Mutation-dependent alteration in cellular distribution of peripheral myelin protein 22 in nerve biopsies from Charcot–Marie–Tooth type 1A

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Summary
The hereditary demyelinating neuropathy Charcot–Marie–Tooth type 1A is caused by duplication or by point mutations of the PMP22 gene. Histopathological differences in these genotypes suggest distinct disease mechanisms. In the present investigation we demonstrate a pathologically altered cellular distribution of PMP22 in sural nerve biopsies of patients with PMP22 point mutations. In these patients, in contrast to findings in patients with PMP22 duplication, PMP22 partially accumulates in the Schwann cells instead of being inserted in the myelin sheath. These findings may explain the different histopathology and may suggest different mechanisms of pathogenesis in these genotypes.

Keywords: CMT1A; PMP22 point mutations; immunohistochemistry; PMP22 localization; genotype–phenotype correlation

Abbreviations: CMT1A = Charcot–Marie–Tooth type 1A; PMP22 = peripheral myelin protein 22

Introduction
The hereditary demyelinating neuropathy Charcot–Marie–Tooth type 1A (CMT1A) is a common inherited neurological disease caused by duplication of the gene for the peripheral myelin protein 22 (PMP22) or by point mutations in the PMP22 gene (Nelis et al., 1996).

CMT1A patients with point mutations show a wide phenotypic spectrum. In comparison with patients with PMP22 gene duplication they generally seem to be clinically more severely affected (Scherer and Chance, 1995) and show higher g-ratios, indicating severe demyelination or hypomyelination, which is in contrast to the hypermyelination found in patients carrying the gene duplication. Furthermore, cases with point mutations show a larger total transverse fascicular area and they develop onion bulbs at earlier disease stages and in larger numbers (Gabreëls-Festen et al., 1995). Even patients with homozygous duplication and a very severe clinical phenotype do not show the histopathological findings of patients with point mutations (Killian and Kloepper, 1979). Thus, although patients with gene duplication are clinically similar to the cases with point mutations they are histopathologically different. This indicates different pathogenetic mechanisms in different genotypes.

In CMT1A with PMP22 duplication, abnormal Schwann cell differentiation has been described (Hanemann et al., 1996, 1997; Hanemann and Muller, 1998). This altered cell differentiation can be explained by the overexpression of PMP22 (Hanemann et al., 1994; Yoshikawa et al., 1994), which has a dual function in myelination and cell growth/differentiation (Zoidl et al., 1995).

Clues to the possible cause of hypomyelination in patients with point mutations come from experiments with cultured cells transfected with PMP22 carrying either the Trembler or TremblerJ mutations. The in vitro data on the two mutations demonstrate cytoplasm accumulation of mutant PMP22 (Naef et al., 1997; D’Urso et al., 1998; Tobler et al., 1999). In normal nerves and in CMT1A with duplication, PMP22 is predominantly found in the compact portion of the myelin sheath (Haney et al., 1996).

Here we have analysed by confocal laser scanning microscopy the distribution of PMP22 in morphometrically different, well-characterized sural nerve biopsies from CMT1A patients carrying defined point mutations (Leu16Pro, Gly107Val, Leu105Arg) and compared the distribution of PMP22 in these cases with the localization of the wild-type protein in patients with the gene duplication.
Material and methods

Nerve specimens

Diagnostic biopsies of the sural nerve were taken from patients diagnosed with CMT1A using clinical, electrophysiological and pathological criteria according to the guidelines of the European consortium for the study of hereditary motor and sensory neuropathy (de Visser et al., 1993). All patients gave informed consent. We analysed two patients with the TremblerJ point mutation leading to Leu16Pro in the first transmembrane domain (Valentijn et al., 1992; Gabreëls-Festen et al., 1995), a patient with a mutation leading to a Gly107Val substitution (Marrosu et al., 1997) and a patient with a Leu105Arg substitution, both in the transmembrane domain III. In addition, sural nerve biopsies from CMT1A patients with a genetically proven PMP22 duplication (Gabreëls-Festen et al., 1995) were used for comparison. Normal control nerves were taken either from multiorgan donors after informed consent or from patients biopsied for diagnostic reasons and proved to have normal nerves during routine examination. Experiments have been approved by the local ethics committee (University Hospital Nijmegen).

