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Regulation of Schwann cell proliferation and apoptosis in PMP22-deficient mice and mouse models of Charcot–Marie–Tooth disease type 1A

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Summary

Charcot–Marie–Tooth disease type 1A (CMT1A) is caused by an increased dosage of the peripheral myelin protein 22 (*PMP22*) gene or by point mutations affecting the same gene. Based on *in vitro* data, PMP22 might be involved, besides in its proven role in the regulation of myelination and myelin maintenance, in the control of Schwann cell proliferation and programmed cell death. In this report, we have used mice lacking PMP22 and mouse models for CMT1A to analyse Schwann cell proliferation and apoptosis *in vivo* during postnatal sciatic nerve development. Our results show that there is no significant change in the number of Schwann cells at postnatal day 1 in the analysed PMP22 mutants compared with the corresponding wild-type animals. Furthermore, the rate of proliferation also was not changed at this early developmental time point. In contrast, cell density and proliferation rates were increased, albeit with different kinetics, in all PMP22 mutants later in development. The increase in proliferation is paralleled by a higher number of apoptotic Schwann cells found in the nerves. Thus, increased Schwann cell proliferation and apoptosis, but only in later development and in adults, are hallmarks of PMP22 mutant mice, regardless of whether increased or decreased *PMP22* gene dosage or point mutations affecting the *PMP22* gene are responsible for the resulting demyelinating, dysmyelinating or amyelinating phenotypes.

Keywords: Schwann cell; PMP22; proliferation; apoptosis; CMT

Abbrevations: BrdU = bromodeoxyuridine; CMT = Charcot–Marie–Tooth disease; HNPP = hereditary neuropathy with liability to pressure palsies; P = postnatal day; PMP22 = peripheral myelin protein 22; PCR = polymerase chain reaction; *Tr* = *Trembler*; TUNEL = d-UTP-digoxigenin nick end-labelling

Introduction

The inherited peripheral neuropathy syndrome Charcot–Marie–Tooth disease (CMT) is the most common genetic disorder in neurology, with an estimated prevalence of 1 : 2500 (Skre, 1974). CMT patients suffering from the most frequently observed subtype CMT1A show slowed nerve conduction velocities, reduced compound motor and sensory nerve action potentials, progressive distal weakness, sensory loss and decreased reflexes (Dyck *et al*., 1993; Krajewski *et al*., 2000). Pathological hallmarks are segmental demyelination of motor and sensory nerves with incomplete remyelination. Genetically, the autosomal-dominant CMT1A is due to an intrachromosomal duplication on the short arm of chromosome 17p12 including the disease-causing, dose-sensitive peripheral myelin protein 22 (PMP22) gene (Raeymaekers *et al*., 1991; Lupski and Garcia, 1992; Patel *et al*., 1992). Intriguingly, the reciprocal deletion of the same DNA fragment is associated with hereditary neuropathy with liability to pressure palsies (HNPP) (Chance *et al*., 1993), which is usually not progressive but rather characterized by temporary palsies after pressure trauma (Windebank *et al.*, 1993). Rare cases of more severe forms of CMT1A can also be due to missense mutations in *PMP22* (Roa *et al*., 1993; Naef and Suter, 1999). Furthermore, HNPP has been found in association with *PMP22* frameshift mutations (Nicholson *et al*., 1994; Young *et al*., 1997).

Several animal models for CMT1A are available. These include the spontaneous mouse mutants *Trembler* (*Tr*) and *Tr-J* which carry point mutations in the *PMP22* gene (Suter *et al*., 1992*a*, *b*). The same mutations have also been found in severely affected CMT1A patients (Valentijn *et al*., 1992; Ionasescu *et al*., 1997). Models for CMT1A due to increased *PMP22* gene dosage have been generated in mice (Huxley

et al., 1996; Magyar *et al*., 1996) and rats (Sereda *et al*., 1996; Niemann *et al*., 2000). Interestingly, the neurological and pathological features of mutant mice correlated well with the findings in human patients and led to a pronounced reappraisal of the axonal defects in CMT1A (Sancho *et al*., 1999; Krajewski *et al*., 2000). Mice lacking PMP22 develop a demyelinating peripheral neuropathy reminiscent of severe CMT1 (Adlkofer *et al*., 1995) while, as expected from human genetics, heterozygous PMP22 knock-out mice revealed a pathology comparable with HNPP (Adlkofer *et al.*, 1997*a*).

Recent findings emphasize the crucial interplay between axons and Schwann cells in the cellular and molecular basis of CMT1A (for a review, see Naef and Suter, 1998). This conceptual framework originally has been derived from normal development of peripheral nerves where neurones regulate proliferation, survival and differentiation of Schwann cells (reviewed by Adlkofer and Lai, 2000). Schwann cells, in turn, support the suvival of neurones during development (reviewed by Jessen and Mirsky, 1999). In addition, Schwann cells are key determinants of the structure of myelinated fibres, in particular the establishment and maintenance of the nodes of Ranvier (reviewed by Arroyo and Scherer, 2000). CMT1 animal models have revealed that the alteration of Schwann cell physiology associated with demyelination and remyelination leads to both altered protein localization in Schwann cell membranes and loss of juxtaparanodal clustering of potassium channels in the axolemma (Neuberg *et al*., 1999). Furthermore, severe axonal atrophy was found in these mouse mutants (Sancho *et al*., 1999).

