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Investigation of AGE, their receptor and NF-κB activation and apoptosis in patients with ATTR and Gelsolin amyloidosis

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Summary. Background: Transthyretin (TTR) and gelsolin amyloidoses represent two types of hereditary amyloidosis in which point mutations in the respective protein lead to conformational changes of the protein with subsequent amyloid fibril formation. Material and methods: Tissues from Finnish gelsolin amyloid patients, Danish, Japanese and Swedish ATTR patients were immunostained for AGE, RAGE, NF-κB, PARP, and caspases 3 and 8. Results: Amyloid was heavily deposited in myocard, kidney and gastrointestinal tract of all patients. Immunoreactive areas to AGE and RAGE were detected in the heart, kidney, rectum, gut and appendix. AGE and RAGE were well co-localised with amyloid deposits. In five out of 14 patients neither NF-κB activation nor induction of apoptosis marked by positive immunostaining for NF-κB, PARP, or caspases 3 and 8 was found, and markers of apoptosis were detected in some samples without accompanying NF-κB activation. Conclusion: Our results suggest that both AGE and RAGE may have a common role in evolution of TTR and gelsolin-related amyloidoses. Apart from AGE-RAGE interactions both amyloid proteins may directly bind to RAGE and result in cellular perturbations; but in view of this study cytotoxic effects other than those triggered by activation of NF-κB or apoptosis should be considered.

Key words: Amyloidosis, NF-κB, Apoptosis, Rage and immunohistochemistry

Introduction

Amyloidosis is not a homogeneous disease, but rather a heterogeneous group of diseases characterized by deposition of proteinaceous fibrils in different tissues. There are many classification systems for amyloidosis, but systemic amyloidoses are often divided into hereditary and non-hereditary forms. The most common types among the non-hereditary are the primary type (AL amyloidosis), caused by monoclonal light chain producing plasma cells, where renal, cardiac and neurological symptoms dominate the clinical picture (Sanchorawala, 2006) and the secondary (AA amyloidosis) that develops as a complication of chronic inflammatory diseases such as rheumatic arthritis (Picken, 2006). The clinical manifestation is often represented by renal symptoms (Picken, 2006).

The inherited amyloidoses are caused by mutations in a specific protein (Benson, 2003), most commonly transthyretin (TTR), but among many other proteins also mutated gelsolin can lead to a systemic amyloidosis. To date, at least 80 amyloid-associated TTR-mutations (ATTR) have been found which typically result in autosomally dominantly inherited systemic TTR amyloidosis (Andrade, 1952). TTR functions as a transport protein for thyroid hormone and, via a complex with retinol-binding protein, vitamin A (Robbins, 1976). The most common neuropathic form of ATTR is familial amyloidosis/amyloidotic polyneuropathy (FAP) which is caused by a point mutation of TTR, where valine is replaced by methionine at position 30 (ATTR Val30Met)

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Abbreviations. AGE: advanced glycation end products; TTR: transthyreti; RAGE: Receptors for AGEs; FAP: familial amyloidosis polyneuropathy.

(Saraiva et al., 1983). Endemic areas with a high prevalence of FAP are found in Portugal, Japan and Sweden. The most dominant clinical symptoms are neuropathy and gastrointestinal symptoms with diarrhoea, constipation, vomiting and nausea (Suhr et al., 1992), but cardiomyopathy (Olofsson, 1983), nephropathy (Lobato et al., 2003) and vitreous opacities (Kawaji et al., 2004) are also common complications.

The Danish type of TTR amyloidosis is caused by a point mutation where methionine replaces leucine at position 111 (ATTR Leu111Met) (Svendsen et al., 1999). The main clinical manifestation is severe restrictive cadiomyopathy which leads to death within a few years due to cardiac failure (Ranløv et al., 1992). In contrast, the Finnish gelsolin amyloidosis is caused by a single amino acid substitution, at position 187, of asparagines for aspartic acid of gelsolin protein, the principal actin-modulating protein expressed in most tissues (Maury et al., 1990). The main clinical manifestations are corneal lattice dystrophy, progressive cranial and peripheral neuropathy and skin changes (Kiuru, 1998).

Advanced glycation end products (AGEs) accumulate in the kidneys of FAP patients and other amyloid types such as beta 2 microglobulin and diabetes patients and are implicated in the development of diabetic nephropathy and vasculopathy and renal failure in FAP (Brownlee et al., 1988; Ruderman et al., 1992; Schmidt et al., 1995; Lobato et al., 1998; Matsunaga et al., 2005). AGE is generated by sequential nonenzymatic glycation and oxidation of amino groups in long-lived proteins, lipids and nucleic acids through a series of reactions forming Schiff bases and Amadori products (Singh et al., 2001). Receptors for AGEs (RAGE) are present on a wide range of cells, such as smooth muscle cells, monocytes, macrophages, endothelial cells, podocytes, astrocytes and microglia (Thornalley, 1998). The binding of AGE to RAGE activates intra-cellular pathways that lead to an increase of the oxidative levels of the cells, and activation of the transcription factor NF-κB (Lander et al., 1997) may induce liberation of cytokines and growth factors by monocytes. Besides the cellular response, AGE can form a pathological cross-link formation with proteins, leading to sclerosis of renal glomeruli, thickening of the capillary basement membrane and development of atherosclerosis (Monnier et al., 1996).

