Short Communication

Deposition of Transthyretin in Early Stages of Familial Amyloidotic Polyneuropathy

Evidence for Toxicity of Nonfibrillar Aggregates

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Familial amyloidotic polyneuropathy (FAP) is a neurodegenerative disorder characterized by extracellular deposition of transthyretin (TTR) amyloid fibrils, particularly in the peripheral nervous system. No systematic immunohistochemical data exists relating TTR deposition with FAP progression. We assessed nerves from FAP patients in different stages of disease progression (FAP 0 to FAP 3) for TTR deposition by immunohistochemistry, and for the presence of amyloid fibrils by Congo Red staining. The nature of the deposited material was further studied by electron microscopy. We observed that early in FAP (FAP 0), TTR is already deposited in an aggregated nonfibrillar form, negative by Congo Red staining. This suggested that *in vivo*, preamyloidogenic forms of TTR exist in the nerve, in a stage before fibril formation. Cytotoxicity of nonfibrillar TTR was assessed in nerves of different FAP stages by immunohistochemistry for macrophage colony-stimulating factor. FAP 0 patients already presented increased axonal expression of macrophage colony-stimulating factor that was maintained in all other stages, in sites related to TTR deposition. Toxicity of synthetic TTR fibrils formed *in vitro* at physiological pH was studied on a Schwannoma cell line by caspase-3 activation assays and showed that early aggregates but not mature fibrils are toxic to cells. Taken together, these results show that nonfibrillar cytotoxic deposits occur in early stages of FAP. (*Am J Pathol* 2001, 159:1993–2000)

Familial amyloidotic polyneuropathy (FAP) is a neurodegenerative autosomal dominant disorder characterized by the extracellular deposition of transthyretin (TTR) fibrils in several tissues, particularly in the peripheral nervous system.¹ The largest focus of the disease is in Portugal (>500 kindreds) where onset of clinical symptoms shows a wide range (17 to 78 years), with > 80% of cases developing symptoms before age 40. Several amyloidogenic point mutations in TTR have been described,² the most common being a Val for Met substitution at position 30 of the protein (Val30Met) present in Portuguese FAP kindreds.³ TTR amyloid deposits can be found in any part of the peripheral nervous system, including the nerve trunks, plexuses, and sensory and autonomic ganglia.⁴ In peripheral nerve, deposition occurs extracellularly, particularly in the endoneurium close to Schwann cells (SCs) and to collagen fibrils. In severely affected nerves, almost the whole endoneurial contents may be replaced by amyloid and very few nerve fibers survive. FAP is characterized by initial axonal loss affecting unmyelinated and small myelinated fibers (MFs) and later affecting the larger fibers.¹ In the autonomic and sensory nervous system, advanced neuronal degeneration that might cause progressive ascending neuropathy (dying back type) is evident in addition to axonopathy.

The cellular effects of TTR deposition on neuronal function in FAP remain however to be elucidated. In the case of Alzheimer's disease, the receptor for advanced glycation end products has been associated with neural toxicity.⁵ We have previously shown that TTR fibrils are able to bind receptor for advanced glycation end products, triggering nuclear factor (NF)- κ B activation.⁶ However, in

Supported by grants from PRAXIS XXI (35785/99 and 35735/99) and fellowships BPD/22027/99 (to M. M. S.), BD/15725/98 (to I. C.), BTI/PL21902 (to R. F.) from the Fundação para a Ciência e Tecnologia from Portugal.

Accepted for publication August 31, 2001.

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FAP no detailed immunohistochemical studies exist on the relationship between amyloid deposition and neurodegeneration. In the present work we investigated in which form TTR is deposited in the nerve, early in FAP, before major nerve fiber degeneration and assessed cytotoxic effects of different stages of TTR fibrillogenesis both *in vitro* and *in vivo*.

