

Fármaco contra a paramiloidose testado em humanos

Estudo português será apresentado na Federation of American Societies for Experimental Biology Journal

Outros destaques deste dia

Duas investigadoras portuguesas testaram com sucesso em ratinhos a aplicação de um antibiótico, a doxiciclina, no tratamento da polineuropatia amiloidótica familiar (PAF) - o nome correcto da doença dos pezinhos, também designada paramiloidose. Como a terapêutica se mostrou eficaz em modelos animais, as investigadoras avançam agora para um ensaio clínico em parceria com um grupo italiano.

O trabalho de Maria João Saraiva e Isabel Cardoso, ambas do Instituto de Biologia Molecular e Celular da Universidade do Porto, deverá ser publicado no início do próximo ano na revista *Federation of American Societies for Experimental Biology Journal*.

MNI -Médicos Na Internet

24 de Novembro de 2005

The FASEB Journal • Research Communication

Doxycycline disrupts transthyretin amyloid: evidence from studies in a FAP transgenic mice model

I. Cardoso* and M. J. Saraiva*,†,1

*Molecular Neurobiology Unit, IBMC, and †Institute for Biomedical Sciences Abel Salazar, Porto University, Porto, Portugal

ABSTRACT Familial amyloidotic polyneuropathy is an autosomal dominant disorder mainly characterized by the extracellular deposition of transthyretin, with special involvement of the peripheral nerve. Several animal models have been generated, including transgenic mice carrying the most prevalent TTR mutation (TTR Val30Met). TTR-Val30Met mice without endogenous TTR (TTR-Val30Met X TTR-KO) were previously analyzed in our laboratory and 60% of the animals over 1 year of age were found to have deposition as amyloid, i.e., with Congo red (CR) -positive material, constituting a good tool to investigate the effect of drugs on TTR deposition and fibrillogenesis. We recently showed that the drug doxycycline acts *in vitro* as a TTR fibril disrupter. In the present work we assessed the activity of this drug *in vivo* in the TTR-Met30Val X TTR-KO mice. Doxycycline was administrated in the drinking water to 23- to 28-month-old mice over a period of 3 months. Immunohistochemistry analyses revealed no differences in nonfibrillar TTR deposition between treated ($n = 11$) and untreated mice ($n = 11$).

However, CR-positive material was observed only in the control group (untreated) whereas none of the animals treated with doxycycline was CR-positive. Immunohistochemistry for several markers associated with amyloid, such as matrix metalloproteinase-9 (MMP-9) and serum amyloid P component (SAP), was performed. MMP-9 was altered with significantly lower levels in treated animals compared with the control group. Mouse SAP was absent in treated animals, being observed only in untreated animals presenting TTR Congo red positive deposits. These results indicate that doxycycline is capable of disrupting TTR Congo red positive amyloid deposits and decreases standard markers associated with fibrillar deposition, being a potential drug in the treatment of amyloidosis. *FASEB J.* 20, 234–239 (2005)

Key Words: metalloproteinase-9 serum amyloid P component amyloid neuropathy treatment

familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disorder characterized by the extracellular deposition of amyloid fibrils, with a special involvement of the peripheral nerve. The age of onset of the disease is usually between 20 and 35 years of age, with fast progression to death within 10 to 15 years.

Clinically, FAP is characterized by early impairment of temperature and pain sensation in the feet, and autonomic dysfunction leading to paresis, malabsorption, and emaciation (1). The amyloid deposits can occur in any part of the peripheral nervous system, including the nerve trunks, plexus, and sensory and autonomic ganglia (2). Other organs commonly affected include the heart, vitreous, and the gastrointestinal tract. In FAP, the major component of the amyloid deposits is transthyretin (TTR); the most common variant has a valine substituted by a methionine at position 30 (TTR Val30Met). About 80 TTR mutations were identified and related to amyloid deposition (3). TTR is a 55 kDa serum homotetramer mainly synthesized in the liver and in the choroid plexus of the brain.

Amyloid deposits bind the dye Congo red (CR) resulting in a characteristic positive green birefringence when viewed under polarized light. The ultrastructural morphology of amyloid deposits is characteristically fibrillar, with an unbranched appearance, consisting of a number of filaments aggregated side-by-side, forming fibers 75–100 Å in diameter and with variable length. There is a predominant β -pleated sheet structure and extensive antiparallel strands with their axes arranged perpendicular to the longitudinal axes of the fiber. It is believed that TTR mutations destabilize the tetrameric fold, increasing the amyloidogenic potential of the protein. The first step of the amyloidogenic cascade is the dissociation of the tetramer into modified monomers, promoting the generation of intermediate species; this triggers a series of events that culminates with the formation of amyloid fibrils. In fact, in vitro, the fibrillar structures formed by TTR are polymorphic, presenting fibrils and oligomers of different diameters. The assembly dynamics evolves from oligomers of 5 or 8 nm wide to short and thin fibrils (4 nm of diameter) that twist over each other to produce mature long fibrils of 8 nm wide (4). Scan transmission electron microscopy (STEM) analysis of synthetic TTR fibrils revealed an increase of 4.7 kDa/nm between TTR fibrils with a different number of protofilaments, corresponding to a 14 kDa molecule (the monomer)