Recently, it was reported that TTR fibrils could interact with RAGE, leading to damage of neuronal cells by promoting inflammatory cytokines, NF-kB and caspase-3 activation (Sousa et al., 2000). We have demonstrated that AGEs and RAGE are found in neural ganglia and blood vessels of the gastrointestinal tract in FAP patients, even though we were unable to find an activation of NF-κB and apoptosis (Matsunaga et al., 2002). However, AGE-RAGE interactions on the cell surface seem to be involved in amyloid toxicity in peripheral nerves, kidneys and gastrointestinal dysfunction of FAP patients, even though this involvement in gastrointestinal tract and kidneys may be through other pathways than those involving NF-κB.

To address the question whether AGEs are involved in the pathogenesis of cardiac failure in patients with different ATTR-mutations, we investigated Danish ATTR Leu111Met patients and Japanese and Swedish ATTR Val30Met patients. AGE involvement in the pathogenesis of kidney failure and gastrointestinal dysfunction in patients with TTR and gelsolin amyloidosis was investigated in heart, kidney and gastrointestinal tissues. Immune staining for AGE, amyloid deposits, RAGE, NF-κB and caspases were performed to disclose the presence of AGE and activation of RAGE, NF-κB and of apoptotic pathways.

Materials and methods

Subjects and specimens

Tissue specimens from four Danish ATTR Leu111Met patients (2 females and 2 males; mean age 51 years, range 48-55), five Finnish gelsolin ASP187Asn amyloidosis patients (3 females and 2 males; mean age 66 years, range 52-79) and 3 Japanese and 2 Swedish ATTR Val30Met patients (2 females and 3 males, mean age 55, range 41-79 years) were available for the study. Histological sections were prepared from heart (9 patients), appendix (1 patient), gut (1 patient), kidney (4 patients), and rectum (2 patients). The clinical features of the subjects are summarised in Table 1.

Histopathological examination

The tissues were fixed in 4% buffered formaldehyde, embedded in paraffin wax and cut at 5 μm. Sections were stained with Mayer's haematoxylin for histopathological examination and with alkaline Congo red for detection of amyloid deposits.

Immunohistochemistry

Specimens were immunostained by the avidin-biotin complex (ABC) method (Dako A/S, Glostrup, Denmark) as described previously (Anan et al., 2000); the employed primary antibodies are listed in detail in Table 2. Briefly, following microwave antigen retrieval (Nyhlin et al., 1997) (for AGE and RAGE only) the hydrogen peroxide (H_2O_2) 0.3% was applied on all sections for 10 minutes in order to inhibit the endogenous peroxidase activity and incubated with 1% bovine serum albumin for 10 min. This was followed by incubation overnight at room temperature with the primary antibodies. The sections were then incubated with secondary antibodies for 30 min, thereafter with the avidin-biotin-peroxidase complex for 30 min, and finally with 3,3'-diamino-benzidine/ H_2O_2 for 7 min at room temperature, and counter-stained with Mayer's Haematoxylin. As a negative control, the primary antibody was replaced by Tris buffer.

To demonstrate the association between amyloid

deposition and RAGE, AGE, NF-κB and apoptotic markers, consecutive sections were stained with alkaline Congo red. Amyloid distribution was examined under polarized light.

Results

Amyloid deposits

Amyloid was heavily deposited in the myocardium and kidney of the ATTR Leu111Met patients. In the kidney amyloid was detected in the glomeruli (Table 3; Fig. 3), tubuli (Table 3, Fig. 4) and blood vessels. In five patients out of nine, amyloid was heavily deposited in the myocardium (Table 3, Fig. 2). The remaining four patients had moderate amyloid deposition in the myocardium (Table 3).

The gelsolin amyloid specimens showed amyloid infiltration in the appendix, rectum, gut and kidney (Table 3, Figs. 1, 3, 4). In the appendix the amyloid deposits were mostly located in the walls of blood vessels. In the gut (Fig. 1) amyloid deposits were detected around blood vessels, whereas the rectum showed amyloid infiltration also in the muscularis mucosae. In the kidney, amyloid was deposited in glomeruli (Fig. 3), tubuli (Fig. 4) and blood vessels, and showed the same pattern as in the ATTR Leu111Met material.

Amyloid infiltration was found in the myocardium of all ATTR Val30Met patients (Table 3). One of the Japanese and both Swedish patients showed massive amyloid infiltration of the myocardium, whereas the remaining two Japanese patients had moderate amyloid depositions in the myocardium (Table 3).