Materials and Methods

Study Participants

Sural nerve biopsy specimens from asymptomatic members of FAP kindreds, from FAP patients as well as normal controls were available in Hospital Geral de Santo Antonio, Porto, Portugal, because this material was obtained as part of the clinical diagnosis and evaluation of polyneuropathy in Portugal, during the period of 1979 to 1989, before the current use of less invasive methods. Initial characterization of clinical material included morphometric studies of nerve fiber density and abundance of amyloid deposits. Amyloid deposition was assessed by standard Congo Red staining and was scored from 0 to 3 depending on the amount of amyloid deposits found (0, no amyloid deposition; 1, discrete amyloid deposition; 2, mildly abundant deposition; and 3, very abundant deposition). Patients were scanned for the Val30Met mutation by immunoblotting.⁷ Morphometric studies were performed on sural nerve biopsy tissue fixed in glutaraldehyde (2.5%) in 0.1 mol/L cacodylate buffer (pH 7.4), postfixed in osmium tetroxide, and embedded in Epon. Quantitation of MFs in semithin sections was performed in an area of at least 0.1 mm² at a magnification of $\times 1000$. Myelinated fibers (MFs) were counted, their diameters measured, and the density was calculated. Unmyelinated fibers (UFs) were counted from thin sections in an area of at least 0.005 mm² and their densities calculated. Evaluation for typical symptoms of FAP⁸ was performed, namely by assessment of sensory impairment.

Immunohistochemistry

For immunohistochemistry, nerve paraffin sections were deparaffinated, dehydrated in a modified alcohol series, and incubated in blocking buffer [1% bovine serum albumin (BSA), and 4% horse serum in phosphate-buffered saline (PBS)] for 30 minutes at 37°C in a moist chamber. Subsequently, incubation with primary antibody at the appropriate dilution in blocking buffer was performed overnight at 4°C. The primary antibodies used were: rabbit polyclonal anti-TTR IgG (1:300; DAKO, Glostrup, Denmark), goat polyclonal anti-macrophage colony-stimulating factor (MCSF) IgG (1:25; Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rabbit anti-neurofilament 200 IgG (1:200; Sigma, Sintra, Portugal). Antigen visualization was performed with either the biotin-extravidin-alkaline phosphatase kits or with the biotin-extravidin-peroxidase kits (Sigma), using Fast Red (Sigma) or 3-amino-9-ethyl carbazole (Sigma), respectively, as substrates. On parallel control sections, primary antibody

was replaced by blocking buffer. Semiquantitative analysis of immunohistochemical images was performed with the Universal Imaging system (NIH). Results shown represent percent occupied area $\pm$ SD.

Immunogold Labeling

Thin sections of glutaraldehyde-osmium tetroxide-fixed, Epon-embedded nerves were mounted onto nickel grids, treated with 14.4% sodium metaperiodate for 30 minutes, and subsequently blocked with blocking buffer (1% BSA in PBS) for 30 minutes. Anti-human TTR (DAKO) was used as primary antibody diluted 1:100 in blocking buffer. In parallel control sections antibody preadsorbed with TTR (1 μ g of TTR/100 μ l diluted primary antibody incubated overnight at room temperature) was used. As secondary antibody, goat anti-rabbit immunoglobulins coupled to 10-nm gold particles (Amersham, Freiburg, Germany), diluted 1:15 in blocking buffer, were used for 45 minutes at room temperature. The grid was subsequently washed in 10% BSA, 3% NaCl, 5% fetal bovine serum, and 0.05% Tween 20 in PBS, four times for 10 minutes each. Finally sections were stained with uranyl acetate for 3 minutes and lead citrate for 40 seconds.

Isolation and Purification of TTR; Preparation of Amyloid Fibrils

Recombinant TTR (either wild type or mutant TTR Leu55Pro) was produced in an *Escherichia coli* expression system, isolated, and purified as previously described.⁹ For preparation of amyloid fibrils, mutant TTR was dialyzed against water, pH 7.0, and concentrated to 5 mg/ml. At this point the preparation was centrifuged at 15,000 $\times g$ for 30 minutes at 4°C. Subsequently, the pellet was washed and resuspended in PBS, pH 7.4, at 2 mg/ml and incubated at 37°C. At given time points ($t = 1$, $t = 6$, and $t = 15$ days), samples were visualized for the presence of amyloid fibrils by transmission electron microscopy (TEM). All preparations were positive by Thioflavin T spectrofluorometric assays. Protein concentration was determined by the Lowry method.

Transmission Electron Microscopy

For visualization by TEM (60 kV; Zeiss), samples were adsorbed to glow-discharged carbon-coated collodion film on 400-mesh copper grids. For negative staining, grids were washed with deionized water and stained with 0.75% uranyl acetate.

Caspase-3 Assay

RN22 cells (rat Schwannoma cell line) were propagated in 25-cm² flasks and maintained at 37°C in a humidified atmosphere of 95% and 5% CO₂. Cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Barcelona, Spain).