¹ Correspondence: Molecular Neurobiology, IBMC, Rua do Campo Alegre, 823, 4150-180, Porto, Portugal. E-mail: mjsaraiv@ibmc.up.pt.

doi: 10.1096/fj.05-4509com

being added to the growing fibril. In vivo, the presence of different TTR amyloidogenic species was also observed (5): early in FAP, TTR is already deposited in an aggregated nonfibrillar form, negative by CR staining; in advanced stages of the disease, these TTR aggregates coexist with mature amyloid fibrils. The same dynamics were observed in transgenic mice for human TTR Val30Met in a TTR null background, with younger animals presenting only nonfibrillar TTR deposits, especially in the gastrointestinal tract and skin but never in the peripheral nervous system; with age, short fibrils are easily observed by TEM, which are CR-positive. Furthermore, TTR initial aggregates are cytotoxic, both in vivo and in vitro, as evidenced by the presence of increased amounts of proinflammatory cytokines and oxidative stress markers, such as nitrotyrosine, in tissues of FAP patients (6) and by apoptotic assays using a Schwannoma cell line (5).

TTR amyloid deposits are not composed entirely of the amyloidogenic protein. Among others, serum amyloid P component (SAP), a universal component of amyloid fibrils, and sulfonated glycosaminoglycans (GAGs) are associated with FAP fibrils (7). During the course of the disease, an extensive remodeling of the connective tissue occurs where amyloid fibrils accumulate. Changes in proteoglycan type and distribution could possibly account at least in part for the derangement of collagen and the altered physical properties of tissues with TTR deposition. Previous findings in our lab using salivary glands and nerves from FAP patients showed by semiquantitative immunohistochemistry an increase in biglycan and matrix metalloproteinase-9 (MMP-9) as compared with normal controls (8), opening new perspectives for the role of matrix remodeling in FAP. In previous studies metalloproteinases have been implicated in other amyloidoses (9–11).

Several small molecules have been assayed with the purpose of inhibiting amyloid fibril formation. In vitro, some of them are effective against fibril formation of different amyloidogenic precursors, suggesting a common mechanism of amyloid formation. For instance, we recently showed that tetracyclines are able to disrupt TTR fibrils in vitro, although they do not inhibit their formation until a certain stage (12). Other studies had already implicated tetracyclines as agents of treatment in amyloidosis (13–15), and since they constitute a group of antibiotics with a safe profile, tetracyclines are potential drugs for the treatment of amyloidosis. In this work, we investigated the effect of doxycycline treatment in transgenic mice for TTR Val30Met.

MATERIALS AND METHODS

Animals

TTR-Val30Met mice in a TTR null background, 23–28 months of age ($n = 11$), kindly provided by Professor Suichiro Maeda from Yamaguchi University, were kept and used strictly in accordance with National and European Union guidelines for the care and handling of laboratory animals. Animals were

treated with doxycycline hydrochloride (Sigma, St. Louis, MO, USA) administrated in the drinking water (40 mg/kg/day) over 3 months. The solution was substituted every 3 days and protected from light. Another group of age-matched animals was given water alone. After 3 months, animals were killed after anesthesia with ketamine/xylazine. Esophagus, stomach, small and large intestines were immediately excised and processed. For light microscopy, tissues were fixed in 4% neutral buffered formalin and embedded in paraffin.

Immunohistochemistry

Sections 5 mm-thick were deparaffinated in xylol and dehydrated in a descendent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol and sections were blocked in 4% bovine serum and 1% bovine serum albumin in PBS. Primary antibodies used were rabbit polyclonal anti-TTR (Dako, Carpinteria, CA, USA, 1:1000), anti-nitrotyrosine (Chemicon, El Segundo, CA, USA 1:1000), anti-MMP-9 (Chemicon, 1:1000), and sheep polyclonal anti-mouse SAP (1:2000), which were diluted in blocking solution and incubated overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma), using 3-amino-9-ethyl carbazole (Sigma) or diaminobenzidine as substrates. On parallel control sections, the primary antibody was replaced by blocking buffer and by nonimmune immunoglobulins corresponding to species used in primary antibodies, namely, rabbit, and sheep; staining was absent in all the cases. For the SAP studies, besides replacing the primary antibody we also preabsorbed anti-mouse SAP (1 g) with excess antigen (10 g SAP from acute phase mouse serum, SAP 300 mg/L), or used the same amount of serum from SAP-deficient mice. These materials were kindly provided by Professor Mark Pepys from London University. Immunohistochemistry analysis was carried out independently by two pathologists unaware of the origin of the tested tissue sections. Semiquantitative immunohistochemistry (SQIHC) analysis was done with the Universal Imaging system (NIH, Bethesda, MD, USA), which performs automated particle analysis in a measured area; that is, the area occupied by pixels corresponding to the immunohistochemical substrate's color is counted and normalized relatively to the total area. In each group, 3–6 animals were analyzed and each slide used in SQ-IHC was quantified in five different selected areas. Results shown represent % occupied area \pm sd.

