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Anti-λ-light chain-peptide antibodies are suitable for the immunohistochemical classification of AL amyloid

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Summary. We aimed to test whether antibodies raised against recombinant peptides corresponding to the variable region of immunoglobulin light chains are suitable for the immunohistochemical classification of amyloid. The Entrez database of the National Center for Biotechnology Information (NCBI) was searched for all protein sequence entries which met the search criteria "amyloid" and "lambda light chain". Sixty-four different λ-light chain-derived amyloid protein sequences were retrieved, aligned and categorized into the V region subgroups of λ-light chain detailed by the NCBI, i.e. subgroup I (21 protein sequences), II (14), III (6), IV (1), V (1) and VI (21). V region subgroup I was chosen for epitope sequence selection and two rabbits were immunized with the following peptides: NH2-ISCSGSSSNIGSNTV-CONH2 and NH2-QRPSG VPDRFSGSKSGTS-CONH2. Sensitivity and specificity of the IgG-purified antibodies was tested by Western blotting using amyloid A- (AA), ALλ- and ALκ-amyloid proteins, and by immunohistochemistry on tissue microarrays with 110 different amyloid containing tissue samples obtained at autopsy from 22 patients, and on 27 biopsy specimens from a series of 24 patients. Our peptide antibodies specifically stained AL amyloid λ-light chain-origin, in both Western blots and formalin-fixed and paraffin-embedded tissue sections, confirming that peptide-antibodies directed against immunoglobulin-derived λ-light chain proteins can be applied for the immunohistochemical classification of amyloid. This offers the opportunity to generate a large set of anti-λ-light chain protein-antibodies for the immunohistochemical classification of amyloid independently from native human tissue sources.

Key words: Amyloid, Immunoglobulin, λ-light chain, Immunohistochemistry

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Introduction

Amyloidosis defines a heterogeneous group of diseases of diverse origin characterized by proteinaceous tissue deposits that show birefringence with green polarization colour in polarized light after Congo red staining (Puchtler et al., 1962; Röcken and Sletten, 2003). More than 26 different proteins and their precursors have been described to form amyloid in humans (Merlini and Bellotti, 2003). Following the histological diagnosis of amyloidosis, the surgical pathologist has to classify the amyloid protein(s) in order to assess patient prognosis and treatment, which may include chemotherapy or organ transplantation (Merlini and Bellotti, 2003; Röcken and Sletten, 2003). A variety of different procedures are used for the classification of amyloid, such as immunohistochemistry, double immunodiffusion, Western blotting, enyzme-linked immunosorbent assay, and amino acid sequencing of purified amyloid fibril proteins (for a review see (Kaplan et al., 2003; Röcken and Sletten, 2003)). Unequivocal identification of the type of amyloid requires isolation of the amyloid protein(s) and determination of the primary structure. Traditionally, biochemical extraction of amyloid proteins has led to the discovery of the various types of amyloid and required large amounts of unfixed amyloid bearing tissue. More recently, microextraction procedures have been developed that allow the biochemical classification of the amyloid protein(s) from minute amounts of tissue, such as provided by a tissue biopsy (Kaplan et al., 1999). These techniques can now be applied to unfixed and formalin-fixed tissue samples, but also have their obstacles (Kaplan et al., 1999). The amount of amyloid present in a given tissue sample may limit feasibility. The microextraction procedures are not highly specific for amyloid proteins and co-extract other proteins. We and others have applied successfully these protein-biochemical procedures to characterize amyloid proteins extracted from unfixed, and formalin-fixed and paraffin-embedded surgical specimens (Layfield et al., 1996; Kaplan et al., 1999; Röcken et al., 2002; Röcken

and Wilhelm, 2005). However, some biopsy and autopsy cases escaped classification, illustrating that a biochemical characterization of amyloid from formalin-fixed specimens is not always successful. Furthermore, protein-biochemical techniques are limited to specialized laboratories and are not available in every surgical pathology department. Using immunohistochemistry to classify amyloid in specimens submitted to histology is an obvious step (Röcken et al., 1996; Röcken and Sletten, 2003). Many studies have confirmed the suitability and use of immunohistochemical classification of amyloid using either unfixed or formalin-fixed and paraffin-embedded tissue sections (Kebbel and Röcken 2006). Amyloid can also be classified by immunoelectron microscopy (IEM). The advantage of IEM is that labelling of the fibrils differentiates from "contaminating" proteins, not related to the amyloid fibrils (Arbustini et al., 1997). However, specimens submitted to surgical pathology are not routinely processed for electron microscopy, unless specifically requested. In addition, the surgical pathologist is often the first to diagnose amyloid, after tissue processing has already been completed and IEM would then require re-embedding.

Irrespective of whether fixed or unfixed tissue specimens are used for immunohistochemical or immunoelectron microscopical classification of amyloid, the diagnosis and classification of AL amyloid, which belongs to the family of monoclonal immunoglobulin deposition diseases (MIDD), poses a particular problem in surgical pathology. MIDD are a group of disorders, which have in common the deposition of monoclonal proteins (M-protein) in organs and tissue. The proteins deposited are intact immunoglobulins or fragments thereof. Due to the deposition of the variable regions of light chains, almost every patient with AL amyloidosis has a unique amyloid protein and hence, AL amyloid stains approximately in only 40% of the cases with commercially available antibodies (Lachmann et al., 2002). In order to circumvent this problem, antibodies can be raised using native human AL amyloid proteins extracted from amyloid-containing tissue (Linke et al., 1986; Bohne et al., 2004). However, this relies on the availability of sufficient amounts of amyloid containing tissue and a representative subtype of AL amyloidosis. Alternatively, antibodies can also be obtained by using recombinant proteins and peptides as immunogen. We and others have successfully raised peptide-antibodies, which are immunoreactive against amyloid proteins originating from apolipoprotein AI and serum amyloid A (Häggqvist et al., 1999; Hoshii et al., 2001; Mucchiano et al., 2001; Gellermann et al., 2005; Gregorini et al., 2005; Röcken et al., 2006).