Table 1. Clinical Pathological Analysis of FAP Patients

Patient	Age at biopsy	Clinical condition at biopsy	FAP symptoms since biopsy	TTR deposition	Amyloid deposition		Fiber density (fibers/mm ²)		Degenerating fibers
					Endo	Epi	MF	UF	
FAP 0									
1	19	AS	AS 22 years later	+	0	0	11,080	25,500	0
2	17	AS	AS 13 years later	+	0	0	7700	41,500	0
3	33	AS	AS 13 years later	+	0	0	10,300	54,800	0
4	25	AS	AS 12 years later	+	0	0	10,800	57,900	0
5	21	AS	AS 12 years later	+	0	0	10,300	80,000	0
6	25	AS	AS 11 years later	+	0	0	7844	nd	nd
7	16	AS	Onset 14 years later	+	0	0	8900	34,000	0
8	34	AS	Onset 13 years later	+	0	0	10,600	nd	nd
9	21	AS	Onset 12 years later	+	0	0	10,470	38,200	0
10	20	AS	Onset 10 years later	+	0	0	9300	49,800	0
11	25	AS	Onset 6 years later	+	0	0	9340	24,000	0
12	17	AS	No follow-up	+	0	0	9680	72,000	0
FAP 1									
13	nd	AS	AS	+	1	1	7270	32,750	+
14	27	AS	Onset 1 year later	+	2	1	2460	4080	+
15	27	S	S	+	2	0	2370	nd	+
16	25	S	S	+	2	0	4360	41,000	+
FAP 2									
17	nd	S	S	+	1	0	1840	9600	+
18	41	S	S	+	2	0	1140	<1000	+
19	58	S	S	+	2	1	1300	<1000	+
20	41	S	S	+	2	1	1620	<1000	nd
FAP 3									
21	43	S	S	+	3	0	720	0	+
22	41	S	S	+	3	1	0	0	+
23	34	S	S	+	3	1	910	0	+
Control 24–27	36–53	—	—	—	0	0	7–11,000	30–70,000	0

Endo, epi-endoneurium and epineurium, respectively; AS, asymptomatic FAP patient; S, symptomatic FAP patient; nd, not determined.

Activation of caspase-3 was measured using the CaspACE colorimetric 96-well plate assay system (Promega), following the manufacturer's instructions. Briefly, 80% confluent cells in Dulbecco's minimal essential medium with 1% fetal bovine serum were exposed for 48 hours to 2 μ mol/L of TTR (either soluble or fibrillar mutant TTR). Subsequently, each well was trypsinized and the cell pellet was lysed in 100 μ l of hypotonic lysis buffer (Promega, Madison, WI) by four cycles of freeze/thawing. Forty μ l of each cell lysate was used in duplicates for determination of caspase-3 activation. The remaining cell lysate was used to measure total cellular protein concentration with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), using BSA as standard. Values shown are the mean of duplicates and the experiment was performed three times.

Results

TTR Is Detected by Immunohistochemistry in Early FAP Stages in Amyloid-Negative Nerves

As no systematic immunohistochemical data existed relating TTR deposition and FAP progression, we started by assessing nerves from asymptomatic Val30Met carriers and FAP patients in different stages of disease progression; TTR deposition by immunohistochemistry and amyloid presence by Congo Red staining were investigated.

The scoring system of patients material was performed by morphometric measurements of MFs and UFs and is summarized in Table 1.

FAP 0 patients ($n = 12$) had no amyloid deposition in nerves and were all asymptomatic at time of biopsy, with ages varying between 16 and 34 years. Presently, up to 22 years after the biopsy, six of the FAP 0 patients remain asymptomatic whereas five have already developed clinical symptoms. No significant reduction in the number of fibers when compared to normal scores was observed (MFs were $\sim 10,000$ fibers/mm²; UFs varied from $\sim 30,000$ to 70,000 fibers/mm²) and no fiber degeneration was found. FAP 1 patients ($n = 4$) had a discrete reduction (MFs varied between 7000 and 2000 fibers/mm²; UFs varied between 4000 and 40,000 fibers/mm²) and presented already TTR amyloid deposition in the nerve and fiber degeneration in contrast to FAP 0. Two of the FAP 1 patients studied here were asymptomatic at the time of biopsy. FAP 2 patients ($n = 4$) showed evident reduction (MFs ~ 1000 fibers/mm²; UFs $< 10,000$ fibers/mm²); and finally, FAP 3 patients ($n = 3$) presented severe reduction (MFs < 1000 fibers/mm²; UFs absent). FAP 2 and FAP 3 patients were all symptomatic and amyloid deposition was in most instances inversely proportional to nerve fiber density.