Congo red binding

The presence of amyloid in tissue sections was investigated after staining with Congo red and observation under polarized light (16). Briefly, deparaffinized tissues sections were incubated for 20 min with 80% ethanol saturated with NaCl followed by 0.5% Congo red in 80% ethanol saturated with NaCl, and analyzed under polarized light. Amyloid was identified by the characteristic green birefringence.

SDS-PAGE zymography

Studies by zymography were performed as described previously (8). Briefly, 10% SDS polyacrylamide gels containing gelatin (Novex Zymogram gels, Invitrogen) were used to identify MMP-9. Protein extracts were obtained by homogenizing stomachs from four doxycycline-treated animals and three untreated mice in PBS pH 7.4. Total protein was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 30 g of each extract was run per lane. After electrophoresis, gels were incubated in Novex Zymogram renaturing buffer for 30 min and then overnight

TABLE 1. TTR deposition in 23- to 28-month-old TTR-Val30Met X TTR-KO mice as evaluated by TTR immunohistochemistry (TTR) and by the presence of fibrillar CR-positive material (CR)^a

TTR-Val30Met X TTR-KO		n 11					
		I	ST	ES	Sk	K	
Sites of TTR deposition							
	Treated with Doxy	TTR	11	11	6	9	4
		CR	0	0	0	0	0
Untreated	TTR	9	11	3	8	8	
	CR	3	9	1	0	0	

^a I, intestine; ST, stomach; ES, esophagus; Sk, skin; K, kidney.

at 37°C in Novex Zymogram development buffer. Gels were then stained with 0.5% Coomassie blue in 40% methanol, 10% acetic acid for 2 h, and destained in 40% methanol, 10% acetic acid for 1 h. MMP-9 was identified based on its molecular weight and its well-established running profile on SDS-PAGE zymography.

RESULTS

Toxicity

We observed no difference in body weight between mice treated with doxycycline and untreated animals, and there was no obvious increase in mortality between the two groups of animals.

Assessment of TTR deposition by immunohistochemistry

We first analyzed the effect of doxycycline on TTR deposition by performing TTR immunohistochemistry in different organs. Untreated animals ($n = 11$) presented widespread TTR staining, particularly in the gastrointestinal tract, confirming previous results obtained in our laboratory (17). All animals had TTR deposition in the stomach, 81% (9/11) presented TTR deposited in the intestine, and 73% (8/11) in the skin and kidney (Table 1, Fig. 1A, middle panel). However, we found no significant differences in TTR load in animals treated with doxycycline: all animals showed TTR deposition in the stomach (11/11), in a similar fashion to the untreated animals (Table 1, Fig. 1A, left panel). We also observed TTR deposits in the intestine of these animals (11/11), esophagus (6/11), skin (9/11), and kidney (4/11) (Table 1).

Assessment of amyloid deposition by Congo red staining

Analysis of TTR deposition by immunohistochemistry refers to the total amount of deposited TTR, both in nonfibrillar (initial aggregates) and fibrillar forms (17). To determine only the fibrillar portion of deposited

TTR, we next performed CR staining, which is specific for mature amyloid fibrils. In the group of untreated animals ($n = 11$), 10 of the animals presented fibrillar CR-positive material in stomach and/or in esophagus and intestine (Table 1, Fig. 1A, right panel). Surprisingly, none of the tissues of the treated animals had CR-positive staining (not shown). These results imply that doxycycline is able to disrupt mature amyloid fibrils but does not destroy TTR aggregates.