AGE immunoreactivity

Immunoreactivity for AGE was detected in the heart (Fig. 2), kidney (Figs. 3, 4), rectum, gut (Fig. 1) and appendix (Table 3) of all patients. In the heart of five patients, AGE immunoreactive areas showed a strong staining pattern in most of the myocard and around the blood vessels. In the other four patients, AGE immunoreactivity was detected in some areas of the

Table 1. Clinical data of patients with Famiilial amyloidotic polyneuropathy, Finnish type, transthyretin Met 111 and Met 30.

Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mutation type	Met 111	Met 111	Met 111	Met 111	Met 30	Met 30	Met 30	Met 30	Met 30	Gelsolin	Gelsolin	Gelsolin	Gelsolin	Gelsolin
Sex	F	M	M	F	F	M	F	M	M	M	F	F	F	M
Age (years)	48	55	51	49	43	61	41	57	75	71	66	79	52	61
Duration of the disease (years)	3	5	3	3	11	9	11	21	6	15	Na	Na	19	16
GI-symptoms	0	0	0	0	D	D	D	C+D	C+D	Na	Na	C	Episodic C+D	0
Orthostatic hypotension	0	0	0	0	+	+	+	0	0	Na	+	+	+	+
Bladder* dysfunction	0	0	0	0	+	+	+	+	0	Na	Na	Na	Na	+
Cardiac symptoms	CHF	CHF	CHF	CHF	Na	Na	Na	0	0	ICC	0	Na	0	ACM
Sensory≠	0	0	0	0	++	+	+++	++	++	++	Na	++	+	++
Motor‡	0	0	0	0	+++	++	+++	++	++	(+)Na	Na	0	0	+
Eye symptoms	0	0	0	0	Dry eye	Dry eye	Dry eye	0	0	CLD Dry eye	CLD, blindness	CLD, Decreased vision	CLD, Decreased vision	CLD, Decreased Vision, dry eye
Kidney symptoms	0	0	0	0	Na	Na	Na	0	0	proteinuria	Proteinuria	Na	0	0
others										CL, CN Confusion Hypothyroidism	CL, CN Paranoia	CL, CN Confusion	CL, CN	CL, CN, AM, CTS

Met: Methionine; F: female; M: male; GI: gastrointestinal; Na: data not available; C: constipation; D: Diarrhoea; CHF: Congestive heart failure; ICC: Impaired cardiac conduction; ACM: Amyloid cardiomyopathy; CLD: Corneal lattice dystrophy; CL: Cutis Laxa; CN: cranial neuropathy; AM: Amyloid myopathy; CTS: carpal tunnel syndrome; * graded into four grades: 0, normal; +, Mild; ++, Moderate; +++, Severe; ≠, Normal; +, Below the noble; ++, Below the face; +++, Including face; ‡: +, Lower limbs; ++, Lower limbs and mild upper; +++, Moderately impaired in four limbs; ++++, Severe impaired in four limbs.

Table 2. Primary antisera used. *RAGE* receptor for advanced glycation end products, *AGE* (*6D12*) advanced glycation end products; NÂ-(carboxymethyl)lysine, *PARP p85* 85kDa caspase-cleaved fragment of human poly (ADP-ribose) polymerase

Antibody against	Dilution	Poly/Monoclonal	Species	Code no.	Source, References
RAGE	1:200	Polyclonal	Goat	AB5484	Chemicon, Temelula, CA
CML	1:300	Monoclonal	Mouse	6D12	TransGenic Inc, Japan
NF-κB	1:200	Monoclonal	Mouse	MAB3026	Chemicon, Temelula, CA
PARP p85	1:100	Polyclonal	Rabbit	G7341	Promega, Madison, WI
Caspase 3	1:200	Polyclonal	Rabbit	9661	Cell signalling Technology
Caspase 8	1:50	Monoclonal	Mouse	9748	Cell signalling Technology

Table 3. Summary of immunostaining for AGE, RAGE, NFκB, PARP and caspases in ATTR Leu111Met, Val30Met and gelsolin amyloid material.

Patient no	Amyloid	AGE	RAGE	PARP	Caspase 8	Caspase 3	NFκB	
Heart	1*'	+++	+++	+++	+	+	++	0
	2*	++	++	++	0	0	++	++
	3*	+++	+++	+++	0	0	0	0
	4*	++	++	++	++	++	++	0
	5#	++	++	++	0	0	++	0
	6#	+++	+++	+++	+	-	+	+
	7#	++	++	++	0	0	0	+
	8#	+++	+++	+++	0	0	0	0
	9#	+++	+++	+++	0	0	0	0
Kidney								
Glomeruli/Tubuli	1*ə	++/++	+++/+++	+++/+++	0/0	0/0	++/++	0/0
	10‡	+++/+++	+++/+++	+++/+++	0/0	0/0	++/++	0/++
	11‡	+++/+++	+++/+++	+++/+++	0/+	0/+	0/+	0/++
	12‡	+++/+++	+++/+++	+++/+++	0/+	0/+	0/+	0/++
Rectum	13‡ə	++	+++	+++	-	-	-	-
	14‡ə	+	++	++	-	-	-	-
Gut	13‡ə	+	++	++	0	0	0	0
Appendix	14‡ə	++	++	++	0	0	0	0

+, very light deposition; ++, intermediate; +++, heavy deposition; 0, no immunoreactivity; -, not investigated; *, ATTR Leu111Met; #, ATT Val30Met; ‡, Gelsolin; ə, from the same patient.