Our current understanding of the regulation of Schwann cell proliferation in CMT1A is less clear and appears somewhat confusing at this time (reviewed by Naef and Suter, 1998; Muller, 2000). Data obtained from *in vitro* studies suggest a direct role for PMP22 in regulating cell growth. Retroviral *PMP22* gene transfer in cultured Schwann cells caused growth suppression, while reduced PMP22 expression had the opposite effect (Zoidl *et al*., 1995). Consistent with these findings, cultivated Schwann cells isolated from nerve biopsies of CMT1A patients carrying the duplication showed decreased proliferation (Hanemann *et al*., 1998). Overexpression of PMP22 in Schwann cells and NIH-3T3 cells using microinjection of expression constructs, however, induced cell death in both cell types (Fabbretti *et al*., 1995; Brancolini *et al*., 1999). If cell death was blocked by co-expression of Bcl-2, these experiments also revealed a potential function for PMP22 in controlling cell morphology, possibly through the modulation of Rho small GTPase (Brancolini *et al*., 1999). Overexpression of *PMP22* point mutations, including the *Tr* mutation, in the same *in vitro* paradigm led to a strongly reduced apoptotic response and the proteins behaved in a dominant-negative manner when co-expressed with wild-type PMP22 (Fabbretti *et al*., 1995).

The molecular and cellular function of PMP22 is of cardinal importance for our understanding of CMT1A. In this report, we have examined the role of PMP22 in Schwann cell proliferation and cell death in early development and disease. To this end, we have analysed and compared peripheral nerves of homozygous PMP22 knock-out mice (Adlkofer *et al*., 1995), the CMT1A mouse model *Tr* (*PMP22* missense point mutation; for a review, see Suter *et al*., 1993) and PMP22 transgenic mice that carry extra copies of the *PMP22* gene (Magyar *et al*., 1996).

Material and methods

Animals and genotype analysis

Homozygous PMP22 knock-out, heterozygous PMP22 transgenic and *Tr* mice were obtained from our own breeding colonies. Wild-type mice from the corresponding genetic backgrounds were used as controls (PMP22 knock-out, Agouti SV129EV/C57BL/6; PMP22 transgenic, B6C3; Tr, CBA). We have taken special care to analyse mice with comparable genetic backgrounds within each group with different PMP22 mutations at each time point. Comparisons between the three groups have to be viewed considering the fact that these strains do not have an identical genetic background. The genotypes were assessed by either Southern blot or polymerase chain reaction (PCR) analysis of mouse tail genomic DNA. Initially, PMP22 knock-out and PMP22 transgenic mice were genotyped by Southern blot as previously described (Adlkofer *et al*., 1995; Magyar *et al*., 1996). PCR protocols were later designed to genotype PMP22 knock-out and PMP22 transgenic mouse progeny. Two PCRs were carried out to distinguish the wild-type from the PMP22 knock-out allele. Oligonucleotide primer 5′-CACGTCTTGC-CTGCTCTGAC-3′, located upstream of the homologous region of the targeting construct, was common to both PCRs. This common primer in combination with the *neo* gene internal oligonucleotide primer 5′-CGCAGCGCATCGCCT-TCTATC-3′ was used to identify the PMP22 knock-out allele. Conditions for hot-start PCRs were 4 min initial denaturation, followed by 35 cycles of 95°C for 30 s, 66°C for 1 min and 72°C for 2 min using a thermal cycler (Perkin-Elmer, GeneAmp PCR System 9600). Oligonucleotide primer 5′-GAGACGAAGAGCAACAC-3′ within exon 1 of the *PMP22* gene together with the common primer allowed the identification of the wild-type allele. Hot-start PCR conditions were as follows: 4 min initial denaturation, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 2 min. PMP22 transgenic progeny were identified using the pTCFupper oligonucleotide primer 5′-CCTCAACCTACTA-TGG-3′ and the PMP22 internal oligonucleotide primer 5′-AGGGCAATAAACACACTG-3′ (Magyar *et al*., 1996). Hot-start PCR conditions were as follows: 4 min initial denaturation, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1.5 min. PCR analysis of *Tr* mice has been reported elsewhere (Adlkofer *et al*., 1997*b*).

Experiments were performed in accordance with the legal requirements of the Eidgenössische Technische Hochschule and Kanton Zürich (Switzerland).

d-UTP-digoxigenin nick end-labelling (TUNEL) assay

PMP22 mutant mice (PMP22 knock-out, PMP22 transgenic and *Tr* mice) and wild-types of the corresponding genetic background at postnatal days 1, 4, 10 and 21, and 10 weeks of age were sacrificed with a lethal dose of sodium pentobarbital (Nembutal®, Abbott, Ill., USA) and their sciatic nerves were removed. Nerves were fixed in 4% paraformaldehyde in PBS (phosphate-buffered saline) for 4 h at 4°C, dehydrated and embedded in paraffin. Longitudinal sections, 5 µm thick, were mounted on slides, dewaxed and rehydrated. Initially, slides were incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase, followed by a 20 min digestion with proteinase K (20 µg/ml) at 37°C. Sections were pre-equilibrated in TdT buffer (30 mM Tris–HCl pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) prior to the addition of the TUNEL reaction mix (0.2 U/µl TdT enzyme, 6 nM biotin-16-dUTP, 6 nM dATP in TdT buffer). The enzymatic reaction was carried out at 37°C for 1.5 h and was terminated by incubating in 2× SSC (standard saline citrate) for 15 min at room temperature. After incubation of the sections in ABComplex (avidin–biotin complex) tagged to horseradish peroxidase (HRP) (Dako, Denmark) for 1 h at 37°C, incorporation of the labelled deoxynucleotide was visualized by applying the diaminobenzidine colour substrate solution for 12 min at room temperature in the dark. Nuclei were counterstained with methyl green (Elias, 1969). Positive and negative controls were run in parallel. Positive controls for the TUNEL reaction were paraffin sections of a E13.5 mouse embryo and sections of mouse small intestine previously digested with DNase I. As negative control, a section of an E13.5 mouse embryo was incubated in TUNEL reaction mix without adding the TdT enzyme.