We observed that in the stage before loss of UFs and MFs and major nerve fiber degeneration (FAP 0), despite the absence of Congo Red birefringence (the hallmark of

Figure 1. A: TTR immunohistochemistry (left) and Congo Red staining (right) of normal nerves (top), FAP 0 patients (middle), and FAP 3 patients (bottom). Original magnifications, $\times 40$. **B:** TTR immunogold labeling and electron microscopy of nerves from a FAP 0 patient (left; original magnification, $\times 84,000$), a FAP 3 patient (middle; original magnification, $\times 84,000$), and a FAP 3 patient using preadsorbed anti-TTR (C, right; original magnification, $\times 66,000$). Scale bar, 100 nm.

amyloid) TTR was present in all 12 cases investigated as revealed by immunohistochemistry with an anti-TTR antibody (Figure 1A, middle left). It is interesting to note that up to 22 years after detection of TTR deposition in the nerve in a nonamyloid form (such as the case of patient 1), no clinical symptoms of FAP were observed. The specificity of the TTR staining was demonstrated by the fact that no TTR deposition occurred in nerves from normal individuals (Figure 1A, top left) and also because no staining was revealed when the anti-TTR antibody was preadsorbed with TTR (data not shown). We hypothesized that TTR might deposit in a nonfibrillar form in early stages of FAP, before assembling into amyloid fibrils, that give the characteristic green-birefringence by Congo Red staining, as observed in stages FAP 1 to 3 (Figure 1A, bottom right).

TTR Deposits in a Nonfibrillar Form in Nerve of Early FAP Stages

To further assess the nature of the deposited TTR in FAP 0 patients, we performed electron microscopy and TTR immunogold labeling of FAP 0 ($n = 2$), FAP 1 ($n = 1$), and FAP 3 ($n = 1$) nerves. In FAP 0 patients, immunolabeling was observed extracellularly in the proximity of SCs, in a nonfibrillar form, as evidenced in Figure 1B by arrows (left panel); very small contiguous fibrillar-like assemblies were noticed, most likely too small to give birefringence with Congo Red staining on histochemistry (Figure 1A, middle right). To ascertain the specificity of the immunogold labeling, we performed similar labeling of normal control nerves. In areas corresponding to sites of TTR deposition in FAP 0 patients, ie, in the extracellular matrix near collagen fibrils, no labeling could be observed in control nerves (not shown). In the FAP 3 patient, the labeled material was clearly present in a fibrillar form (Figure 1B, middle panel) and the dimensions of the fibrils (8 to 10 nm) were typical of amyloid fibrils. When we performed similar gold immunogold labeling using preadsorbed anti-TTR in the same FAP 3 nerve, no labeling was observed, thus showing the specificity of our results (Figure 1B, right). In FAP 1, both forms of deposition, ie, nonfibrillar and fibrillar were detected (not shown).

Nerve Cytotoxicity of Nonfibrillar TTR in Early Stages of FAP

To assess the possible cytotoxicity of nonfibrillar TTR deposits and because previous studies demonstrated activation of NF- κ B by TTR in FAP tissues,⁶ we assessed cellular stress in early-stage FAP. Because NF- κ B activation might cause increased expression of proinflammatory cytokines, namely MCSF, we analyzed the presence of MCSF in affected nerves from FAP patients in relation to deposition of TTR.

Semiquantitative analysis of immunohistochemical images for immunoreactive MCSF in FAP nerve biopsies, compared with control individuals, demonstrated early an

increased expression of this cytokine (Figure 2). Although normal nerve showed virtually no detectable antigen (Figure 2, top right), FAP 0 individuals (ie, before amyloid was present) already displayed increased MCSF antigens (Figure 2, top left). Increased levels of this cytokine were also evident in FAP 3 individuals (Figure 2, top middle panel). In each case, the level of cytokine appeared to increase by approximately twofold, compared with controls, and was statistically significant (Figure 2, bottom right) ($P < 0.03$). Endoneurial axons seemed responsible for this increase in MCSF expression based on co-localization with the neuron-specific marker N200 (Figure 2, bottom left). The presence of an inflammation marker in FAP 0 nerves suggests that nonfibrillar TTR deposition is already cytotoxic.