Immunohistochemistry and morphometry

Nerves were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5–10 µm thick serial sections. Sections were deparaffinized and incubated with the following primary antibodies: mouse monoclonal anti-78kD glucose regulated protein (binding protein, BIP) (StressGen, Victoria, Canada; diluted 1:300), mouse monoclonal anti-neurofilament panaxonal marker (Biotrend/SMI, Köln, Germany; diluted 1:800), mouse monoclonal anti-P0 (undiluted) (Bollensen et al., 1990) and rabbit polyclonal antibody (diluted 1:200) against synthetic oligopeptides (amino acids 118–130) of PMP22 (D’Urso and Muller, 1997). Appropriate secondary antibodies labeled with Cy3-conjugated, fluorescein-labelled or biotinylated anti-rabbit rat absorbed IgG, followed by fluorescein coupled to avidin (Vector, Burlingame, Calif., USA) were used.

All sections were analysed by confocal laser scanning microscopy (MRC 1024, BioRad, Munich, Germany). Samples which were double stained were analysed simultaneously. In merged images colocalization of green (fluorescein) and red (Cy3) fluorochromes appear yellow.

For quantitative morphometry using light and electron microscopic examination, standard techniques were applied, as described previously (Gabreëls-Festen et al., 1992).

Results

PMP22 distribution was analysed in sural nerve biopsies of four different genotypes. The genotypes included two patients with the TremblerJ mutation (Leu16Pro) allowing comparison with in vitro and animal data on this mutation, and a patient with a Gly107Val substitution. From the latter patient both a sural nerve biopsy as well as an L5 root were available. In addition, a biopsy carrying a Leu105Arg mutation and patients with duplication of the PMP22 gene were analysed. Nerves from multiorgan donors showing normal myelin staining with antibodies to PMP22 were used as controls (data not shown).

Sural nerves carrying the Gly107Val mutation showed massive PMP22 immunoreactivity in onion bulbs (Fig. 1A–C). PMP22 onion bulb staining could also be demonstrated in proximal nerve segments, i.e. the L5 roots (data not shown). While many of the onion bulbs are denervated and lack a central axon, others show axonal sprouting in the outer layers (Fig. 1A). In the onion bulbs PMP22 immunoreactivity did not colocalize with either the marker for endoplasmatic reticulum, BIP, (Fig. 1B) or with P0 (Fig. 1C). Antibodies to P0 weakly stained the residual myelin sheath suggesting that the mutant PMP22 (Gly107Val) does not prevent targeting of P0 into the myelin sheath (Fig. 1C, arrow).

In a biopsy carrying the Leu16Pro (TremblerJ) mutation we found a residual thin myelin rim (Fig. 1E and F) stained weakly with PMP22 antibody (Fig. 1E, arrow) indicating that either both the mutant and wild-type protein or, more likely, only wild-type PMP22 is targeted into the myelin sheath. Interestingly, we detected significant staining of PMP22 in periaxonal Schwann cell cytoplasm (half-moon-like staining in Fig. 1E, marked by asterisk) indicating accumulation of PMP22 in the cytoplasm. In the cytoplasm, PMP22 immunoreactivity partially overlapped with the endoplasmic reticulum marker BIP resulting in yellow fluorescence (Fig. 1E). In addition, electron micrographs of the same biopsy revealed distensions of the endoplasmic reticulum and especially of the Golgi apparatus (Fig. 1F, arrowhead). Different antibodies to identify Golgi and lysosomal compartments did not give satisfactory staining. Onion bulbs, which were abundantly present in these biopsies (Table 1), did not show PMP22 staining. Using a P0 antibody we could not detect any P0 accumulation in the cytoplasm (data not shown). A second independent biopsy with the Leu16Pro (TremblerJ) mutation gave identical results (data not shown).

In contrast to patients who had the Leu16Pro and Gly107Val PMP22 mutations, no staining with PMP22 antibody was found in the biopsy carrying the Leu105Arg substitution (Fig. 1D). In the sural nerve of the latter patient a similar number of onion bulbs to those in the biopsies from the other two patients were detected. In patients with gene duplication we detected strong PMP22 immunoreactivity in the myelin sheath (Fig. 2). While cytoplasm accumulation of PMP22 was marked in Schwann cells carrying the TremblerJ mutation or in onion bulb Schwann cells carrying the Gly107Val substitution, we found no clear evidence for accumulation of PMP22 in patients with the PMP22 duplication at the different disease stages studied (Fig. 2, and numerous biopsies shown in Hanemann et al., 1994). It should be noted that the late disease stages of patients with
Localization of PMP22 mutations in nerve biopsies