In this study, we aimed to test whether antibodies can be raised for the immunohistochemical classification of amyloid by using recombinant peptides directed against the variable region of λ-light chain as immunogen. This would offer the opportunity to generate anti-λ-light chain protein-antibodies independently from native human tissue sources. Furthermore, the possibility of mixing peptide-antibody-cocktails with antibodies directed against varies subtypes of λ-light chain proteins might improve the sensitivity of the immunohistochemical classification of amyloid.

Materials and methods

Patient selection

110 formalin-fixed, paraffin-embedded tissue specimens obtained from an autopsy series of 22 patients (8 women, 14 men, mean age 72 years, range 35-81 years) with generalized AL amyloidosis (κ or λ-light chain origin) or AA amyloidosis were used in this study for the generation of a tissue microarray (see below). Twenty-seven amyloid containing biopsy samples obtained from another set of 24 patients (8 women, 16 men, mean age 60 years, range 11-85 years) with AL- (κ- or λ-light chain origin), AA- or transthyretin- (ATTR) derived amyloidosis were retrieved from the archive of the Department of Pathology. Variable amounts of amyloid were present as vascular and interstitial deposits in all the specimens, as shown by Congo red staining and high-intensity cross-polarization microscopy (Puchtler et al., 1962). The classification of amyloid was based on immunohistochemistry, protein biochemical characterization and clinical history as described elsewhere (Kebbel and Röcken 2006). Only unequivocal cases were included in this series. This study was in accordance with the guidelines of the Ethics Committee of the University of Magdeburg. Data were encoded to ensure patient protection.

Materials

Amyloid proteins were obtained from post mortem tissues (spleen) of two patients (patient No. 1 and 2) and from a biopsy of subcutaneous tissue of the lower leg from a third patient. Patient No. 1 (GS), a 50-year-old woman had suffered from multiple myeloma and generalized AL amyloidosis. Patient No. 2 (EK) a 61-year-old man had suffered from rheumatoid arthritis and generalized AA amyloidosis. The amyloid proteins of both patients had been classified by amino acid sequencing or mass spectrometry (Bohne et al., 2004; Röcken et al., 2005). Patient No. 3 (CT), a 42-year-old woman, had suffered from an extramedullary κ-light chain plasmocytoma with local AL amyloidosis (Daigeler et al., 2006). The tumor cells adjacent to the amyloid deposits showed the same light chain restriction as the amyloid deposits.

Tissue microarrays

Tissue microarrays were generated from 110 donor paraffin blocks with amyloid-containing tissues of diverse origin. The donor paraffin blocks were obtained from an autopsy series of 22 patients with AL (λ)

amyloidosis (7 patients; 41 donor paraffin blocks), AL (κ) amyloidosis (4 patients; 7 donor paraffin blocks) or AA amyloidosis (11 patients; 62 donor paraffin blocks). One tissue cylinder of 1.2 μm diameter was punched from the amyloid-containing areas of the donor block and placed into recipient block using a precision instrument (Beecher Instruments, Silver Spring, MD, USA). 3-4 μm thick paraffin sections were cut from each recipient block and stained with H&E and Congo red to confirm successful transfer of amyloid-containing tissue.

Data search

The Entrez database of the National Center for Biotechnology Information (NCBI) was searched for all protein sequence entries, which met search criteria "amyloid" and "lambda light chain". The search was carried out during January 2004, and entries submitted after that date were not included.

Generation of polyclonal antibodies

For the epitope-search, the secondary protein structure was analysed as described by Parker et al. (Parker et al., 1986) using the Peptide Companion software (CoshiSoft/PeptiSearch; SanDiego, CA). Synthetic peptides were purified by high pressure liquid chromatography (HPLC) and had a purity of 86% and 89%, respectively. Two female 3-4 month old New Zealand white rabbits were both immunized with a 2-peptide-cocktail according to a standardized immunization protocol (Pineda Antibody-Service, Berlin, Germany). IgG was purified with a HiTrap Protein G HP affinity column (Amersham Biosciences AB, Freiburg, Germany).

SDS-PAGE and Western Blotting

SDS-PAGE and Western blotting were carried out as described elsewhere (Röcken and Wilhelm, 2005). Immunostaining of the transferred proteins was performed with AL3 as the primary antibody [dilution 1st-day-IgG 1:30,000 (i.e. pre-immune serum), 61st-day-IgG 1:100 (i.e. 61st day after starting immunization protocol), 90th-day-IgG 1:30,000 (i.e. 90th day after starting immunization protocol), room temperature, 60 minutes]. Blocking was carried out in Tris-buffered saline, containing 3% bovine serum albumin and 0.05% Tween-20. The membrane was incubated with the secondary biotinylated goat-anti-rabbit antibody for 60 min at room temperature (dilution 1:1000; both DAKO). Immunostaining was visualized with the NBT/BCIP substrate (p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; Pierce Biotechnology, Bonn, Germany).

Immunohistochemistry

Immunohistochemistry was performed with commercially available monoclonal antibodies directed against AA amyloid (1:400) and polyclonal antibodies directed against amyloid P-component (1:1600), fibrinogen (1:1000), lysozyme (1:3000), transthyretin (1:500), λ-light chain (λ-DAKO; 1:10000), κ-light chain (κ-DAKO; 1:10000) and ß2-microglobulin (ß2M; 1:2000; all DAKO, Hamburg Germany), and non-commercially available polyclonal antibodies directed against apolipoprotein AI (anti-apo AI; dilution 1:1500) (Gregorini et al., 2005), and λ-light chain-derived amyloid proteins (anit-AL1 antibody; dilution 1:1000) (Bohne et al., 2004), as described elsewhere (Kebbel and Röcken 2006). The specificity of immunostaining was verified using specimens containing known classes of amyloid (AA amyloid, apolipoprotein AI, ß2 microglobulin, transthyretin, ß-light chain), using positive controls recommended by the manufacturers (remaining antibodies), by omitting the primary antibodies, by using pre-immune serum instead of the primary antibody, or by pre-absorption of the AL3-antibody with the peptides used for immunization.