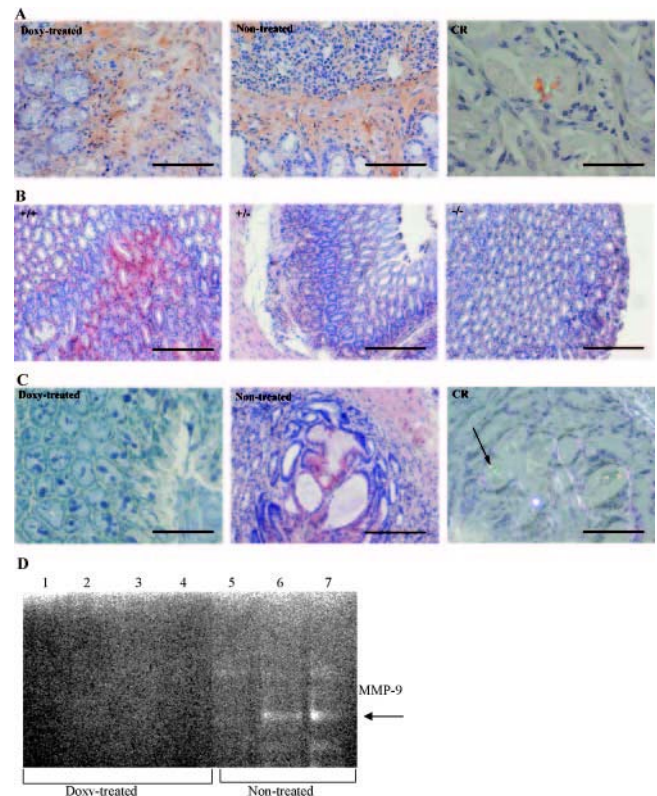


Figure 1 A) Immunohistochemistry for human TTR and CR binding in treated and nontreated transgenic mice. *Left panel*) Stomach slide representative of mice treated with doxycycline 40 mg/Kg/day for 3 months: TTR deposition presents similar load to the nontreated animals (*middle panel*). Congo red-positive TTR immunoreactive deposits were only observed in the control nontreated group (*right panel*). Scale bar: 100 μ m (*left and middle panels*); 50 μ m (*right panel*). B) MMP-9 levels in treated and nontreated transgenic mice. *Left panel*) Significant levels of MMP-9 were observed by immunohistochemistry in stomach of transgenic mice with TTR amyloid deposits (/) whereas animals with nonfibrillar TTR deposition (/ , *middle panel*) or no deposition (/ , *right panel*) reveal no significant MMP-9 amounts. Scale bar: 200 μ m. C) After treatment with doxycycline 40 mg/Kg/day for 3 months, MMP-9 levels are drastically reduced (doxy-treated, *left panel*) whereas nontreated animals of the same age showed very strong staining for MMP-9 (nontreated, *middle panel*) which colocalizes with TTR amyloid deposits (CR, arrow, *right panel*). Scale bar: 100 μ m (*left panel*); 200 μ m (*middle and right panels*). D) Gelatin zymography of protein extracts obtained from stomachs of TTR transgenic mice treated with doxycycline (lanes 1–4) and nontreated animals (lanes 5–7). 30 μ g of total protein were loaded on the gel.

Metalloproteinase-9 (MMP-9)

Studies of the levels of MMP-9 in tissues from FAP patients with amyloid deposits indicated an increased amount of this metalloproteinase when compared with normal individuals (8) but not in tissues with nonfibrillar deposits. Thus, we investigated a possible correlation between nonfibrillar TTR/amyloid deposition and MMP-9 levels in the transgenic mouse strain under study.

First, tissues of transgenic mice were selected for TTR deposition by immunohistochemistry and for CR binding and classified as (/) if TTR and CR positive; (/) if TTR positive but CR negative, and (/) if neither TTR deposition nor green birefringence after CR binding were observed. We next evaluated the levels of MMP-9 and found significant levels of this metalloproteinase associated with stomachs of (/) animals (Fig. 1B, left panel) whereas stomachs of mice (/) and (/) presented negligible levels of the metalloproteinase (Fig. 1B, middle and right panels). Thus, MMP-9 up-regulation occurred only in amyloid laden tissues, confirming data on clinical samples.

We next assessed the levels of MMP-9 in the stomachs of the doxycycline-treated and untreated animals, where differences in CR staining were previously observed (see Table 1). In the control group, MMP-9 stained intensely in the stomach (Fig. 1C, middle panel), particularly at sites of amyloid deposition (Fig. 1C, right panel), whereas animals treated with doxycycline presented negligible levels of MMP-9 throughout the analyzed tissues ($n = 11$), (Fig. 1C, left panel). The presence of higher amounts of MMP-9 in the stomachs of untreated animals was further confirmed by zymography (Fig. 1D).