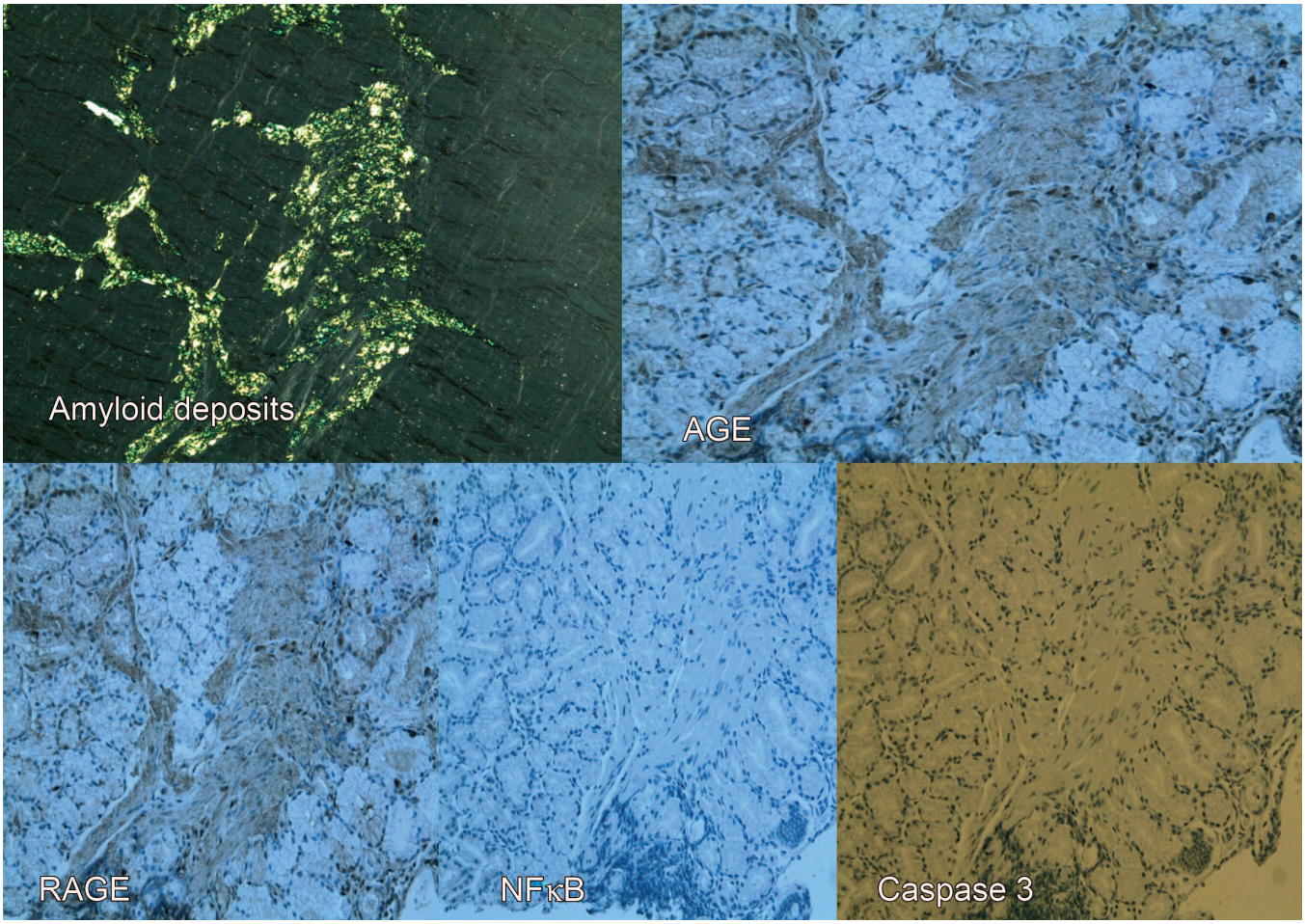

Fig 1. Immunohistochemical staining (ABC-method) and Congo red staining of AGE, RAGE, *NF-κB* and caspase 3 showing small intestine from gelsolin patient. x 114

myocard and in the blood vessels, although it was not as strong staining as for the above mentioned patients. In the kidney AGE immunoreactivity was presented in the glomeruli and tubuli of all patients (Table 3, Figs. 3, 4). In the gut (Table 3), AGE immunoreactivity was localised to circular and longitudinal muscle layers, and many neurons in the ganglia were positively stained for AGE. AGE immunoreactivity was detected in the mucosae and submucosae of rectum (Table 3). In appendix (Table 3) AGE immunoreactivity was detected in the neurons. AGE was found in both gelsolin and transthyretin amyloid patients with similar intensity (Table 3).