Results

Results of sequence search

Previous studies have shown that the primary structure of the variable domain of immunoglobulin-derived λ-light chains is different at particular residues and positions between amyloid and non-amyloid-forming λ-light chains (Hurle et al., 1994; Stevens et al., 1995). Thus, in order to minimize the risk of generating peptide-antibodies that would not detect amyloid proteins we searched the database only for λ-light chain derived protein sequences that had been obtained from AL amyloid deposits. Sixty-four different amyloid protein sequences were retrieved from the NCBI protein database using "amyloid" and "lambda light chain" as search criteria. These sequences were categorized into the six V region subgroups of λ-light chain as they were specified by the NCBI: 21 amyloid protein sequences belonged to the λ-light chain subgroup I, 14 to subgroup II, 6 to subgroup III, 1 to subgroup IV, 1 to subgroup V, and 21 to subgroup VI. Among the two most prevalent subgroups, i.e. I and VI, subgroup I was chosen arbitrarily for further analysis. The 21 amino acid sequences of λ-light chain subgroup I-derived amyloid proteins were aligned and showed considerable variability (Fig. 1), making a rational selection difficult. Therefore a practical approach was applied. The different side chains were counted in each "row" and a hypothetical λ-light chain subgroup I-sequence was created from the most common amino acid residues (Fig. 1). Antigenic epitopes were searched using this hypothetical λ-light chain subgroup I amyloid protein and the secondary protein structure was analysed as described by Parker et al. (1986) using the Peptide Companion software (CoshiSoft/PeptiSearch; SanDiego, CA). Two peptide epitopes sequences were selected for

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                    1        10        20        30        40        50        60        70        80        90       100       110       120       130
                    |        |         |         |         |         |         |         |         |         |         |         |         |         |
(1)MTCSPLLLTLLIHCTGSWAQSVLTQPPSVSAAPGQKVTISCSGNRYNIGNNY VSWYQQFPGTAPKLLIYDDNE RPSGIPDRFSGSKSGTSATLGITGLQTGDEADYFCGTWDSGLTAV  VFGGGTKLTVLG
(2)                 QSVLTQPPSVSAAPGQKVTISCSGSSSDIGNNY VSWYQQLPGAAPKLLIYDTSK RPSGVPDRFSGSNSGTSATLGITGLQTGDEADYYCGTWDGSLSAI   FGGGTKLTVLRQPKAGPSV TLF  PPSS
(3)                 QSVLTQPPSVSAAPGQNVSISCSGSSSNIGNNH VSWYQQFPGTAPKLLTYDNNE RPSGIPDRFSGSKSGTSATLVITGLQTGDEADYFCGTWDYTLSW   VFGGGTKLTVLGQPKAAPSV TLS  PPSS
(4)                 QSVLTQPPSVSAAPGHKVTISCSGSSSNIGKNY VSWYQQIPGTSPKLLIYDNNK RPSGIPARFSGSKSGTSATLVITGLQSGDEADYYCGTWDSSLSDGDVVFGGGTKVTVLGQPKAGPSV TLF  PPSS
(5)                 QSVLTQPPSASGTPGQRVIISCSGSSSNIGRNT VNWYLHLPGTAPKLVIYSNDQ RPSGVPDRFSGSKSVTSASLAISGLQSGDEAHSYCAAWDDSLRGP  VFGGGTELTVLSQPKAAPSV TLF  PPSS
(6)                 QSVLTQPPSASGTPGQRVTISCSGSNSNIGSNS VNWYQHLPGTAPKLLIYSQSQ RPSGVPDRFSGSRSGTSASLAISGLQSEDEAEYYCAAWDDSLNGP  VFGGGTKLTVLGQPKAAPSV TLF  PPSS
(7)                  SELTQPASVSVSPGQTARITCSGDALTKKYPY   WYQQKLGQAPVLVIYEDSK RPSGIPERFSGSSSGTTATLTISRAQVEDEGDYYCYSTDSSGNQRF  FGGGTKLTVLGQPKAAPSV TLF  PP
(8)                 QSVLTQPPSVSVSQGQTARIACSGDQLGNYYSS   WYQQKPGQSPLLIIYQDTR RSSGIPERFSGSNSGNTATLTITGTQSMDEADYFCQTWDGSTVM    FGGGTKLTVLSQPKAAPLGTLF  PPSS
(9)                 SYELTQPPSVSVSPGQTASITCSGDRLGDKFAC   WYQQKPGQSPVLVIYEDTK RPSGIPERFSGSNSGNTATLTISGTQAMDEGDYYCQSWDSSTA     VFGGGTKLTVLGQPKAAPLRSLCSHPP
(10)                QSVLTQPPSVSVSPGQTVGITCFGNKLGGEYAS   WYQLKPGQSPLLVIYQDSK RPSGIPERFSGSNSGNTATLTISGTLALDEAYYYCQAWDNGAPT     FGGGTKLTVLGQPKAAPSVTLF  PPSS
(11)                 SELTQPASVSGSPGQSITISCTGTSSDVGGYNYVTWYQQHPGKAPNVLIYEVTY RASGVSNRFSGSKSGNTASLTISGLQAEDDADYYCSSYTINSTR   VFGTGTKVTVLGLPKANPTDTLF  PPSS
(12)                 SELTQPASVSGSPGQSITISCTGPSSDVGDYNYISWYQQHPDKAPKLIIYDVSH RPSGISDRFSGSKSGNTASLTISGLQTEDGADYYCTSYTSTTTY   VFGTGTKVTVLGQPKANPTVSLF  PPSS
(13)                 SELTQPASVSGSPGQSITISCTGTSSDVAIYNYVSWYQQYPTKAPKLTIYEVNN RPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTITGTQ   VFGGGTKLTVLGQPKAAPSVTLF  PPSS
(14)                QSVLTQPPSASGTPGQRVSISCSGSTSNIGSNT VNWYLHLPGTAPKLLIYSNNQ RPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLDGV   VFGGGTKLTVLGQPKAAPFGTLF  PPSS
(15)                QSVLTQPPSASGTPGQRVTISCSGSRTNIASNT VNWYQHLPGMAPKLLIYTNDQ RPSGVPDRISGSKSGTSASLGISGLQSEDEADYYCAAWDDRLNGP   VFGGGTKLTVLGQPKAAPSVTLF  PPSS
(16)                QSVLTQPPSASGTPGQRVTISCSGSSSNIGSNT VTWYQHLPGAAPKLLIYSNDQ RPSGVPDRFSGSKSGTSASLAISGLQSVDEAHYYCSAWDDRLNGP   VFGGGAKLNVLSQPKAAPSVTLF  PPSS
(17)                QSVLTQPPSASGTPGQRVTISCSGSNSNIGINI VNWYQQVPGRAPKLLIYSNNQ RPSGVADRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLHGP   VFGGGTQLTVLGQPKAAPSVTLF  PPSS
(18)                QSVLTQPPSVSAAPGQKVTISCSGSYSNIGKNY VSWYQHIPGTAPRLLIFENKK RPSGIPDRMSASKSGPSATLDIAGLQTGDEADYYCATWENSLTAGSW VFGAGTRVTVLSQPKAAPSVTLF  PPSS
(19)                SYELTQPPSVSGAPRQRVTISCSGTSSNIGNNV VNWYQHFPGKAPKLLIYYDDEL ASGVSDRFSGSKSDTSASLAISGLQSEDEADYYCSTWDHNLNVV   VFGGGTKVTVLGQPKAAPSVTLF  PPSS
(20)                QSVLTQPPSLSAAPGQRVSISCSGSSSNIGKNY VDWYQQLPGTAPKLLIFNNNK RPSGIPDRFSGSKSGTSATLGITGLQTGDEAIYYCGTWDNRRS     VFGGGTNVTVVG
(21)                             GQRVTISCSGSSSNIGSNT VNWYQKLPGTAPKLLIYSTNQW PSGVPDRFSGSKSGTSASLAISELQSEDEGDYYCASWDDTMDFP  VF