To quantify apoptosis, sciatic nerve longitudinal sections were visualized in a Zeiss Axiophot with the ×100 lens. A grid with 100 divisions mounted in one of the microscope objectives helped to define the field of analysis. The nuclei counting proceeded from the proximal to the distal end of the sciatic nerve sections until 1000 or 500 nuclei, depending on the age of the animals, were counted in consecutive microscopic fields. All TUNEL-labelled cells from the total number of counted nuclei were recorded at the same time. The cell death index was calculated as the percentage of nuclei that were TUNEL positive. At least four animals per genotype and age group were analysed. Statistical analysis was performed with the Mann–Whitney *U*-test (two-side test paradigm) using Statview, version 4.0 software. *P* values $\leqslant 0.05$ were considered to be statistically significant.

BrdU incorporation assay

Mice of the same genotype and age as for the TUNEL assay were injected intraperitoneally with bromodeoxyuridine (BrdU; 100 µg/g of body weight) in 0.9% NaCl/7 mM NaOH.

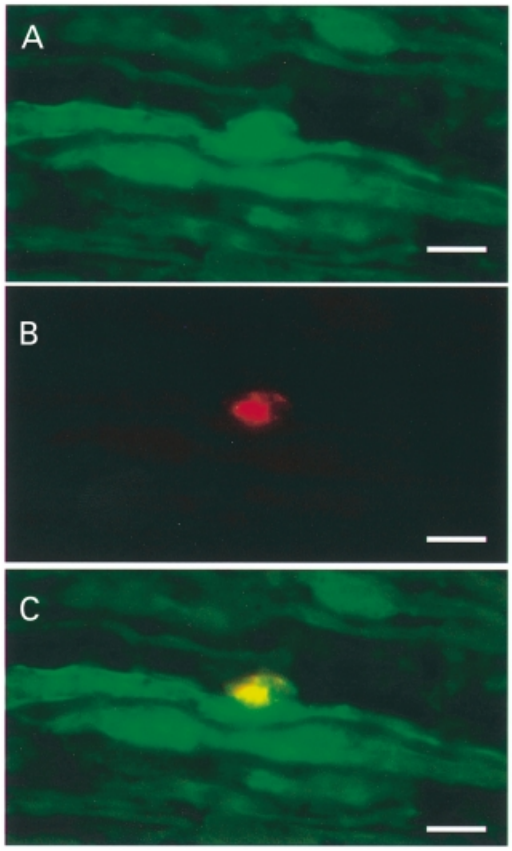
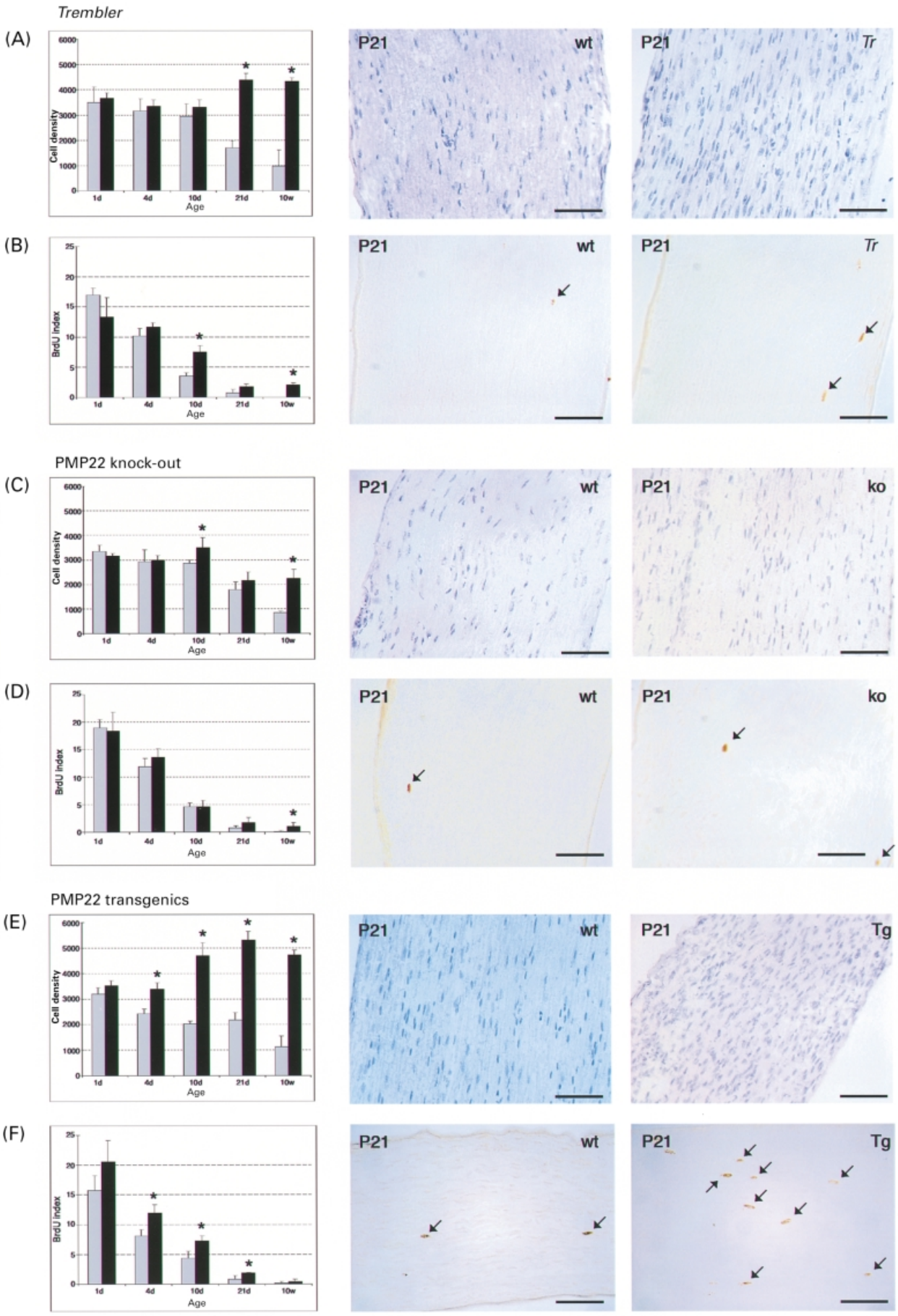