RAGE immunoreactivity

Immunoreactivity for RAGE could be detected in the heart (Fig. 2), kidney (Figs. 3, 4), rectum, gut (Fig. 1) and appendix of all patients included in this study (Table 3). RAGE showed strong immunoreactivity in the heart of five patients, and moderate reactivity in the remaining four patients (Table 3). In all heart samples, RAGE were localised to the myocard and blood vessels. In the kidney (Table 3), RAGE immunoreactivity was found in the glomeruli (Fig. 3) and tubuli (Fig. 4). In the gut, RAGE immunoreactivity was noted in the circular and longitudinal muscle layers, as well as in the neurons of ganglia and in the mucosae (Fig. 1) (Table 3). In the rectum and appendix, RAGE immunoreactivity was located in the mucosae, submucosae and in the neurons. RAGE was found in both gelsolin and TTR amyloid patients and with similar intensity (Table 3).

PARP and Caspase 3 and 8 immunoreactivity

PARP and caspase 8 immunoreactivities were detected in the myocard of three patients and in the tubuli of 2 patients (Table 3). The immunoreactivity was not correlated to the intensity of immunoreactivity to AGE or RAGE. There was no immunoreaction for PARP and caspase 8 detected in the gut or appendix (Table 3). Caspase 3 showed more intensive immunoreaction than capase 8. Caspase 3 was positively immunostained in myocards of 6 patients (Table 3), in glomeruli of two

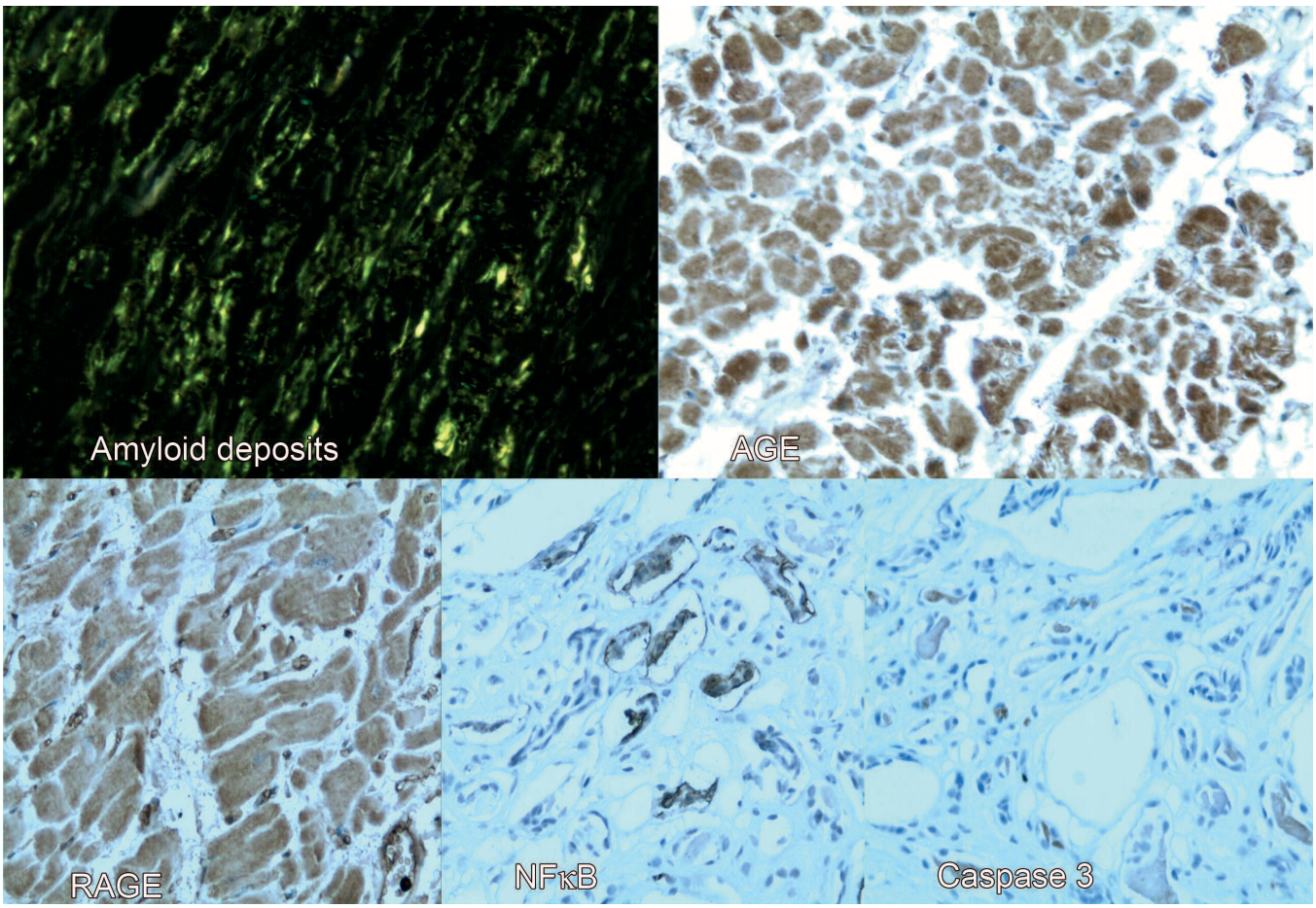

Fig. 2. Immunohistochemical staining (ABC-method) and Congo red staining of AGE, RAGE, *NF-κB* and caspase 3 showing myocard from FAP, Leu111Met patient. x 200

patients (Table 3, Fig. 3) and tubuli of four patients (Table 3, Fig. 4). Furthermore, the gut of one patient showed immunoreaction for caspase 3 (Table). In 3 samples, PARP or caspase immunoreactivity was noted without any corresponding detectable NFκB reactivity (Table 3). PARP and caspase reactivity was found in tissues from both TTR and gelsolin amyloid patients.