Hypothetical sequence QSVLTQPPSVSGSPGQRVTISCSGSSSNIGSNT VNWYQQLPGTAPKLLIYSNNQ RPSGVPDRFSGSKSGTSASLTISGLQSEDEADYYCAAWDDSLNGV   VFGGGTKLTVLGQPKAAPSVTLF  PPSS
                                 PEPTIDE #1                                                PEPTIDE #2
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Fig. 1. Sequence alignment of all AL amyloid proteins of λ-light chain subgroup I origin found in the Entrez database of the National Center for Biotechnology Information (NCBI). “Amyloid” and “lambda light chain” were used as search criteria. Two peptide epitopes sequences were considered as being promising immunogens (peptide #1 and peptide #2).

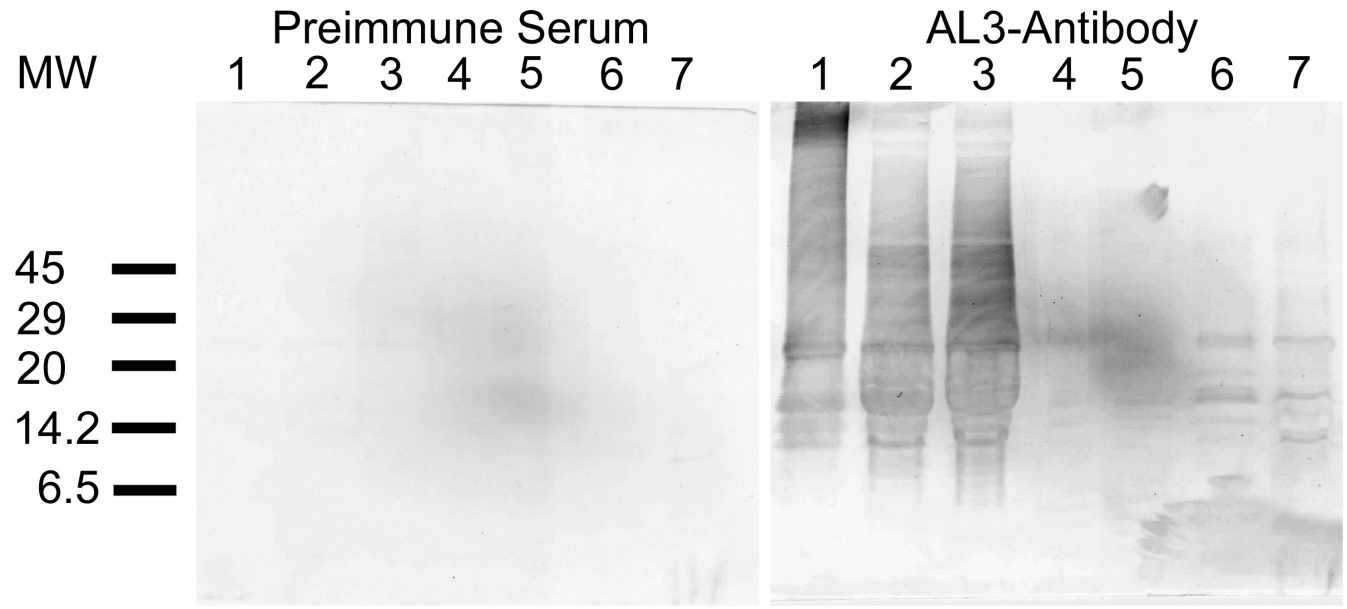

Fig. 2. Amyloid proteins were extracted from unfixed tissue samples, or tissue samples that had been fixed in phosphate buffered 4% p-formaldehyde, separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. Lane 1-3 shows proteins extracted from the amyloid-laden spleen of patient No. 1, who had suffered from generalized AL amyloidosis of λ-light chain origin. Lanes 4 and 5 show proteins extracted from amyloidotic tissue of patient No. 3, who had suffered from extramedullary κ-light chain plasmacytoma. Lanes 6 and 7 show protein bands extracted from amyloid-laden spleenic tissue of patient No. 2, who had suffered from generalized AA amyloidosis. Western blotting was performed with the AL3-antibody and the corresponding preimmune serum obtained from the same rabbit.

the generation of the peptide antibodies (Fig. 1). Finally two female New Zealand rabbits were immunized, both with each of the following two most promising antigenic peptides, i.e. NH2-ISCSGSSSNIGSNTV-CONH2 and NH2-QRPSGVPDRFSGSKSGTS-CONH2. IgG-antibodies obtained from the first rabbit were named AL3-antibodies. The second rabbit died during the immunization protocol.