Fig. 1 A proliferating Schwann cell on a longitudinal section of a sciatic nerve from a PMP22 transgenic mouse at P21. The immunoreactivity of S100 labels Schwann cells in green (**A**). BrdU labelling is shown in red (**B**), corresponding to a Schwann cell nucleus on the overlay (**C**, yellow). Scale bar = 10 µm.

One hour after BrdU injection, animals were sacrificed and tissues of interest were removed. Tissue-Tek-embedded sciatic nerves were frozen in liquid nitrogen-cooled isopentene and sectioned with a cryostat. Six consecutive longitudinal sections, 5 µm thick, were mounted alternatively onto two Superfrost slides, air dried and fixed for 5 min with 2% paraformaldehyde in PBS. Because our DNA denaturation protocol precluded the simultaneous identification of BrdU-labelled cells and staining of nuclei in the same section, one slide was stained with haematoxylin to count nuclei whereas the second slide was processed to reveal BrdU-positive cells. After blocking endogenous peroxidase activity, DNA denaturation was achieved by incubating the sections in 2 M HCl for 15 min at 37°C followed by neutralization in 0.1 M sodium borate pH 8.5 for 10 min. Nuclei that incorporated BrdU were labelled with a biotinylated anti-BrdU monoclonal antibody (Caltag Laboratories, Calif., USA) diluted 1 : 20 and detected by the ABComplex–horseradish peroxidase method as described above. Sections of small intestine from the same animals were used as positive controls.

BrdU-labelled cells were counted in three alternated consecutive longitudinal sections. Starting from the proximal


Trembler
(A)
Cell density
Age
1d
4d
10d
21d
10w
P21
wt
P21
Tr
(B)
BrdU index
Age
P21
wt
P21
Tr
PMP22 knock-out
(C)
Cell density
Age
P21
wt
P21
ko
(D)
BrdU index
Age
P21
wt
P21
ko
PMP22 transgenics
(E)
Cell density
Age
P21
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P21
Tg
(F)
BrdU index
Age
P21
wt
P21
Tg

end of the sciatic nerve, 4–6 consecutive microscopic fields, as defined by the grid mounted in the microscope objective, were examined per section with a ×100 lens. All BrdU-labelled cells present within the defined fields were counted. An identical quantification method was used to assess the number of haematoxylin-stained nuclei in the other three alternated sections. The proliferation index was calculated as the percentage of BrdU-labelled cells in relation to the total number of haematoxylin-stained nuclei present in the same area. Cell density was defined as the total number of haematoxylin-stained nuclei per mm^2. Usually five animals per genotype and age group were analysed. Statistical analysis was performed with the Mann–Whitney *U*-test (two-side test paradigm) using Statview, version 4.0 software. *P* values $\leqslant 0.05$ were considered to be statistically significant.

Immunohistochemistry

To demonstrate that the BrdU- and TUNEL-positive cells were Schwann cells, selected nerves were immunostained with a polyclonal rabbit antiserum against S100 protein (Dako, Denmark) combined with either BrdU or TUNEL labelling. For these experiments, sciatic nerve sections were embedded in Tissue-Tek and 5 μm thick cryosections obtained. Thereafter, identical protocols to those described above were used to process sections for either BrdU or TUNEL labelling, except that Texas red-tagged streptavidin (Jackson ImmunoResearch Laboratories, Pa., USA) was used as detection system. S100-labelled cells were identified with a fluorescein-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories).

Results

Earlier analysis of PMP22 mouse mutants indicated that PMP22 plays a key role in the initial steps of myelination and in the maintenance of the myelin sheath (reviewed by Carenini *et al*., 1999; D'Urso *et al*., 1999; Naef and Suter, 1998). Since demyelination induces proliferation of Schwann cells in various mouse mutants, regardless of the underlying molecular defect (Giese *et al*., 1992; Anzini *et al*., 1997; Neuberg *et al*., 1999), we decided to start our analysis at postnatal day (P) 1, prior to the onset of appreciable myelination. Since PMP22 is expressed by peripheral glia, motor neurones and sensory neurones before birth (Baechner *et al*., 1995; Parmantier *et al*., 1995, 1997; Hagedorn *et al*., 1999), this strategy should allow the detection of alterations in Schwann cell proliferation and cell death in early development without the interference of demyelination.