NF-κB immunoreactivity

In the heart (Table 3, Fig. 2), NF-κB showed no immunoreactive reactions in six samples. The myocard of 3 patients showed immunoreactivity to NF-κB; one moderately and two weakly. NF-κB reactivity in the kidney was found in 3 of 4 patients' samples and in the tubuli only (Table 3, Fig. 4). Appendix and gut showed no immunoreactivity for NF-κB (Fig. 1) (Table 3). In all tissue samples, except one heart sample with NF-κB immunoreactivity, a corresponding activity of PARP or caspases was detected (Table 3). NF-κB activation was noted in both gelsolin and TTR amyloid samples.

AGE/RAGE/TTR

AGE and RAGE showed the same pattern of immunoreactivity in all tissues investigated in this study. AGE and RAGE were co-localised to the same areas, as well as AGE/ TTR and RAGE/ TTR.

Discussion

To our knowledge, this is the first investigation of the presence and relationship between amyloid deposits and AGE, RAGE, NF-κB and caspases in TTR- and gelsolin amyloidosis. Accumulation of AGE and binding to its receptor was noted in ATTR and gelsolin amyloidosis investigated, and in some cases was found together with an activation of NF-κB, which in turn appeared to have activated apoptotic cellular events that could contribute to organ dysfunction. However, most of