SAP levels

SAP undergoes calcium-dependent binding to amyloid fibrils in vitro (18) and binds to amyloid deposits in vivo (19). It is also known that SAP protects amyloid fibrils (20), including TTR fibrils (8), from proteolytic cleavage. We evaluated the presence of mouse SAP in TTR deposits in animals treated with doxycycline and com-

pared with the untreated group, using stomach and intestine slides; we first evaluated SAP levels by immunohistochemistry in control mice with and without TTR deposition. Only animals with amyloid (/) showed significant levels of SAP in the stomach whereas animals with nonfibrillar (/) and without (/) deposition presented negligible levels of SAP (Fig. 2A, upper panels). We further pursued this investigation by performing colocalization studies of deposited TTR and SAP. Our results showed colocalization of SAP only with TTR amyloid deposits, which generate green birefringence after CR binding, but not with nonfibrillar TTR deposits. The specificity of SAP immunoreactivity was assessed in preabsorption assays using either acute phase mouse serum or serum from SAP-deficient mice (not shown).

As for the mice under study, doxycycline-treated mice showed no significant amounts of SAP in tissues (Fig. 2B). In contrast, untreated animals presented levels of SAP highly increased in the stomach comparable to control mice classified as (/). The observation that SAP is present only in amyloid-laden tissues agrees with the literature, defining SAP as a universal amyloid component.

Since doxycycline-treated mice did not present SAP in the stomach (or in any other organ), the results indicate that amyloid fibrils were disaggregated and lost their ability to bind SAP.

DISCUSSION

In FAP, TTR deposits in several organs, leading to dysfunction and death. TTR accumulates extracellularly as aggregates and, in latter stages of disease progression, as amyloid fibrils. The initial aggregates (prefibrillar material, CR-negative) were shown to induce caspase-3 activation and apoptosis in vitro (5). Furthermore, authors have also demonstrated that in FAP, TTR aggregates are cytotoxic (5, 6) and signs of increased oxidative stress are observed early in FAP (5). Several transgenic mice carrying the human TTR Val30Met gene have been created with the aim of studying the mechanism of amyloid formation and

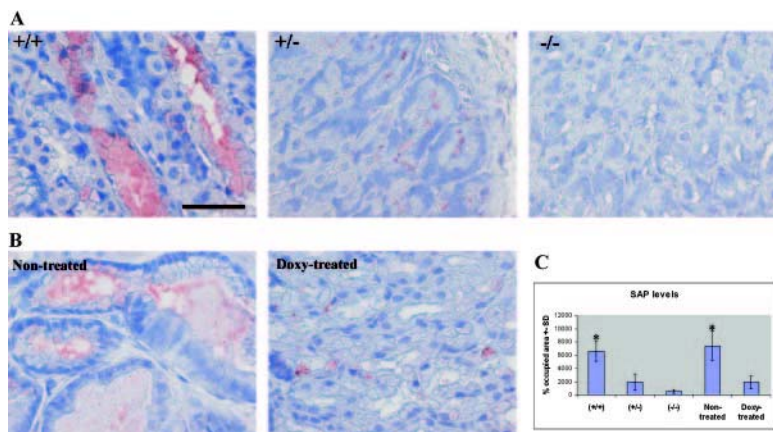


Figure 2 Evaluation of SAP levels in treated and nontreated transgenic mice. *Upper panels*) SAP staining was performed in stomachs of TTR transgenic mice and shown to be present only in animals with fibrillar TTR deposition (/ , *left panel*), whereas mice with nonfibrillar TTR deposition (/ , *middle panel*) or without deposits (/ , *right panel*) had no significant levels of SAP. *Lower panels*) Nontreated transgenic mice also showed great amounts of SAP (nontreated, *left panel*) which lowered to background levels in animals treated with doxycycline (Doxycycline-treated, *right panel*). Scale bar: 50 μ m. Histogram: Quantification of SAP staining in stomachs of the analyzed animals: (/ , $n = 3$), (/ , $n = 5$) and (/ , $n = 3$) and in animals nontreated ($n = 6$) or treated with doxycycline (doxy-treated, $n = 6$). * $P < 0.05$.

factors affecting TTR deposition. Sousa et al. studied TTR deposition in TTR Val30Met transgenic mice showing the presence of prefibrillar TTR, deposited mainly in the gastrointestinal tract and skin (17), which evolved to amyloid CR-positive deposits with age. Thus, this animal model is suitable for the study of TTR amyloidosis and factors affecting its development, including drugs that revealed active in vitro.

Tetracyclines affect many mammalian cell functions including proliferation, migration, apoptosis, and matrix remodeling (21). It was recently shown that minocycline, a derivative of tetracycline able to cross the blood-brain barrier, exerts a neuroprotective effect in a transgenic model of Huntington disease (22) and in mice with amyotrophic lateral sclerosis (ALS) (23). We had shown that tetracyclines interfere with TTR fibrillogenesis in vitro (12), acting as fibril disrupters, and that the resulting species are nontoxic as evaluated by caspase-3 quantification in a Schwannoma cell line. Prior to that work, other groups reported doxycycline activity against A-beta (14), prion protein (13). In the present study, tissue analysis by CR staining indicated that doxycycline was able to disaggregate TTR amyloid mature fibrils since no CR green birefringence was observed in tissues from animals treated with the drug. In contrast, in the untreated animals 81% presented CR positive material, namely, in the stomach. Our results also suggest that doxycycline is not able to act on nonfibrillar deposits, as the overall TTR load was similar in both groups of animals. These findings agree with the in vitro results showing that doxycycline was not capable of inhibiting TTR fibril formation until a certain fibril length but was very efficient at disrupting already formed fibrils (12).