We analysed three groups of PMP22 mutants to assess the influence of *PMP22* gene dosage (PMP22 transgenic mice and PMP22 knock-out mice) and the effect of altered PMP22 protein (*Tr* mutant) on the proliferation and apoptosis of Schwann cells in comparison with wild-type controls. To follow the development and to evaluate the probable onset of alteration of proliferation and/or apoptosis, we examined sciatic nerves of P1, P4, P10 and P21 animals. In addition, 10-week-old young adult mice with completed peripheral myelination were also examined.

Schwann cell proliferation

S100 was used as a Schwann cell marker in combination with BrdU incorporation (for example, see Fig. 1A–C). BrdU-positive cells without S100 immunoreactivity were not observed (data not shown).

First, we compared the cell density in sciatic nerves of wild-type and mutants at P1. No significant differences between mutant mice and their corresponding control animals were observed within the three paired groups that were analysed (Fig. 2A, C and E). In all three wild-type control groups, cell density in the sciatic nerve decreased from P1 until the age of 10 weeks, to approximately one-third of the initial value (Fig. 2A, C and E). In the mutant animals, we observed a significant increase in cell density in *Tr* animals from P21 onwards (Fig. 2A) and in the transgenic PMP22 animals as early as P4 (Fig. 2E).

Next, we determined the proliferation rate by calculating the number of cells with BrdU incorporation in relation to the total cell number (BrdU index). In wild-type mice, this index ranged from 15.8 ± 2.5% to 18.9 ± 1.5% at P1 and was reduced to undetectable levels in 10-week-old animals. In the *Tr* mutants, we found a strong increase of Schwann cell proliferation at P10 and in 10-week-old animals, while proliferation of Schwann cells at P21 did not reach significance in comparison with the age-matched wild-type controls with comparable genetic background (Fig. 2B). These findings are consistent with pathology data indicating that significant myelination occurs initially in these mice (Ayers and Anderson, 1976) followed by demyelination and, as shown here and suggested earlier (Perkins *et al*., 1981), accompanied by Schwann cell proliferation leading to cellular onion bulb structures. In PMP22 knock-out animals, a significant increase in the rate of proliferation was found

Fig. 2 Qualitative and quantitative analysis of cell densitiy and proliferation in normal and PMP22 mutant sciatic nerves. **A**, **C** and **E** refer to the cell density per mm^2; **B**, **D** and **F** show the BrdU labelling index of proliferating cells in three groups of PMP22 mutant animals and their corresponding wild-type controls (wt). Asterisks indicate statistical significance ($P < 0.025$). Arrows show BrdU-positive nuclei. Scale bar = 20 μm. Genotypes are given in the upper right corner of each section: *Tr* = *Trembler*; ko = PMP22 knock-out; Tg = PMP22 transgenic.