Cytotoxicity of Nonfibrillar and Fibrillar TTR in Cell Culture

TTR Leu55Pro is associated with a very aggressive form of FAP and X-ray data on this mutant revealed important structural changes that might be representative of amyloid precursor forms;¹⁰ this variant thus constitutes an important tool to follow fibrillogenesis. We studied TTR Leu55Pro fibril dynamic assembly throughout time at physiological conditions (PBS, pH 7.4) by TEM and tested the toxicity of the different species formed during that process. After 1 day we could observe aggregates and very short prefibrillar structures 7 to 8 nm wide (Figure 3A, top right). Longer incubations at 37°C showed that these structures elongated and formed fibrils with widths of ~8 nm (Figure 3A, bottom panels) morphologically similar to amyloid fibrils present in FAP amyloid deposits.

Toxicity of the different species observed during the TTR Leu55Pro fibril assembly process, including the initial aggregated nonfibrillar form of TTR (1-day incubation), and fibrillar forms of different lengths (6- and 15-day incubations) was assessed by caspase-3 activation on a Schwannoma cell line. Caspase-3 activation was observed only with the initial aggregates, whereas the soluble counterpart and the longer fibrils did not produce statistically significant caspase-3 activation (Figure 3B). These results confirm that nonfibrillar TTR is already toxic. Furthermore, our results showed that mature amyloid fibrils are not toxic for the cell line used.

Discussion

The aim of this work was to gain insights on the relationship between TTR deposition and FAP progression and also to evaluate the possible cytotoxicity of preamyloid forms of the protein. Abnormal self-assembly of TTR into fibrils has been implicated as the causative agent in FAP and it has been postulated that amyloid physically displaces normal elements of peripheral nerves, ultimately resulting in neuronal loss. However, it is interesting to note that in some cases, axonal and SC degeneration were reported not to be associated topographically with

Figure 2. MCSF immunohistochemistry of nerves of FAP 0 patients ($n = 4$) (**top left**; original magnification, $\times 60$); FAP 3 patients ($n = 3$) (**top middle**; original magnification, $\times 40$), and from normal individuals (**top right**; original magnification, $\times 40$). Co-localization of MCSF antigens in FAP 0 with the neuronal marker neurofilament 200 (**bottom left**; original magnification, $\times 60$). Quantitation of immunohistochemical images corresponding to M-CSF staining in control individuals and FAP patients (**bottom right**). Data are represented as percent occupied area $\pm$ SD (*, $P < 0.03$; †, $P < 0.005$).

amyloid deposition.^{1,5} The observation that axon degeneration and neuronal dysfunction may precede amyloid fibril deposition suggested a possible role to other intrinsic factors such as cytotoxicity of early nonfibrillar deposits of TTR but their presence was never demonstrated in FAP.

A previous study¹¹ of 31 FAP asymptomatic carriers was conducted, in which sural nerve biopsies were assessed for amyloid deposition, for the presence of degenerating fibers and morphometric studies of UFs and MFs. Half of the asymptomatic carriers already presented amyloid deposition and/or fiber degeneration. No clear relationship between the amount of endoneurial amyloid deposition and degree of fiber loss was observed in these individuals. Also, no evidence of nerve fiber degeneration caused by close contact with amyloid deposits was demonstrated. Therefore, there seems to be no cause-effect relationship between amyloid deposition and FAP, suggesting the importance of other factors in this disorder and/or of toxicity of small preamyloid aggregates of TTR.

In this report we show deposition of TTR in the form of small toxic nonfibrillar aggregates occurring locally before amyloid formation. Individuals with this nonfibrillar form of TTR deposition (FAP 0) are asymptomatic, have normal fiber density, and no fiber degeneration. It is interesting to note that in some FAP 0 patients, even 11 to 22 years after detection of nonfibrillar TTR in the nerve, no clinical symptoms related to FAP are detected. Given the

demonstrated toxic nature of these small aggregates, the mechanism by which axons are able to survive this injury should be further addressed in the future.