SDS page and western blotting

Successful immunization was tested by Western blotting using λ-light chain-derived AL amyloid proteins as a positive control, κ-light chain-derived AL amyloid proteins and AA amyloid proteins as a negative control. Amyloid proteins from the control samples were either retrieved from formalin fixed tissue using the extraction procedure described by Layfield et al. (1996) or from unfixed native tissue using the extraction procedure described by Kaplan et al. (1999) Amyloid proteins were extracted from unfixed amyloid-laden spleenic tissue. Following gel electrophoresis and Western blotting with the AL3-antibodies, samples from patient No. 1 (GS; ALl amyloidosis) showed three faint bands at around 14.2 kDa (Fig. 2), which were interpreted as differently sized AL amyloid proteins. One single strongly immunoreactive band was found above 20 kDa, probably representing intact immunoglobulin light chain. Minor smearing was observed in the molecular weight range above 45 kDa and has been found previously (Röcken and Wilhelm, 2005). Immunoreactivity was observed irrespective of whether amyloid proteins were extracted from native or formalin-fixed amyloid-laden tissue samples. AL3 did not stain k-light chain derived amyloid proteins (patient No. 3) or AA amyloid proteins (patient No. 2). AA amyloid proteins of patient No. 2 were previously shown to be in the range of 6.2 kDa. The few faint bands well above 6.5 kDa probably represent immunoglobulin light chains, which were co-extracted with AA amyloid proteins.

No immunostaining was observed with any amyloid protein, when AL3 was replaced by preimmune serum or pre-incubated with the peptides used for immunization (Fig. 2).

Immunohistochemistry

We next validated the AL3-antibodies using tissue microarrays (TMA) with 110 samples of amyloid containing tissue samples obtained from 22 patients. The amyloid deposits of these tissue samples had been classified by immunohistochemistry as described elsewhere (Müller et al., 2000; Kebbel and Röcken 2006). Using TMA, the sensitivity of the AL3-antibodies was compared with two other antibodies, i.e. AL1 and λ-DAKO. The AL1-antibody had been generated by using native human λ-light chain amyloid proteins as immunogen (Bohne et al., 2004). λ-DAKO is a commercially available antibody directed against λ-light chain. The amyloid deposits of 33 tissue samples obtained from 7 patients showed unequivocal staining with AL3 (80%; Figs. 3, 4). In these cases AL1 and λ-DAKO (except 4 samples) showed unequivocal staining. Two samples of one patient (patient No. 11) showed a spotty staining with AL3 and κ-light chain, whilst λ-DAKO and AL1 were immunonegative. In 5 samples from 3 patients AL1, AL3 and λ-DAKO were immunonegative whilst the anti-κ-light chain antibody showed staining of the amyloid deposits. In all 62 samples from 11 patients with systemic AA amyloidosis, no immunostaining was observed with either AL1 or AL3. Figure 4 summarizes the results of the immunohistochemistry.

Finally, we tested sensitivity and specificity on 27 amyloid-containing biopsy specimens obtained from

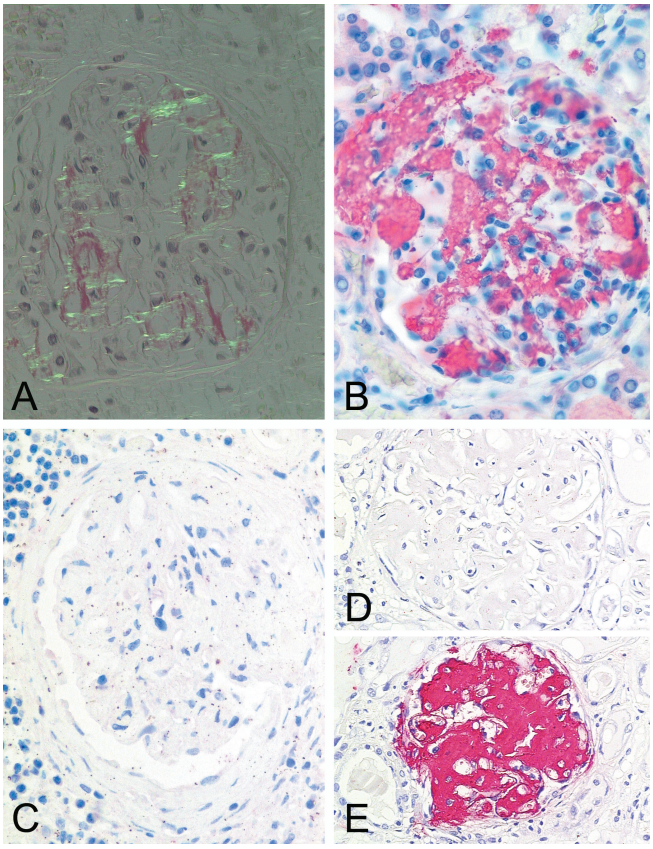

Fig. 3. Tissue microarrays were used to test immunostaining of two anti-λ-light chain peptide antibodies (AL3). **A.** Green birefringent amyloid deposits in the glomerulus of patient No. 1, who had suffered from AL amyloidosis of λ-light chain origin (Bohne et al., 2004). These AL amyloid deposits showed intense staining with AL3 **(B)**. No immunostaining of AL amyloid was found with the corresponding preimmune serum **(C)**. AL3 also showed no immunostaining of AA amyloid **(D)**, which stained only with a monoclonal antibody directed against AA amyloid **(E)**. Congo red staining **(A)**, immunostaining with AL3 **(B, D)**, immunostaining with the corresponding pre-immune serum **(C)**, immunostaining with anti-AA amyloid antibody **(E)**. Original magnifications A-C, x 400; D,E, x 200

another series of 22 patients. This series included also patients suffering from ATTR amyloidosis. Figure 5 summarizes the results. Five tissue samples with AL amyloidosis showed an unequivocal staining with AL3 (42%). AL3-antibodies did not stain AA-, ALκ- or ATTR amyloid.

Furthermore, the amyloid deposits of every patient were immunoreactive for amyloid P component.

Fig. 4. Tissue microarrays were used to test sensitivity, immunostaining of the anti-λ-light chain peptide antibody AL3 was compared with immunostaining using a commercially available antibody directed against λ-light chain (λ-DAKO) and an antibody that had been raised using native human AL amyloid proteins of λ-light chain origin (AL1). AA- and ALκ amyloid served as a control. Each line represents a single core cylinder of an amyloidotic tissue sample. Black square denotes positive immunostaining; open square denotes no immunostaining found. Patients 1-9 had suffered from AL amyloidosis λ-light chain, patients 10-13 had suffered from AL amyloidosis κ-light chain and patients 14-23 had suffered from AA amyloidsois. The amyloid deposits did not stain with antibodies directed against apolipoprotein AI, fibrinogen, lysozyme, transthyretin and ß2-microglobulin (data not shown).