Fig. 1 Immunohistochemistry and electron micrograph of sural nerve biopsies from patients with PMP22 point mutations. A–C show a biopsy from a patient with a Gly107Val substitution, D from a patient with a Leu105Arg substitution, and E and F from a patient with a Leu16Pro substitution, corresponding to patients in Table 1. Green indicates staining with a PMP22 antibody in all sections, red indicates staining with neurofilament antibody (A and D), an antibody to endoplasmic reticulum marker BIP (B and E) and a P0 antibody (arrow in C). Note, PMP22 onion bulb staining in A–C, colocalization of PMP22 and BIP in the Schwann cytoplasm (yellow fluorescence) and residual myelin staining in E (arrow), and distended Golgi cisternae in the electron micrograph (arrowhead). Bar indicates 100 μm (A–C), 10 μm (D, including inset), 5 μm (E), 500 nm (F).
Fig. 2 Examples of immunohistochemistry of sural nerve biopsies from patients with PMP22 duplication corresponding to the 4-year-old (A) and 26-year-old (B) patients from Table 1. Green indicates staining with a PMP22 antibody and red indicates staining with an antibody to the endoplasmatic reticulum marker BIP. Bar indicates 5 µm. (A) shows an example of a young patient with PMP22 duplication and very mild disease (>50% normal myelinated fibres and only 27% onion bulbs in morphometric analysis) to demonstrate that there is no cytoplasmic accumulation in the early stages of CMT1A with gene duplication.

Table 1 Morphometric analysis of sural nerves of patients with different PMP22 mutations

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Leu16Pro</th>
<th>Leu16Pro</th>
<th>Gly107Val</th>
<th>Leu105Arg</th>
<th>PMP22 duplication</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>CMT1A</td>
<td>CMT1A</td>
<td>CMT1A</td>
<td>CMT1A</td>
<td>CMT1A</td>
</tr>
<tr>
<td>% onion bulbs</td>
<td>90</td>
<td>97</td>
<td>100</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>Density of myelinated fibres</td>
<td>14.3</td>
<td>18.6</td>
<td>1.2</td>
<td>18.6</td>
<td>31</td>
</tr>
<tr>
<td>TTFA*</td>
<td>3.3 ± 0.05</td>
<td>3.9</td>
<td>2.66</td>
<td>2.4</td>
<td>1.73</td>
</tr>
<tr>
<td>Mean g-ratio†</td>
<td>0.88 ± 0.05</td>
<td>0.8 ± 0.08</td>
<td>0.81 ± 0.06</td>
<td>0.98 ± 0.05</td>
<td>0.52</td>
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*Mean of age-matched controls: 0.61 (2–5 years, n = 4), 0.72 (6–10 years, n = 5), 0.87 (11–30 years, n = 5); †mean of age-related controls: 0.73 (2–5 years, n = 2), 0.67 (6–10 years, n = 2), 0.67 (11–20 years, n = 3), 0.66 (21–50 years, n = 3). TTFA = total transverse fascicular area.

Discussion

In this paper we have demonstrated differential cellular localization of PMP22 in human sural nerve biopsies depending on the underlying genetic defect of CMT1A patients.

In human nerve biopsies of patients with mutations equivalent to TremblerJ (Leu16Pro) we demonstrated accumulation of PMP22 immunoreactivity in the cytoplasm and were able to show distended Golgi apparatus and partial colocalization of PMP22 with an antibody to the endoplasmatic reticulum. Thus, our in vivo data are consistent with those from in vitro studies showing accumulation of PMP22 carrying this mutation (D’Urso et al., 1998; Tobler et al., 1999). Our findings extend the in vitro data, because in the previous studies transfected cultures of heterologous cells or rat Schwann cells without axons were used and hence no myelin was formed. However, while in vitro studies using an antibody to tagged mutant protein allow one to differentially stain mutated and wild-type PMP22 proteins, a distinction between these two protein forms is not possible in human nerves.

