myelin alterations frequently seen with axonal atrophy of myelinated fibers of rat peroneal nerves after eight months galactose feeding. A. Infolded loop of myelin. ×4,650. B. Myelin cleavage and infolding. ×5,200.

These findings are similar to those observed in the permanent axotomy model.

In the permanent axotomy model, we demonstrated that a reduction in axonal caliber correlated with a proportionate decrease in the number of NF (24). Similar alterations were found in the sural nerves of patients with hereditary motor and sensory neuropathy, type I (HMSN-I) (22, 23). We suggested that this reduction in the number of NF may be the result of decreased NF synthesis, assembly or transport and that the decrease in NF proteins may cause the distal axonal atrophy. It has
been proposed that NF content may play an important role in the determination of axonal caliber (31, 32). The NF proteins are synthesized exclusively in the cell body and delivered to the axon by slow axonal transport, component a (SCa) (33). Distal axonal atrophy, which is a direct result of proximal blockade of NF proteins, has been shown in experimental β,β-iminodipropionitrile (IDPN) neuropathy (34, 35). Hoffman and colleagues (36) have demonstrated that a reduction in axonal caliber correlated with a proportional decrease in the number of NF in the proximal stump of transected rat sciatic nerves. These findings suggest that NF content, controlled by SCa, is a major primary determinant of axonal caliber.

**TABLE 2**
Frequency of Myelinated Fiber Alterations in Transverse Electron Micrographs of Peroneal Nerves from Galactose-fed and Control Rats

<table>
<thead>
<tr>
<th>Grade*</th>
<th>Frequency (%; mean ± SD)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galactose-fed (n = 5)</td>
<td>Control (n = 6)</td>
</tr>
<tr>
<td>MI</td>
<td>7.9 ± 2.6</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>MO</td>
<td>2.5 ± 0.8</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>MCC</td>
<td>11.9 ± 3.4</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>MCP</td>
<td>7.6 ± 1.1</td>
<td>4.6 ± 1.5</td>
</tr>
<tr>
<td>FMS</td>
<td>3.2 ± 0.9</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>MR</td>
<td>6.0 ± 2.9</td>
<td>2.3 ± 0.8</td>
</tr>
</tbody>
</table>

* Descriptive grading of myelin alterations; see Dyck et al (24).

MI = myelin infolding; MO = myelin outfolding; MCC = myelin clefts, complete; MCP = myelin clefts, partial; FMS = focal myelin separation; MR = myelin reduplication.
Fig. 6. The upper panel consists of four consecutive lengths of a teased peroneal nerve fiber without abnormality. The lower panel consists of four consecutive lengths of a teased fiber showing myelin wrinkling typically seen in axonal atrophy and characteristic of experimental galactose neuropathy, as described in the text.

Possible mechanisms leading to axonal atrophy in EGN are 1) endoneurial edema and elevated EFP, 2) endoneurial hyperosmolarity, and 3) endoneurial hypoxia. It is well known that marked endoneurial edema and elevated EFP are concomitant in EGN and may be present for months before the development of definite nerve fiber lesions (5, 6, 11–13). Endoneurial edema results from an accumulation of galactitol, so that endoneurial fluid osmotic pressure is likely to be elevated (13). Axonal atrophy has been demonstrated with the use of hyperosmolar fixative (37) and in acute hyperosmolar hyperglycemia (38). In EGN, EFP falls with continued intoxication and at a time when morphological changes are increasing (6, 12, 13,

| TABLE 3 |
|-----------------|------------------|------------------|
| Frequency of Various Pathological Conditions in Single Teased Fibers from Peroneal Nerves of Galactose-fed and Control Rats |

<table>
<thead>
<tr>
<th>Grade*</th>
<th>Frequency (%), mean ± SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galactose-fed (n = 5)</td>
<td>Control (n = 6)</td>
</tr>
<tr>
<td>A</td>
<td>64.8 ± 12.9</td>
<td>97.0 ± 4.9</td>
</tr>
<tr>
<td>B</td>
<td>29.1 ± 13.6</td>
<td>2.6 ± 1.4</td>
</tr>
<tr>
<td>C + D + F + G</td>
<td>3.4 ± 4.2</td>
<td>0.6 ± 0.9</td>
</tr>
<tr>
<td>E + H</td>
<td>0.7 ± 1.0</td>
<td>0.4 ± 0.9</td>
</tr>
</tbody>
</table>

* Descriptive grading of teased single fibers; see Dyck et al (21).
A = normal; B = myelin wrinkling; C + D + F + G = segmental de- and remyelination; E + H = axonal de- and regeneration.
15), so that elevated EFP seems to be unlikely to be the major mechanism responsible for axonal atrophy. However, since EFP was measured at a single point, transitory or focal elevated EFP could conceivably have contributed to axonal atrophy.

An alternative hypothesis, which we favor, is that axonal atrophy in EGN may be due to endoneurial hypoxia. Low et al (14) have recently demonstrated a significant reduction in endoneurial oxygen tension associated with reduced local oxygen consumption in galactose-intoxicated rat sciatic nerves. This was paralleled by a significant increase in intercapillary distance secondary to endoneurial edema. A mild reduction in nerve blood flow at four months and a significant decline at six months after starting galactose feeding have been reported (19, 20). Impaired axonal transport of NF proteins, a process which is oxygen dependent, may be a potential mechanism for axonal atrophy. Slow axonal transport, particularly of NF triplet proteins, has not yet been critically evaluated in EGN, although Sidenius and Jakobsen (39) reported that the slow axonal transport velocity of structural proteins was reduced. In experimental diabetic neuropathy, axonal atrophy (40–43) and endoneurial hypoxia related to a reduction in nerve blood flow and microangiopathy (19) have been demonstrated, although the role of endoneurial hypoxia in the production of axonal atrophy is uncertain. Results of slow axonal transport studies in experimental diabetes are conflicting (44), but an impaired retrograde axoplasmic transport which may cause a reduced signal to the cell body to synthesize structural proteins (45), a reduction in protein synthesis in the retinal ganglion cells which results in axonal atrophy (46), and a reduced amino acid uptake in the dorsal root ganglia (47) have been described.

In conclusion, we were able to demonstrate: 1) a proportionate decrease of axonal caliber and the number of NF/axon relative to myelin spiral length, and 2) excessive myelin wrinkling, indicative of axonal atrophy, in EGN. It is conceivable that factors in the microenvironment, such as endoneurial hypoxia, may be responsible for the axonal atrophy of MF in EGN.

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