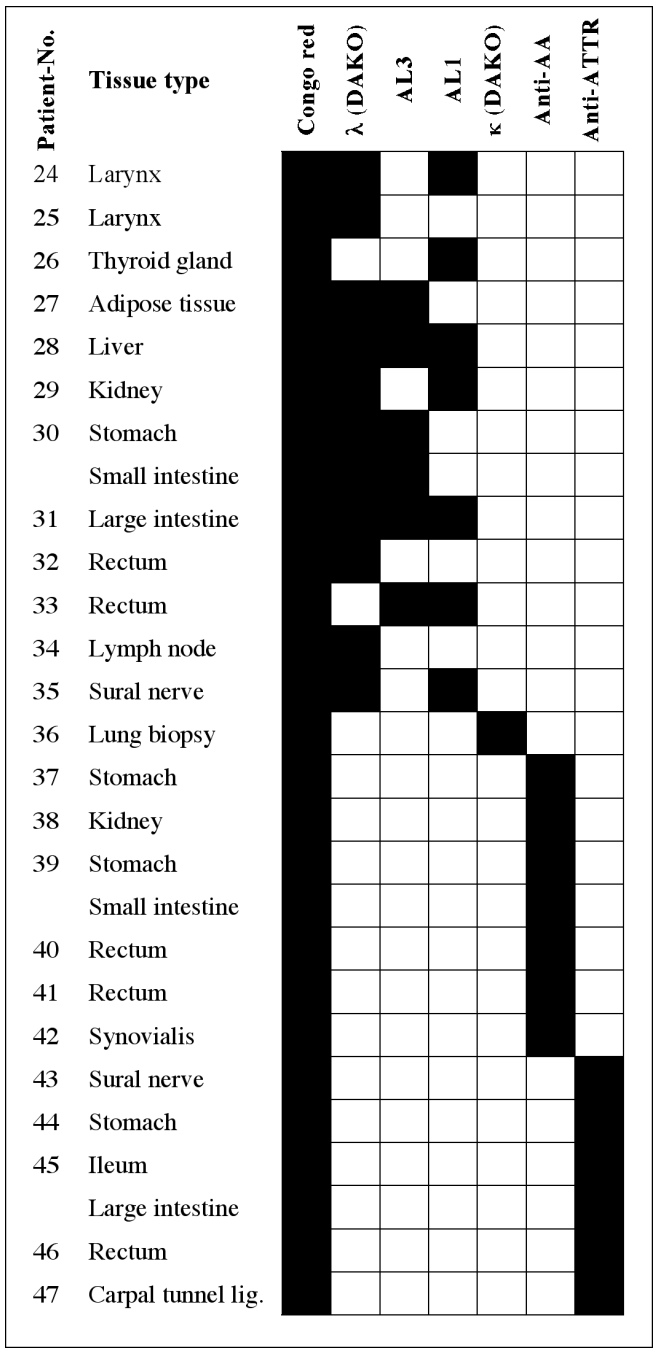

Fig. 5. Using amyloid containing biopsy specimens, immunostaining of the anti-λ-light chain peptide antibody AL3 was compared with immunostaining using a commercially available antibody directed against λ-light chain (λ-DAKO) and an antibody that had been raised using native human AL amyloid proteins of λ-light chain origin (AL1). AA-, ALκ-, and ATTR amyloid served as a control. Each line represents tissue sections obtained from biopsies or resection specimens of the indicated origin and had not been included in the tissue microarrays. Black square denotes positive immunostaining; open square denotes no immunostaining found. Patients 24-35 had suffered from AL amyloidosis λ-light chain origin, patient 36 had suffered from AL amyloidosis κ-light chain origin. Patients 37-42 had suffered from AA amyloidosis and patients 43-47 from ATTR amyloidosis. The amyloid deposits did not stain with antibodies directed against apolipoprotein AI, fibrinogen, lysozyme and ß2-microglobulin (data not shown)

Amyloid did not stain with antibodies directed against apolipoprotein AI, fibrinogen, lysozyme and ß2-microglobulin (data not shown). No immunostaining was observed with preimmune serum, after the omission of the primary antibody or after pre-absorption of the AL3-antibody with the peptides used for immunization (Fig. 3).

Discussion

Following the diagnosis of amyloidosis, the surgical pathologist has to classify the amyloid deposits. Tissue specimens submitted to histology are usually formalin-fixed and paraffin-embedded and first using immunohistochemistry to classify amyloid in these specimens is an obvious step (Röcken et al., 1996; Röcken and Sletten, 2003). While immunohistochemical classification of AA- and ALys amyloid is usualy uncomplicated, since antibodies directed against these amyloid proteins never show unspecific staining with other types of amyloid, the diagnosis and classification of AL amyloid poses a particular problem in surgical pathology. Due to the deposition of the variable regions of light chains, almost every patient with AL amyloidosis has a unique amyloid protein, which is further demonstrated in this study (Fig. 1). Using "amyloid" and "λ-light chain" as search criteria we found 64 different AL amyloid proteins of λ-light chain origin. Commercially available λ- and κ-light chain antibodies are usually targeted against the constant region of the intact immunoglobulin light chain molecules, while AL amyloid proteins are proteolytical fragments consisting, with some exceptions (Kaplan et al., 2004), mainly of the variable region. Thus, commercially available antibodies have an intrinsically limited sensitivity.