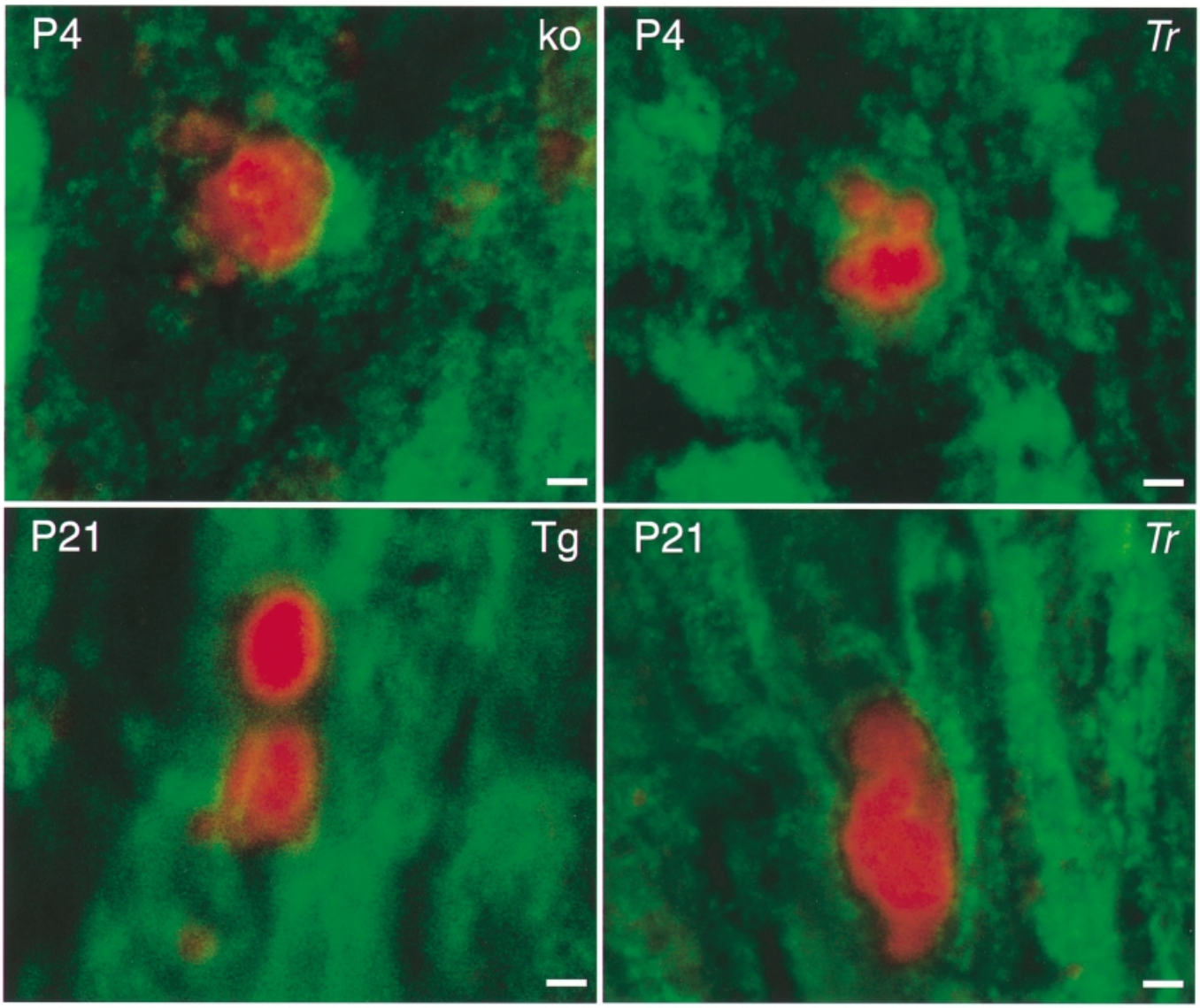

Fig. 3 Confocal microscopy of TUNEL immunoreactivity on longitudinal sections of sciatic nerves from PMP22 mutant sciatic nerves. Green fluorescence (FITC) indicates S100-positive cells and red fluorescence marks TUNEL-positive nuclei. Note the pronounced nuclear fragmentation. Scale bar = 2 μm. Age is indicated in the upper left corner, while genotypes are given in the upper right corner of each section. *Tr* = *Trembler*; Tg = transgenic; KO = PMP22 knock-out.

only in 10-week-old animals (Fig. 2D). This result is consistent with the described pathology in these mice which are affected by focal hypermyelination (tomacula formation) early in life, followed by demyelination including onion bulb cell formation later (Adlkofer *et al*., 1995; Sancho *et al*., 1999). PMP22 transgenic mice showed a constant, significantly elevated proliferation index from P4 onwards in relation to the wild-type control group, consistent with the observed almost complete amyelination of the peripheral nerves of these animals throughout development (Fig. 2F; Magyar *et al*., 1996; Sancho *et al*., 1999). In contrast to wild-type animals, proliferating cells were detectable in the sciatic nerves of all three mutants at 10 weeks of age, but the rate was reduced to 1–2%.

Cell death of Schwann cells

Cell death was analysed by TUNEL staining in combination with S100 immunohistochemistry. Examples of dying Schwann cells in the sciatic nerves of various PMP22 mutant mice, also characterized by fragmented nuclei, are shown in Fig. 3. Furthermore, a representative overview of apoptosis in a sciatic nerve of a *Tr* mutant at 10 weeks of age and the corresponding wild-type animal is shown (Fig. 4). In wild-type mice, considerable programmed cell death was observed from P1 onwards, peaking between P4 and P10, with very low levels at P21, and no detectable dying cells at the age of 10 weeks (Fig. 5). These findings are consistent with earlier suggestions that axon–Schwann cell interactions regulate the correct Schwann cell number during early postnatal

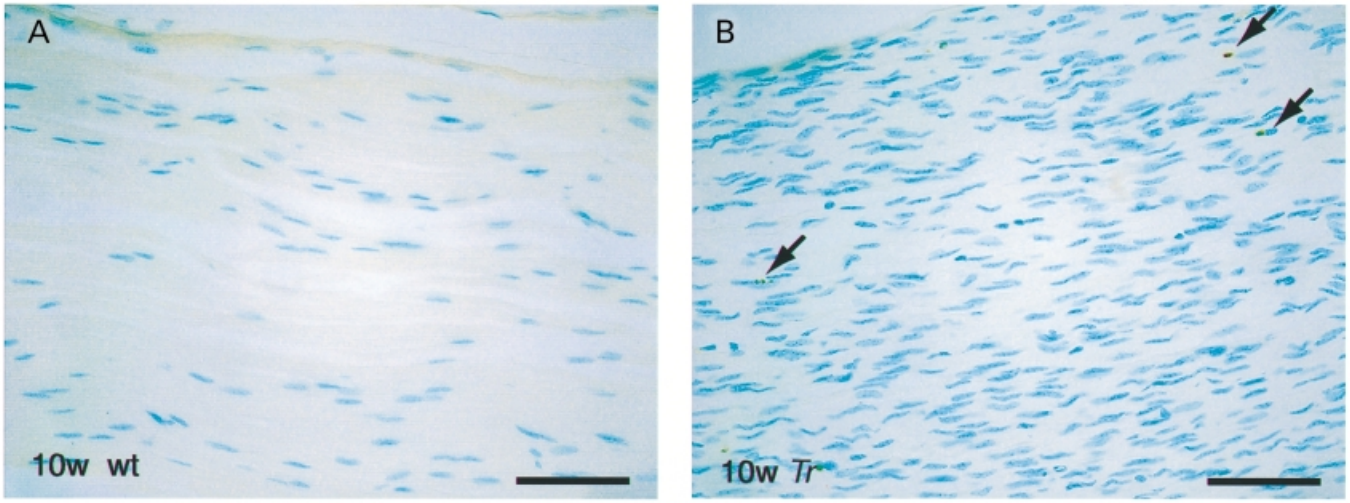

Fig. 4 Longitudinal sections of sciatic nerves showing TUNEL-positive nuclei in a 10-week-old *Trembler* (*Tr*) mouse and an age-matched control animal (wt). Arrows indicate TUNEL-positive, partially fragmented nuclei. Scale bar = 20 μm.

development through apoptosis (Grinspan *et al*., 1996; Syroid *et al*., 1999).