We investigated levels of nitrotyrosine (NT) staining in untreated and in doxycycline-treated animals and found no significant differences (data not shown). Because animals treated with doxycycline showed comparable levels of TTR load (nonfibrillar, CR-negative) to untreated animals, the NT results were somehow expected. Moreover, it further supports that doxycycline and/or doxycycline-treated fibrils are nontoxic to cells, which is also in accordance with the in vitro data (12).

SAP is a universal component of amyloid deposits. It is thought that this molecule contributes to amyloidogenesis, probably by stabilizing amyloid fibrils and retarding their clearance (24). SAP efficiently protects amyloid fibrils from proteolysis in vitro and may contribute to persistence of amyloid in vivo (20, 25). Furthermore, several reports indicate that although not essential in amyloid deposition, SAP significantly accelerates the reaction (26, 27). In line with these observations, SAP has been proposed as a possible target in therapeutic strategies in amyloidosis (20, 24, 28, 29). In our study, the evaluation of SAP levels in mice treated with doxycycline revealed a drastic decrease relative to untreated animals, suggesting that fibrils have been disrupted as a result of the treatment. We concluded that SAP binds to TTR fibrillar deposits but not to

prefibrillar aggregates, further suggesting that fibrils had been disaggregated in the tissues of treated mice. MMP-9 degrades extracellular matrix (ECM) components and regulates the activity of a number of soluble proteins, thus indicating a role in several physiological processes, including degeneration in the peripheral nervous system (PNS) (30). In Alzheimer's disease, MMP-9 is increased and detected in close proximity to extracellular amyloid plaques, in the cytoplasm of neurons, and in neurofibrillar tangles and vascular walls (30); amyloid- β (A β) peptide induces the synthesis, release, and activation of MMP-9 in murine cerebral endothelial cells, resulting in increased extracellular matrix (ECM) degradation (31). In FAP tissues it has been observed that biglycan, NGAL, and MMP-9 were increased relative to control samples (8). While biglycan was already increased in FAP0 human nerves (with prefibrillar TTR aggregates), NGAL and MMP-9 were only evident in tissues with fibrillar material. In a similar fashion, in our study, stomachs presenting fibrillar TTR showed increased levels of MMP-9 relative to animals exhibiting only prefibrillar TTR or no TTR deposition. Furthermore, MMP-9 was particularly overexpressed in sites of amyloid deposition (Fig. 1C), suggesting a relationship between amyloid formation and metalloproteinases expression, in particular of MMP-9. Regarding mice treated with doxycycline, a great decrease in MMP-9 levels was observed, indicating to some extent matrix remodeling, probably due to degradation of amyloid fibrils by doxycycline. However, since several reports indicate doxycycline as a MMP-9 inhibitor (32–35), we cannot ascertain whether the observed reduced MMP-9 activity was due to the direct action of doxycycline on the metalloproteinase, or to fibril disaggregation by this drug, with consequent matrix recovery and MMP-9 decrease.

Altogether, our findings reveal for the first time the beneficial effects of doxycycline in vivo in a FAP model, promoting this drug as a promising molecule in the treatment of this and other amyloidosis, and paving the way for future clinical trials. Moreover, our results showed that the animal model used mimics the human disease features, concerning MMP-9 and SAP markers.



We thank Rossana Correia for tissue processing, Margot Fairbanks and Ruben Fernandes for their help in immunohistochemical analysis. We are also very grateful to Dr. Glennys Tennent for helpful discussions concerning the SAP experiments. This work was supported by the Portuguese Foundation for Science and Technology (FCT) through a fellowship to Cardoso I (SFRH/BPD/9416/2002) and a POCTI grant, and by the Gulbenkian Foundation.