To circumvent this problem, anti-AL amyloid protein antibodies had been raised using native human AL amyloid proteins extracted from amyloid containing tissue (Bohne et al., 2004). However, this approach does not address the problem of sequence variability. One possibility of circumventing sequence variability might be to collect amyloidotic tissue of all different types of AL amyloid and extract amyloid proteins from these tissue sources for the generation of antibodies. Even so, it is very unlikely that a single pathologist or amyloid researcher will ever have a sufficient number of amyloid containing tissues of all different types of AL amyloid in order to raise these antibodies. Alternatively, monoclonal urinary light chains and serum immunoglobulins have been used to raise monoclonal and polyclonal antibodies and have shown in two patients to be capable of detecting AL amyloid proteins (Solomon et al., 1990; Abe et al., 1993). However, saving large amounts of amyloid-containing tissue from e.g. autopsies or urinary and serum samples for the generation of antibodies might raise ethical concerns and requires patient consent. Instead of using native human proteins, antibodies can also be raised by using small recombinant peptides

spanning 15 to 25 amino acids. We and others have previously successfully raised peptide-antibodies, directed against amyloid proteins such AA-, AApoAI- and medin-derived (AMed) amyloid proteins (Häggqvist et al., 1999; Mucchiano et al., 2001; Gellermann et al., 2005; Gregorini et al., 2005). While Hoshii et al. (2001) previously generated peptide-antibodies directed against the constant region of immunoglobuin light chains, we believe that we are the first to generate peptide antibodies by using recombinant peptides corresponding to the variable region of λ-light chain as immunogen. We show here that these antibodies can be used for immunostaining of tissue sections obtained from formalin-fixed and paraffin-embedded tissue blocks, irrespective of the anatomical origin of the tissue sample and whether it was a biopsy specimen or obtained at autopsy. Thus, immunostaining was not influenced by autolysis. In addition, these antibodies were capable of detecting ALλ amyloid proteins. However, as expected, AL3 did not stain the AL amyloid deposits of every patient. Considering the distribution of the different ALλ amyloid proteins among the six subgroups, one would expect, as observed in our study, that at least 30-40% of the patients with AL amyloid should stain with an antibody directed against λ-light chain subgroup I. Among the 21 amino acid sequences of λ-light chain subgroup I amyloid proteins 7 to 10 differed significantly within the amino acid region of peptide #1 (Fig. 1). This might have further reduced the sensitivity of the antibody and illustrates the difficulty of finding a suitable antigenic sequence for the generation of peptide-antibodies. In this study we also did not systematically address the influence of the amount of amyloid on the immunostaining pattern. However, in general, we did not observe an obvious difference between small and large amyloid deposits.

The AL3-antibody was also applicable to Western blotting of amyloid proteins extracted either from unfixed or formalin-fixed tissue, demonstrating its broad applicability. Western blotting is particularly interesting, since false positive immunostaining of amyloid is as much a problem as false negative immunostaining. Serum proteins, immunoglobulins among them, commonly contaminate amyloid deposits and positive staining for λ- or κ-light chain does not necessarily indicate AL amyloidosis (Röcken et al., 1996). Thus, when amyloid stains immunohistochemically with antibodies directed against immunoglobulins or their respective light chains, one may wish to confirm this by further biochemical analyses, in order to prevent false positive diagnosis of AL amyloidosis (Kaplan et al., 2004; Röcken and Wilhelm 2005). Immunohistochemistry is unable to provide any distinct information about the molecular weight(s) of the antigens detected in a tissue section, and extraction and separation by SDS-PAGE can provide valuable information about the size of the respective antigens: detection of several light chain fragments most likely represents amyloid protein(s).

Smearing, as observed in Western blotting (Fig. 2), does not demonstrate lack of specificity. In the present study it occurred only in cases with AL amyloidosis of λ-light chain origin, and has, in general, been observed previously (Röcken and Wilhelm, 2005). It was interesting to note that smearing was most prominent in the high molecular weight range. As amyloid proteins form oligomers, supposedly of a huge number of different molecular weight sizes, smearing might represent all the different numbers of cross-linked amyloid protein oligomers present in a tissue sample. However, smearing may also result from cross-linking of amyloid proteins with other, non-amyloidotic tissue components. While κ-light chain derived amyloid completely lacked any immunostaining, distinct bands were found in extracts of AA amyloid-containing tissue. However, each of these bands was well above the molecular weight of AA amyloid proteins (approx. 6.5 kDa), and even above the molecular weight of serum amyloid A (approx. 12 kDa), and thus neither of these faint bands represent an amyloid protein. It is more likely that these bands represent λ-light chain proteins co-extracted with the AA amyloid proteins and has been observed previously (Röcken and Wilhelm, 2005). Neither the method described by Layfield et al. (1996) nor the one described by Kaplan et al. (1999) specifically extract only amyloid proteins from amyloidotic tissue. Obviously, the organic solvents enclosed in the extraction buffers extract also proteins other than amyloid proteins. Histologically, we occasionally observed spotty λ-light chain immunoreactivity in AA amyloid deposits, and k-light chain immunoreactivity in λ-light chain derived amyloid deposits (Fig. 4), which illustrates that immunoglobulins are present in amyloid-laden tissue without representing amyloid proteins.

In summary, we show here that small synthetic peptides can be used to successfully raise antibodies directed against human immunoglobulin-derived λ-light chain proteins that detect AL amyloid proteins by conventional immunohistochemistry as well as in Western blots. This offers the opportunity to generate a large set of anti-λ-light chain-antibodies independently from native human tissue sources, limited sequence variability and ethical restrictions for the immunohistochemical classification of AL amyloid.

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References

Abe M., Goto T., Kennel S. J., Wolfenbarger D., Macy S.D., Weiss D.T. and Solomon A. (1993). Production and immunodiagnostic applications of antihuman light chain monoclonal antibodies. Am. J. Clin. Pathol. 100, 67-74.

Arbustini E., Morbini P., Verga L., Concardi M., Porcu E., Pilotto A.,

Zorzoli I., Garini P., Anesi E. and Merlini G. (1997). Light and electron microscopy immunohistochemical characterization of amyloid deposits. Amyloid 4, 157-170.

Bohne S., Sletten K., Menard R., Bühling F., Vockler S., Wrenger E., Roessner A. and Röcken C. (2004). Cleavage of AL amyloid proteins and AL amyloid deposits by cathepsins B, K, and L. J. Pathol. 203, 528-537.

Daigeler A., Kuci H., Altmann S., Schneider W. and Röcken C. (2006). Extramedullary multiple myeloma associated with amyloidomas of the lower limb. Plast. Reconstruct. Surg. 118, 298-301.