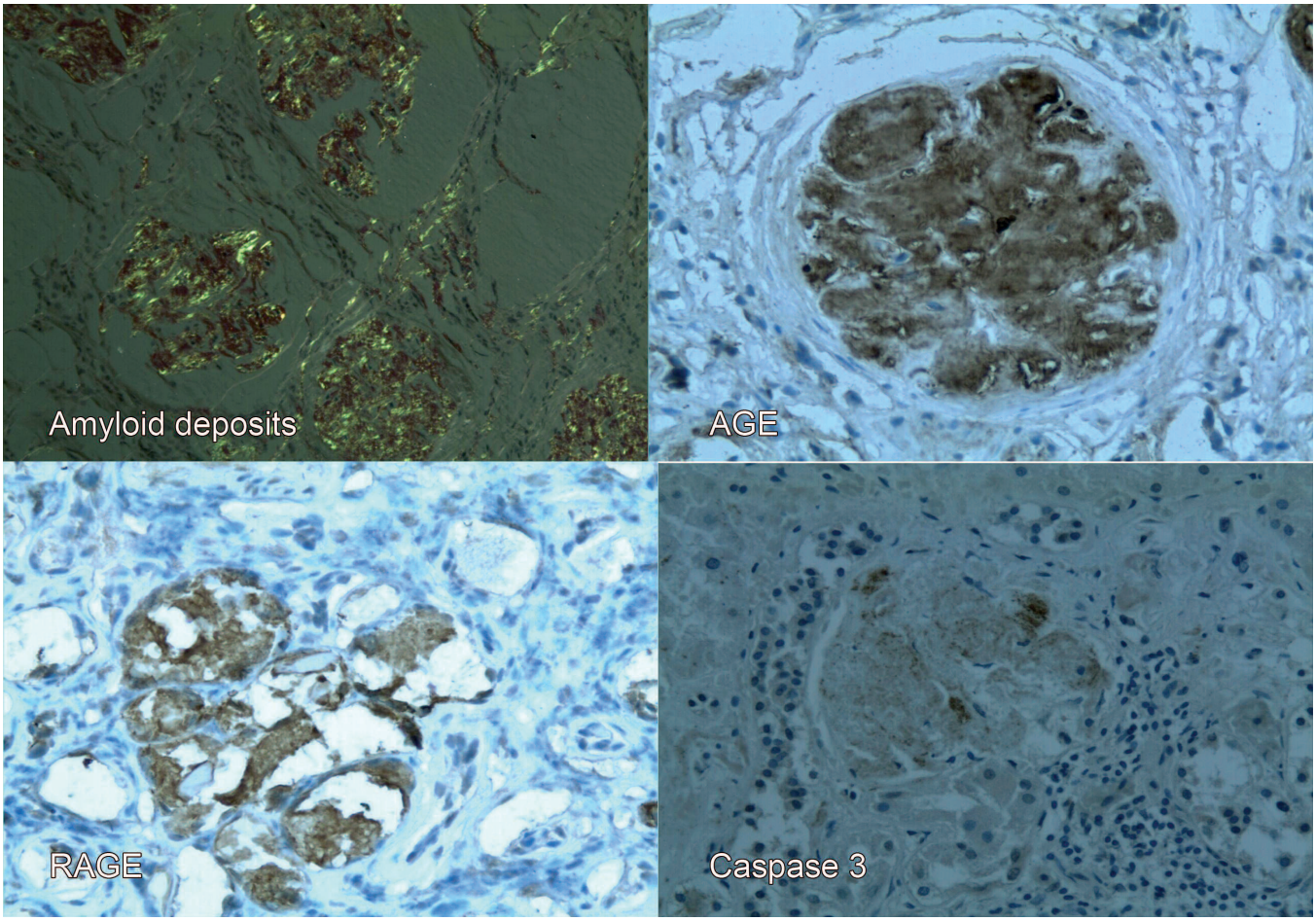

Fig. 3. Immunohistochemistry using ABC-method and Congo red staining showing the deposits of AGE, RAGE, amyloid and *NF-κB* in the glomeruli of FAP, gelsolin patient. x 400

the tissues investigated showed, in spite of heavy accumulation of AGE and RAGE, no activation of NF-κB or presence of markers for apoptosis. This suggests that alternative pathway(s) for cell damage than that mediated through NF-κB activation may be operating.

In a previous study (Matsunaga et al., 2005) we demonstrated that AGE and RAGE were deposited in the kidneys of FAP patients with a distribution similar to that observed in diabetes nephropathy. The present study shows that the distribution of amyloid deposits, AGE and RAGE in the kidneys of gelsolin amyloid patients show the same pattern as that of ATTR Val30Met patients. Tanji et al. found that carboxymethyl lysine (CML) is the major constituent of AGE in the basement membranes of podocytes in diabetic nephropathy and that it is associated with up-regulation of RAGE (Tanji et al., 2000). Patients with diabetes have increased levels of AGEs, and it is likely that the proximal tubular metabolism of AGEs is involved in the pathogenesis of diabetic nephropathy (Saito et al., 2005). In patients with chronic renal failure, impaired metabolism of AGEs leads to AGE accumulation in serum, and AGEs are associated with the development of uremic complications (Henle and Miyata, 2003). Maury described the presence of gelsolin-amyloid deposits in homozygous Finnish gelsolin amyloid patients' kidneys, and concluded that this contributed to their severe nephropathy (Maury, 1993). The similarities between the clinical presentation of kidney failure in diabetes, ATTR Val30Met and gelsolin amyloid patients and the heavy accumulation of AGE found in their kidneys support the suggestion that AGE plays a central role in the pathogenesis of kidney failure in amyloid and diabetes nephropathy.

Several pathways may operate in amyloid nephropathy: one in which AGE and/or amyloid fibrils binds to RAGE in the tubuli and activates NF-kB, which in turn activates a cascade of cellular events which leads to cell death, and another in which AGE-modified proteins cause direct tubular cell hypertrophy (Xiang et al., 2001) which leads to tubular dysfunction. However, RAGE may also mediate its toxic bioactivities through