The rate of apoptosis was similar in the three mutant groups and in the corresponding wild-type animals at P1 (Fig. 5A–C). In *Tr* animals, significantly increased cell death was observed starting at P21 and continuing at 10 weeks of age (Figs 4A and 5A). The results in PMP22 knock-out animals were qualitatively similar to *Tr* (Fig. 5B). In PMP22 transgenic mice, the rate of apoptosis was already doubled compared with wild-type at P4, and this strongly increased apoptosis continued up to adulthood (Fig. 5C). Earlier studies have indicated that the low affinity neurotrophin receptor p75 is involved in the induction of apoptosis in transected nerves (Syroid *et al*., 2000). Interestingly, p75 is also upregulated in peripheral nerves of PMP22 transgenic mice (Magyar *et al*., 1996). However, a potential causative function for p75 in the increased Schwann cell apoptosis of these animals remains to be established.

In summary, all three groups of PMP22 mutant animals showed a significant increase of apoptotic Schwann cells at P21. At the age of 10 weeks, none of the corresponding wild-type groups showed any apoptotic Schwann cells, while apoptosis was still prominent in the mutant mice.

Discussion

In this study, we have examined Schwann cell proliferation and apoptosis in three different mouse strains with altered expression of the *PMP22* gene. The spontaneous mouse mutant *Tr* carries a point mutation in the *PMP22* gene which leads to intracellular retention of the mutated protein *in vitro* and *in vivo* (Naef *et al*., 1997; Colby *et al*., 2000). Since PMP22 appears to form dimers, it is conceivable that wild-type PMP22 is also retained, at least partially, within the cell, suggesting a dominant–negative disease mechanism (Naef *et al*., 1997; Naef and Suter, 1999; Tobler *et al*., 1999; Brancolini *et al*., 2000). However, a contribution by a gain-of-function effect, possibly due to overloading of intracellular compartments, has also been demonstrated by genetic analysis (Adlkofer *et al*., 1997*b*). Similar to observations in animal models of Pelizaeus–Merzbacher disease where disrupted proteolipid protein trafficking appears to result in oligodendrocyte apoptosis (Gow *et al*., 1998), we show that a substantial increase in Schwann cell apoptosis is observed in *Tr* at P21 and at the age of 10 weeks. In support of a causative action of the *Tr* protein in the induction of apoptosis, we have also observed strongly increased programmed cell death in adult homozygous *Tr* mice compared with the heterozygous animals used in our study (preliminary observation). In addition, increased levels of apoptosis have also been described in the *Tr-Ncnp* mouse which carries an in-frame deletion in the *PMP22* gene (Suh *et al*., 1997). The most straightforward interpretation of these data is that excessive intracellular accumulation of mutated PMP22 cannot be tolerated by Schwann cells *in vivo* and, consequently, the cells die. A similar mechanism may also explain the increased apoptosis in PMP22 transgenic mice, consistent with the abundant empty basal lamina present in the nerves of these animals (Magyar *et al*., 1996). The substantially earlier onset of apoptosis in PMP22 transgenic mice compared with *Tr* could be due to the high *PMP22* gene dosage in these animals which may lead to fast intracellular accumulation of large amounts of misfolded PMP22 and immediate activation of the apoptosis pathway (Pareek *et al*., 1993). Alternative interpretations, however, are also possible. In the process of this study, we have confirmed that Schwann cell apoptosis occurs naturally in developing nerves of wild-type mice during the first two postnatal weeks (Grinspan *et al*., 1996; Syroid *et al*., 2000). It has been suggested that axon–Schwann cell interactions in this way regulate the correct Schwann cell numbers by competition for axonally derived neuregulins (Grinspan *et al*.,