REFERENCES

1. Andrade, C. (1952) A peculiar form of peripheral neuropathy; familial atypical generalized amyloidosis with special involvement of the peripheral nerves. *Brain* **75**, 408–427
2. Coimbra, A., and Andrade, C. (1971) Familial amyloid polyneuropathy: an electron microscope study of the peripheral nerve in five cases. *Brain* **94**, 199–206

3. Saraiva, M. J., Sousa, M. M., Cardoso, I., and Fernandes, R. (2004) Familial amyloidotic polyneuropathy: protein aggregation in the peripheral nervous system. *J. Mol. Neurosci.* **23**, 35–40
4. Cardoso, I., Goldsbury, C. S., Müller, S. A., Olivieri, V., Wirtz, S., Damas, A. M., Aebi, U., and Saraiva, M. J. (2002) Transthyretin fibrillogenesis entails the assembly of monomers: A molecular model for in vitro assembled transthyretin amyloid-like fibrils. *J. Mol. Biol.* **317**, 683–695
5. Sousa, M. M., Cardoso, I., Fernandes, R., Guimaraes, A., and Saraiva, M. J. (2001) Deposition of transthyretin in early stages of familial amyloidotic polyneuropathy: evidence for toxicity of nonfibrillar aggregates. *Am. J. Pathol.* **159**, 1993–2000
6. Sousa, M. M., Du Yan, S., Fernandes, R., Guimaraes, A., Stern, D., and Saraiva, M. J. (2001) Familial amyloid polyneuropathy: receptor for advanced glycation end products-dependent triggering of neuronal inflammatory and apoptotic pathways. *J. Neurosci.* **21**, 7576–7586
7. Inoue, S., Kuroiwa, M., Saraiva, M. J., Guimaraes, A., and Kisilevsky, R. (1998) Ultrastructure of familial amyloid polyneuropathy amyloid fibrils: examination with high-resolution electron microscopy. *J. Struct. Biol.* **124**, 1–12
8. Sousa, M. M., do Amaral, J. B., Guimaraes, A., and Saraiva, M. J. (2005) Up-regulation of the extracellular matrix remodeling genes, biglycan, neutrophil gelatinase-associated lipocalin, and matrix metalloproteinase-9 in familial amyloid polyneuropathy. *FASEB J.* **19**, 124–126
9. Helbecque, N., Hermant, X., Cotel, D., and Amouyel, P. (2003) The role of matrix metalloproteinase-9 in dementia. *Neurosci. Lett.* **350**, 181–183
10. Campistol, J. M., Shirahama, T., Abraham, C. R., Rodgers, O. G., Sole, M., Cohen, A. S., and Skinner, M. (1992) Demonstration of plasma proteinase inhibitors in beta 2-microglobulin amyloid deposits. *Kidney Int.* **42**, 915–923
11. Stix, B., Kahne, T., Sletten, K., Raynes, J., Roessner, A., and Rocken, C. (2001) Proteolysis of AA amyloid fibril proteins by matrix metalloproteinases-1, -2, and -3. *Am. J. Pathol.* **159**, 561–570
12. Cardoso, I., Merlini, G., and Saraiva, M. J. (2003) 4'-iodo-4'-Deoxydoxorubicin and tetracyclines disrupt transthyretin amyloid fibrils in vitro producing noncytotoxic species. Screening for TTR fibril disrupters. *FASEB J.* **17**, 803–809
13. Tagliavini, F., Forloni, G., Colombo, L., Rossi, G., Girola, L., Canciani, B., Angeretti, N., Giampaolo, L., Peressini, E., Awan, T., et al. (2000) Tetracycline affects abnormal properties of synthetic PrP peptides and PrP(Sc) in vitro. *J. Mol. Biol.* **300**, 1309–1322
14. Forloni, G., Colombo, L., Girola, L., Tagliavini, F., and Salmona, M. (2001) Anti-amyloidogenic activity of tetracyclines: studies in vitro. *FEBS Lett.* **487**, 404–407
15. De Felice, F. G., Houzel, J. C., Garcia-Abreu, J., Louzada, P. R., Jr., Afonso, R. C., Meirelles, M. N., Lent, R., Neto, V. M., and Ferreira, S. T. (2001) Inhibition of Alzheimer's disease beta-amyloid aggregation, neurotoxicity, and in vivo deposition by nitrophenols: implications for Alzheimer's therapy. *FASEB J.* **15**, 1297–1299
16. Puchtler, H., and Sweat, F. (1965) Congo Red as a stain for fluorescence microscopy of amyloid. *J. Histochem. Cytochem.* **13**, 693–694
17. Sousa, M. M., Fernandes, R., Palha, J. A., Taboada, A., Vieira, P., and Saraiva, M. J. (2002) Evidence for early cytotoxic aggregates in transgenic mice for human transthyretin. *Am. J. Pathol.* **161**, 1935–1948
18. Pepys, M. B., Dyck, R. F., de Beer, F. C., Skinner, M., and Cohen, A. S. (1979) Binding of serum amyloid P-component (SAP) by amyloid fibrils. *Clin. Exp. Immunol.* **38**, 284–293