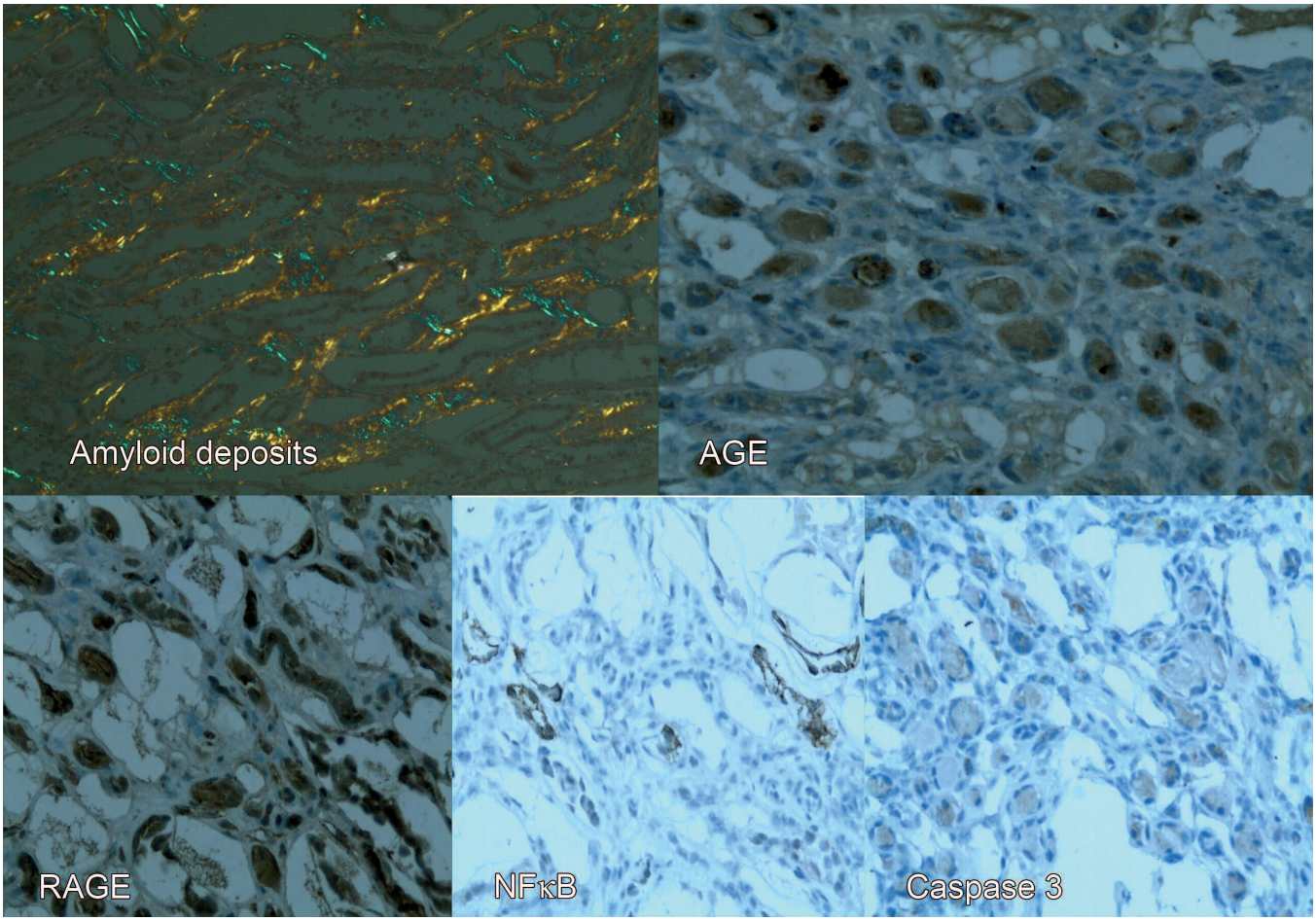

Fig. 4. Immunohistochemical staining (ABC-method) and Congo red staining showing the deposition of amyloid and immunoreaction of AGE, RAGE, *NF-κB* and caspase 3 in the tubuli of a patient with familial amyloidosis with polyneuropathy. x 150

other pathways than that of NF-kB activation, since RAGE is a multiligand receptor, i.e. its ligands may recognize several other receptors and mediates its damaging effects through hereto-unknown pathways.

Experimental and clinical studies have suggested that oxidative stress increases in heart failure and causes structural and functional disintegration, leading to contractile dysfunction and structural re-modelling of the myocardium (Tsutsui, 2004). Several studies suggest that AGEs are related to the development and progression of heart failure (Bucciarelli et al., 2006; Koyama et al., 2007). This investigation revealed that amyloid deposits in the heart are associated with an accumulation of AGE and RAGE in TTR amyloidosis. However, in only three of nine patients was AGE/RAGE associated with NF-κB activation, and even though markers of apoptosis were observed in five out of nine cases, only two of those also showed NF-κB-activation. Thus, myocardial dysfunction is not consistently related to NF-κB activation or to apoptosis. We have previously shown the presence of markers of oxidative stress in amyloid rich tissues (Ando et al., 1997). Therefore, oxidative stress and activation of intracellular signalling leading to production of cytokines and of inflammatory mediators are probably also implicated in the process. In addition, restriction of the heart function is also caused by the sheer amount of amyloid deposits in the myocardium. Met 111 patients have severe restrictive cardiomyopathy, which leads to death within a few years due to cardiac failure (Svendsen et al., 1998), whereas the cardiomyopathy in ATTR Val30Met-patients appears to develop more slowly, and is often found in patients with late onset without symptoms of cardiomyopathy (Suhr et al., 2006). In the heart, activated NF-κB plays a dual role – one as a promoter for ischemic injury through inflammation, and the other as a protector against apoptosis and inflammation through an upregulation of protective substances, such as manganese superoxide dismutase, inducible cyclo-oxygenase and inducible NO synthase (Valen et al., 2001; Valen, 2004). It could be speculated that lack of activation of NF-κB in some patients' myocards could contribute to heart failure and cardiomyopathy.

In a previous study (Matsunaga et al., 2002) we showed that AGE and RAGE were present in the gastrointestinal tract of ATTR Val30Met patients, and that AGE and RAGE correlated well with amyloid deposits, even though activation of RAGE neither lead to NF-κB activation nor to apoptosis. Similarly, in the present study, kidney glomeruli and intestines from gelsolin amyloidosis patients showed amyloid deposits and accumulation AGE and RAGE, which correlated well to amyloid deposits, but did not lead to activation of NF-κB or induction of apoptosis.

In conclusion, AGE and RAGE were associated with amyloid deposits in all samples, irrespective if it was ATTR or gelsolin amyloidosis. However, apoptosis and NF-κB activation were not uniformly found in amyloid rich tissue; thus, additional pathways for AGE/RAGE and amyloid toxicity may operate besides those activated by NF-κB and caused by apoptosis.

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