Our data indicates that neuronal stress in patients with FAP begins at a very early stage; increased expression of MCSF in peripheral nerve axons was observed before amyloid deposits were detected based on Congo Red staining (FAP 0). The increased expression of MCSF is probably related to the previously reported activation of NF- κ B by TTR fibrils and up-regulation of p50, one of the NF- κ B subunits, in FAP nerves,⁶ as MCSF is one of the targets of the NF- κ B transcription factor.¹²

We further demonstrated the cytotoxicity of prefibrillar TTR structures in cell culture assays by determining the activation of caspase-3. Supporting the concept of toxicity of nonfibrillar TTR found by immunohistochemistry, caspase-3 activation occurred only with the initial TTR aggregates and not with the soluble protein and longer fibrils. This observation resembles other amyloid-related disorders such as Alzheimer's disease. In Alzheimer's disease, the mean level of soluble A β is increased and correlates highly with markers of disease severity.^{13,14} In contrast, the level of insoluble A β is found only to discriminate Alzheimer's disease patients from controls¹³ and does not correlate with disease severity or number of amyloid plaques. These findings supported the concept of several interacting pools of A β : a large relatively static insoluble pool that is derived from a constant turning over

Figure 3. A: TEM of negatively stained TTR Leu55Pro: soluble, initial aggregates, 6- and 15-day fibrils. Scale bar, 100 nm. **B:** Activation of caspase-3 in RN22 cells exposed for 48 hours to 2 mmol/L of the different species shown in **A**: soluble TTR (sol. TTR), initial aggregates (I. aggreg.), 6-day fibrils (6d fibrils), and 15-day fibrils (15d fibrils). *, $P < 0.03$; ‡, $P < 0.003$.

of the smaller soluble pool.¹³ In the case of FAP, it has been shown that soluble TTR plasma levels are decreased in patients,¹⁵ despite an equal expression in the liver.¹⁶ Whether this decrease is because of the extracellular deposition of the mutated protein or an altered metabolism remains to be elucidated.

TTR is synthesized mainly by the liver and the choroid plexus of the brain,¹⁷ but not by the nerve, its major site of deposition. It is presently not known whether the deposited TTR derives from the plasma pool, of liver origin, and/or from the cerebrospinal fluid pool, originated by the choroid plexuses of brain, neither the mechanisms by which TTR crosses these barriers. It is possible that sensory and sympathetic nerve ganglion cells and their axons are probably constantly exposed to serum or cerebrospinal fluid proteins, even in normal cases and this fact would account for TTR access to the nerve.

The reason why this protein preferentially aggregates in this environment is however not known. TTR is a tetrameric protein of four identical subunits.¹⁸ The molecular mechanisms that convert soluble TTR tetramers into insoluble amyloid fibrils are still unknown. Dissociation of the tetramer is thought to be a prerequisite for amyloid formation *in vitro* and involvement of monomers and/or

dimers in fibril formation has been suggested.^{19,20} Quintas and colleagues²¹ observed that on dilution, at TTR concentrations compatible with the interstitial milieu where aggregation occurs, tetrameric TTR dissociates into monomeric species that then aggregate.

The close association of TTR aggregates and amyloid fibrils with SCs observed in FAP¹ might result in cellular activation and altered gene expression, as we document here for the case of MCSF. In axons of Guillain-Barre syndrome, another form of peripheral neuropathy, increased expression of molecules implicated in immune-mediated processes, such as cytokines, has already been reported.²² We hypothesize that despite the apparent lack of a primary direct deleterious effect on the SC itself, subsequent axonal degeneration may occur as a consequence of the disturbed axon-SC interaction. Interactions of SCs and axons have been the focus of much attention in other neuropathies such as Charcot-Marie-Tooth disorders.²³ It is well known that disturbed SC-axon crosstalk has an high impact on the normal physiology and survival of these two cell types. It is possible that in FAP, as a consequence of the toxicity of nonfibrillar TTR in contact with SCs, abnormal SC-axon interactions ultimately occur reflecting the pathophysiological changes associated with this disorder. However, further studies are needed to address this issue.

The study here reported is relevant to understanding molecular events leading to neurodegeneration in FAP, and as a model for neurodegeneration in other peripheral axonal neuropathies.

Acknowledgments

We thank Paul Moreira for the production and purification of recombinant TTR and Teresa Barandela for tissue processing.