One previous in vivo study of the TremblerJ animal model also shows accumulation of PMP22 in the cytoplasm (Notterbeck et al., 1997). However, these authors demonstrated increased myelin breakdown and colocalization with an antibody to the lysosomal compartment but not with an endoplasmatic reticulum marker. We tried different antibodies to the lysosomal compartment all of which gave unsatisfactory staining in the human biopsies. The difference between the data from the TremblerJ animal model (lysosomal localization of PMP22), the in vitro data and our present data (endoplasmatic reticulum/Golgi compartment localization of PMP22) may be, in part, explained by the fact that the data in the TremblerJ mice were derived from adult mice at an advanced disease stage, where pronounced myelin degradation has occurred (Notterbeck et al., 1997). We cannot exclude that, at more advanced disease stages, increased
myelin breakdown may give rise to lysosomal accumulation in human nerves as well.

From the present data it cannot be inferred how PMP22 carrying the TremblerJ mutation interferes with myelin formation or maintenance. The fact that PMP22 partially accumulates in the Schwann cells instead of being inserted into the myelin sheath could explain the hypomyelination found in the biopsies of patients with PMP22 point mutations. However, the in vivo cytoplasm accumulation of PMP22 demonstrated in this paper could represent either the mutated or wild type, or both forms of PMP22. In vitro data suggest that most of the wild-type protein is targeted to the membrane (D’Urso et al., 1998; Tobler et al., 1999) and is not restrained by the mutant protein. This is in accordance with the thin myelin staining in Fig. 1E and F. An alternative pathogenetic mechanism, a loss of function, is unlikely since this should give rise to a hereditary neuropathy with liability to pressure palsies like phenotype seen with deletion of the PMP22 gene.

In a biopsy carrying the Gly107Val mutation we found massive staining of onion bulbs distally in the sural nerve as well as proximally in the L5 root. We could not detect such a marked onion bulb staining in any of the other genotypes including numerous patients with the PMP22 duplication (Hanemann et al., 1994), the TremblerJ mutation and an additional PMP22 missense mutation (Leu105Arg) indicating that the Gly107Val substitution leads to clear accumulation of PMP22 in the onion bulbs. Applying immuno-electron microscopy, Haney and colleagues previously observed prominent PMP22 staining in the myelin sheath but very weak PMP22 labelling in the cytoplasm and in onion bulbs of patients with the PMP22 duplication as well as in the Schwann cell cytoplasm of normal nerves; there was no evidence of accumulation in the endoplasmic reticulum of patients with the PMP22 duplication (Haney et al., 1996). Using the paraffin material available for the patients analysed here, this weak staining seems to be below our detection limit. Nishimura and colleagues also found some onion bulb staining in CMT1 patients with duplicated and non-duplicated PMP22 genes (Nishimura et al., 1996). Unfortunately, the PMP22 antibody used by these authors also stained axons, so raising questions about the antibody specificity. However, the Gly107Val substitution clearly leads to a very marked accumulation of PMP22 in onion bulbs.

Since we have chosen histopathologically comparable phenotypes (see Table 1) of the various genotypes, differences in disease stages cannot explain the differential cellular distribution of PMP22 shown here. With respect to the Gly107Val mutation, we were even able to analyse distal and proximal nerves showing the same PMP22 localization. In the TremblerJ genotype we analysed two different patients showing the same PMP22 localization. Thus, it seems that each mutation causes a unique biological effect reflected in different PMP22 localization. Our observations obtained in human nerves are in agreement with classical examples of different phenotypes caused by distinct PMP22 missense mutations, such as in the Trembler and TremblerJ mice. Our data are also in line with data from diseases caused by distinct mutations in the myelin proteins P0 and proteolipid protein. Mutations in the latter proteins give rise to a heterogeneous group of demyelinating disorders (Warner et al., 1996) partly due to impaired protein trafficking (Gow et al., 1998). It is interesting to note that the Gly107Val mutation, which leads to accumulation in onion bulbs, is a conservative amino acid substitution as opposed to the TremblerJ (Leu16Pro) mutation in which a helix breaking amino acid is introduced into a hydrophobic transmembrane domain leading to accumulation in the Schwann cell cytoplasm. That we did not find PMP22 staining in the biopsy from the patient with the Leu105Arg substitution is in agreement with the fact that this patient had early disease onset and a very thin myelin sheath, as indicated by the high g-ratio. A possible explanation for this could be that the Leu105Arg mutation in the transmembrane domain III leads to a substitution of a non-polar by a polar amino acid.

However, the intriguing question for the future is the mechanisms by which certain point mutations cause PMP22 to accumulate in the cytoplasm while in others it does so in the onion bulb. The answer cannot be provided by studies of human biopsies alone, but needs further in vitro experiments to be carried out.

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