19. Baltz, M. L., Caspi, D., Evans, D. J., Rowe, I. F., Hind, C. R., and Pepys, M. B. (1986) Circulating serum amyloid P component is the precursor of amyloid P component in tissue amyloid deposits. *Clin. Exp. Immunol.* **66**, 691–700
20. Pepys, M. B., Tennent, G. A., Booth, D. R., Bellotti, V., Lovat, L. B., Tan, S. Y., Persey, M. R., Hutchinson, W. L., Booth, S. E., Madhoo, S., et al. (1996) Molecular mechanisms of fibrillogenesis and the protective role of amyloid P component: two possible avenues for therapy. *Ciba Found. Symp.* **199**, 73–78
21. Bendeck, M. P., Conte, M., Zhang, M., Nili, N., Strauss, B. H., and Farwell, S. M. (2002) Doxycycline modulates smooth muscle cell growth, migration, and matrix remodeling after arterial injury. *Am. J. Pathol.* **160**, 1089–1095
22. Chen, M., Ona, V. O., Li, M., Ferrante, R. J., Fink, K. B., Zhu, S., Bian, J., Guo, L., Farrell, L. A., Hersch, S. M., et al. (2000) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat. Med.* **6**, 797–801
23. Zhu, S., Stavrovskaya, I. G., Drozda, M., Kim, B. Y. S., Ona, V., Li, M., Sarang, S., Liu, A. S., Hartley, D. M., Wu, D. C., et al. (2002) Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature (London)* **417**, 74–78
24. Pepys, M. B. (2001) Pathogenesis, diagnosis and treatment of systemic amyloidosis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**, 203–210
25. Tennent, G. A., Lovat, L. B., and Pepys, M. B. (1995) Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis. *Proc. Natl. Acad. Sci. USA* **92**, 4299–4303
26. Maeda, S. (2003) Use of genetically altered mice to study the role of serum amyloid P component in amyloid deposition. *Amyloid Suppl.* **1**, 17–20
27. Botto, M., Hawkins, P. N., Bickerstaff, M. C., Herbert, J., Bygrave, A. E., McBride, A., Hutchinson, W. L., Tennent, G. A., Walport, M. J., and Pepys, M. B. (1997) Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid P component gene. *Nat. Med.* **3**, 855–859
28. Pepys, M. B., Herbert, J., Hutchinson, W. L., Tennent, G. A., Lachmann, H. J., Gallimore, J. R., Lovat, L. B., Bartfai, T., Alanine, A., Hertel, C., et al. (2002) Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. *Nature (London)* **417**, 254–259
29. Gillmore, J. D., Madhoo, S., Pepys, M. B., and Hawkins, P. N. (2000) Renal transplantation for amyloid end-stage renal failure—insights from serial serum amyloid P component scintigraphy. *Nucl. Med. Commun.* **21**, 735–740
30. Hughes, P. M., Wells, G. M., Perry, V. H., Brown, M. C., and Miller, K. M. (2002) Comparison of matrix metalloproteinase expression during Wallerian degeneration in the central and peripheral nervous systems. *Neuroscience* **113**, 273–287
31. Lee, J. M., Yin, K. J., Hsin, I., Chen, S., Fryer, J. D., Holtzman, D. M., Hsu, C. Y., and Xu, J. (2003) Matrix metalloproteinase-9 and spontaneous hemorrhage in an animal model of cerebral amyloid angiopathy. *Ann. Neurol.* **54**, 379–382
32. Fernandez, F. G., Campbell, L. G., Liu, W., Shipley, J. M., Itoharu, S., Patterson, G. A., Senior, R. M., Mohanakumar, T., and Jaramillo, A. (2005) Inhibition of obliterative airway disease development in murine tracheal allografts by matrix metalloproteinase-9 deficiency. *Am. J. Transplant.* **5**, 671–683
33. Koistinaho, M., Malm, T. M., Kettunen, M. I., Goldsteins, G., Starckx, S., Kauppinen, R. A., Opdenakker, G., and Koistinaho, J. (2005) Minocycline protects against permanent cerebral ischemia in wild type but not in matrix metalloproteinase-9-deficient mice. *J. Cereb. Blood Flow Metab.* **25**, 460–467
34. Lee, C. Z., Xu, B., Hashimoto, T., McCulloch, C. E., Yang, G. Y., and Young, W. L. (2004) Doxycycline suppresses cerebral matrix metalloproteinase-9 and angiogenesis induced by focal hyperstimulation of vascular endothelial growth factor in a mouse model. *Stroke* **35**, 1715–1719
35. Duijvenvoorden, W. C., Hirte, H. W., and Singh, G. (1997) Use of tetracycline as an inhibitor of matrix metalloproteinase activity secreted by human bone-metastasizing cancer cells. *Invasion Metastasis* **17**, 312–322

Received for publication June 14, 2005.
Accepted for publication October 13, 2005.