Gellermann G.P., Appel T.R., Tannert A., Radestock A., Hortschansky P., Schroeckh V., Leisner C., Lütkepohl T., Shtrasburg S., Röcken C., Pras M., Linke R.P., Diekmann S. and Fändrich M. (2005). Raft lipids as common components of human extracellular amyloid fibrils. Proc. Natl. Acad. Sci. USA 102, 6297-6302

Gregorini G., Izzi C., Obici L., Tardanico R., Röcken C., Viola B.F., Capistrano M., Donadei S., Biasi L., Scalvini T., Merlini G. and Scolari F. (2005). Renal Apolipoprotein A-I Amyloidosis: A rare cause of hereditary tubulo-interstitial nephritis. J. Am. Soc. Nephrol. 16, 3680-3686.

Häggqvist B., Naslund J., Sletten K., Westermark G.T., Mucchiano G., Tjernberg L.O., Nordstedt C., Engstrom U. and Westermark P. (1999). Medin: An integral fragment of aortic smooth muscle cell-produced lactadherin forms the most common human amyloid. Proc. Natl. Acad. Sci. USA 96, 8669-8674.

Hoshii Y., Setoguchi M., Iwata T., Ueda J., Cui D., Kawano H., Gondo T., Takahashi M. and Ishihara T. (2001). Useful polyclonal antibodies against synthetic peptides corresponding to immunoglobulin light chain constant region for immunohistochemical detection of immunoglobulin light chain antibodies. Pathol. Int. 51, 264-270.

Hurle M.R., Helms L.R., Li L., Chan W. and Wetzel R. (1994). A role for destabilizing amino acid replacements in light-chain amyloidosis. Proc. Natl. Acad. Sci. USA 91, 5446-5450.

Kaplan B., Hrncic R., Murphy C.L., Gallo G., Weiss D.T. and Solomon A. (1999). Microextraction and purification techniques applicable to chemical characterization of amyloid proteins in minute amounts of tissue. Methods Enzymol. 309, 67-81.

Kaplan B., Martin B.M., Livneh A., Pras M. and Gallo G.R. (2004). Biochemical subtyping of amyloid in formalin-fixed tissue samples confirms and supplements immunohistologic data. Am. J. Clin. Pathol. 121, 794-800.

Kaplan B., Shtrasburg S. and Pras M. (2003). Micropurification techniques in the analysis of amyloid proteins. J. Clin. Pathol. 56, 86-90.

Kebbel A. and Röcken C. (2006). Immunohistochemical classification of amyloid in surgical pathology revisited. Am. J. Surg. Pathol. 30, 673-683.

Lachmann H.J., Booth D.R., Booth S.E., Bybee A., Gilbertson J.A., Gillmore J.D., Pepys M.B. and Hawkins P.N. (2002). Misdiagnosis of hereditary amyloidosis as AL (primary). amyloidosis. N. Engl. J. Med. 346, 1786-1791.

Layfield R., Bailey K., Lowe J., Allibone R., Mayer R.J. and Landon M. (1996). Extraction and protein sequencing of immunoglobulin light chain from formalin-fixed cerebrovascular amyloid deposits. J. Pathol. 180, 455-459.

Linke R.P., Nathrath W.B.J. and Eulitz M. (1986). Classification of amyloid syndromes from tissue sections using antibodies against various amyloid fibril proteins: report of 142 cases. In: Amyloidosis. Glenner G.G., Osserman E.F., Benditt E.P., Calkins E., Cohen A.S. and Zucker-Franklin D. (eds). Plenum Publishing Corporation. New York, London. pp 599-605.

Merlini G. and Bellotti V. (2003). Molecular mechanisms of amyloidosis. N. Engl. J. Med. 349, 583-596.

Mucchiano G.I., Haggqvist B., Sletten K. and Westermark P. (2001). Apolipoprotein A-1-derived amyloid in atherosclerotic plaques of the human aorta. J. Pathol. 193, 270-275.

Müller D., Roessner A. and Röcken C. (2000). Distribution pattern of matrix metalloproteinases (MMP-1, -2, -3, and -9)., tissue inhibitors of matrix metalloproteinases (TIMP-1 and -2)., and a2-macroglobulin in cases of generalized AA- and AL amyloidosis. Virchows Arch. 437, 521-527.

Parker J.M., Guo D. and Hodges R.S. (1986). New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. Biochemistry 25, 5425-5432.

Puchtler H., Sweat F. and Levine M. (1962). On the binding of Congo red by amyloid. J. Histochem. Cytochem. 10, 355-364.

Röcken C. and Sletten K. (2003). Amyloid in surgical pathology. Virchows Arch. 443, 3-16.

Röcken C. and Wilhelm S. (2005). Influence of tissue fixation on the microextraction and identification of amyloid proteins. J. Lab. Clin. Med. 146, 244-250.

Röcken C., Kronsbein H., Sletten K., Roessner A. and Bässler R. (2002). Amyloidosis of the breast. Virchows Arch. 440, 527-535.

Röcken C., Menard R., Bühling F., Vöckler S., Raynes J., Stix B., Krüger S., Roessner A. and Kähne T. (2005). Proteolysis of serum amyloid A and AA amyloid proteins by cysteine proteases: cathepsin B generates AA amyloid proteins and cathepsin L may prevent their formation. Ann. Rheumatic Dis. 64, 808-815.

Röcken C., Schwotzer E., Linke R.P. and Saeger W. (1996). The classification of amyloid deposits in clinicopathological practice. Histopathology 29, 325-335.

Röcken C., Tautenhahn J., Bühling F., Sachwitz D., Vöckler S., Goette A. and Bürger T. (2006). Prevalence and pathology of amyloid in atherosclerotic arteries. Arterioscler. Thromb. Vasc. Biol. 26, 676-677.

Solomon A., Weiss D.T., Macy S.D. and Antonucci R.A. (1990). Immunocytochemical detection of kappa and lambda light chain V region subgroups in human B-cell malignancies. Am. J. Pathol. 137, 855-862.

Stevens F.J., Myatt E.A., Chang C.H., Westholm F.A., Eulitz M., Weiss D.T., Murphy C., Solomon A. and Schiffer M. (1995). A molecular model for self-assembly of amyloid fibrils: immunoglobulin light chains. Biochemistry 34, 10697-10702.

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