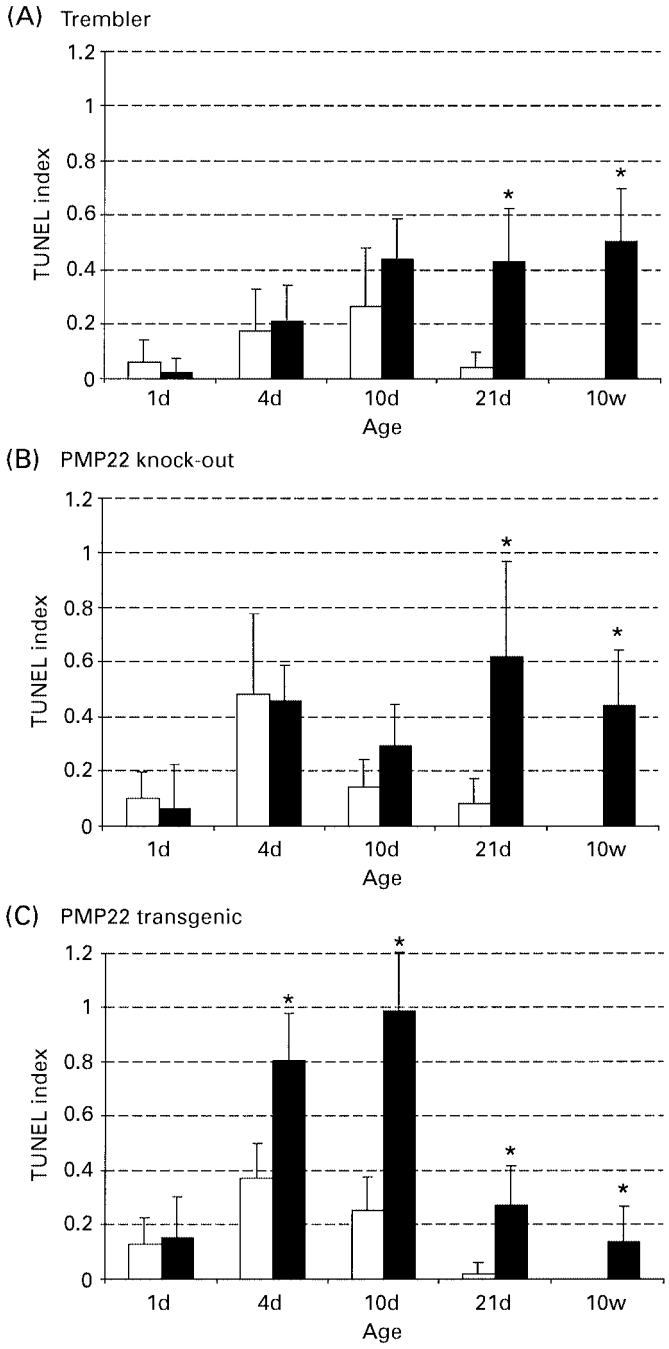

Fig. 5 Quantification of apoptosis in the sciatic nerves of PMP22 mutant mice (black bars) and the corresponding wild-type control groups (grey bars). Asterisks indicate statistical significance ($P < 0.025$).

1996; Syroid *et al*., 1996). Sciatic nerves of PMP22 transgenic animals contain virtually no myelin and generate an excess of Schwann cells starting already at P4, presumably since they are exposed continuously to mitogenic axolemma proteins. Thus, the early phase of increased Schwann cell apoptosis in PMP22 transgenic mice from P4 to P10, which is not present in *Tr*, may also involve processes regulating correct Schwann cell numbers in early development, including the availability of and spatial exposure to limited amounts of survival factors (reviewed by Garratt *et al*., 2000). Importantly, there is a window of susceptibility of Schwann cells to apoptosis during development since programmed cell death could be enhanced by axotomy only during early development (Grinspan *et al*., 1996). Thus, the Schwann cell death observed from P21 onwards in PMP22 mutants is likely to be due to other mechanisms.

Toxification through intracellular accumulation may contribute to the late phase of apoptosis in *Tr* and PMP22 transgenic mice, but the increased levels of Schwann cell apoptosis seen in PMP22 knock-out mice require other explanations. Based on the fact that this increase is correlated with morphological signs of abundant deymelination, remyelination and onion bulb formation (Adlkofer *et al*., 1995; Sancho *et al*., 1999), it is likely that dying supernumerary Schwann cells are responsible for our findings. The same mechanisms may also contribute to the apoptosis observed in *Tr* late in development since onion bulb formation associated with increased cell density is found within peripheral nerves (Ayers and Anderson, 1976; Perkins *et al*., 1981).

Experiments in cell culture have suggested that PMP22 is directly involved in the regulation of the cell cycle (Zoidl *et al*., 1995, 1997) and the induction of cell death (Fabbretti *et al*., 1995; Brancolini *et al*., 1999). To address this issue, we have analysed the PMP22 mutants at P1. We reasoned that the function of PMP22 in myelination and myelin maintenance is not a substantially interfering factor at this time point since the sciatic nerve is still largely unmyelinated. Our analysis revealed no significant alterations in Schwann cell density, proliferation index or the rate of apoptosis in any PMP22 mutant compared with wild-type mice at this early time point. Thus, normal PMP22 expression is dispensable for correct Schwann cell proliferation and cell death up to P1. An influence of PMP22 in these processes later in development, however, cannot be excluded. This is difficult to assess due to the underlying myelin breakdown (demyelination) or amyelination pathologies which alter axon–Schwann cell interactions and, consequently, proliferation and apoptosis in ways that are not fully understood.

How do our data compare with the human disease? In nerve biopsies of young CMT1A patients carrying the *PMP22* duplication on chromosome 17p.12, Schwann cells show no signs of increased proliferation (Hanemann *et al*., 1997). Consistent with our observations in PMP22 transgenic mice, the authors concluded that there is no evidence for altered initial Schwann cell proliferation prior to the process of de- and remyelination. Intriguingly, however, human Schwann cells obtained from CMT1A patients carrying the chromosomal duplication show a decrease in proliferation *in vitro* (Hanemann *et al*., 1998). This difference between *in vitro* and *in vivo* studies may reflect age-related changes and/or the course of disease. Alternatively, culturing CMT1A Schwann cells may unmask a difference in their potential for decreased proliferation that cannot be observed *in vivo*,

a common emerging theme in developmental biology (Anderson, 2001).

Evidence for apoptosis of Schwann cells in CMT1A and HNPP nerve biopsies from patients with proven *PMP22* duplications and deletions has been provided by Erdem and colleagues using the TUNEL method (Erdem *et al*., 1998). Quantitative studies showed a significantly reduced number of total Schwann cells in CMT1A compared with controls indicating a loss of Schwann cells. In HNPP, the number of total Schwann cells was increased and significant Schwann cell apoptosis was observed. The authors suggested on the basis of further morphological analysis that this Schwann cell apoptosis might be related to the regenerative state of the nerve resulting from the process of sprout pruning (Erdem *et al*., 1998).

In summary, we show that altered expression of PMP22 does not affect the generation of the correct number of early Schwann cells. Furthermore, the proliferation index is not changed in PMP22 mutants in early postnatal development. However, increased proliferation and apoptosis are prominent features in all mutants at later developmental stages. Based on the differences in the kinetics of these alterations during the development of the different mutants, it is likely that overloading of intracellular compartments and altered access to trophic factors due to myelination deficiencies are responsible for the increased cell density, proliferation and apoptosis observed in PMP22 mutant peripheral nerve.

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