References

- Coimbra A, Andrade C: Familial amyloid polyneuropathy: an electron microscope study of peripheral nerve in five cases. II. Nerve fibril changes. *Brain* 1971, 94:207-212
- Saraiva MJ: Molecular genetics of familial amyloidotic polyneuropathy. *J Peripher Nerv Syst* 1996, 1:179-188
- Saraiva MJ, Birken S, Costa PP, Goodman DS: Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type. Definition of molecular abnormality in transthyretin (prealbumin). *J Clin Invest* 1983, 74:104-119
- Coimbra A, Andrade C: Familial amyloid polyneuropathy: an electron microscope study of peripheral nerve in five cases. I. Interstitial changes. *Brain* 1971, 94:199-206
- Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM: RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature* 1996, 382:685-691
- Sousa MM, Yan SD, Stern D, Saraiva MJ: Interaction of the receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor κ B (NF- κ B) activation. *Lab Invest* 2000, 80:1101-1110
- Saraiva MJ, Costa PP, Goodman DS: Biochemical marker in familial amyloidotic polyneuropathy, Portuguese type. Family studies on the transthyretin (prealbumin)-methionine-30 variant. *J Clin Invest* 1985, 76:2171-2177

8. Andrade C: A peculiar form of peripheral neuropathy: familial atypical generalized amyloidosis with special involvement of the peripheral nerves. *Brain* 1952, 75:408–427
9. Almeida MR, Damas AM, Lans MC, Brower A, Saraiva MJ: Thyroxine binding to transthyretin Met 119. Comparative studies of different heterozygotic carriers and structural analysis. *Endocrine* 1997, 6:309–315
10. Sebastiao MP, Saraiva MJ, Damas AM: The crystal structure of amyloidogenic Leu55 → Pro transthyretin variant reveals a possible pathway for transthyretin polymerization into amyloid fibrils. *J Biol Chem* 1998, 273:24715–24722
11. Guimarães A, Viana Pinheiro A, Leite I: Sural nerve biopsy in familial amyloidotic polyneuropathy: a morphological and morphometric polyneuropathy. *Amyloid and Amyloidosis*. Edited by JB Natvig, O Forre, G Husby, A Husenbekk, B Skogen, K Sletten, P Westermark. London, Kluwer Academic Publishers, 1990, pp 493–498
12. Yao GQ, Sun BH, Insogna KL, Weir EC: Nuclear factor-kappaB p50 is required for tumor necrosis factor-alpha-induced colony-stimulating factor-1 gene expression in osteoblasts. *Endocrinology* 2000, 141:2914–2922
13. McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL: Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann Neurol* 1999, 46:860–866
14. Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, Buxbaum JD: Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *JAMA* 2000, 283:1615–1617
15. Saraiva MJ, Costa PP, Goodman DS: Studies on plasma transthyretin (prealbumin) in familial amyloidotic polyneuropathy, Portuguese type. *J Lab Clin Med* 1983, 102:590–603
16. Mita S, Maeda S, Shimada K, Araki S: Analyses of prealbumin mRNAs in individuals with familial amyloidotic polyneuropathy. *J Biochem* 1986, 100:1215–1222
17. Dickson PW, Aldred AR, Marley PD, Tu GF, Howlett GJ, Schreiber G: High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. *Biochem Biophys Res Commun* 1985, 127:890–895
18. Blake CC, Geisow MJ, Oatley SJ, Rerat B, Rerat C: Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 Å. *J Mol Biol* 1978, 121:339–356
19. Blake C, Serpell L: Synchrotron X-ray studies suggest that the core of the transthyretin amyloid fibril is a continuous-sheet helix. *Structure* 1996, 4:989–998
20. Inouye H, Domingues FS, Damas AM, Saraiva MJ, Lundgren E, Sandgren O, Kirschner DA: Analysis of X-ray diffraction patterns from amyloid of biopsied vitreous humor and kidney of transthyretin (TTR) Met30 familial amyloidotic polyneuropathy (FAP) patients: axially arrayed TTR monomers constitute the protofilament. *Amyloid* 1998, 5:163–174
21. Quintas A, Vaz DC, Cardoso I, Saraiva MJ, Brito RM: Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. *J Biol Chem* 2001, 276:27207–27213
22. Putzu GA, Figarella-Branger D, Bouvier-Labit C, Liprandi A, Bianco N, Pellissier JF: Immunohistochemical localization of cytokines, C5b-9 and ICAM-1 in peripheral nerve of Guillain-Barre syndrome. *J Neurol Sci* 2000, 174:16–21
23. Griffin JW, Sheikh K: Schwann cell-axon interactions in Charcot-Marie-Tooth disease. *Ann NY Acad Sci* 1999, 883:77–90