ELSEVIER

Journal of Chromatography B, 000 (2001) 000–000

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Molecular heterogeneity of amyloid β_2-microglobulin and modification with advanced glycation end products

Roumyana Mironova[a], Toshimitsu Niwa[b,*]

[a]*Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[b]*Department of Clinical Preventive Medicine, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan*

Abstract

By using liquid chromatography–electrospray ionization mass spectrometry, Western blotting and N-terminal amino acid sequence analysis, we characterized the molecular heterogeneity and advanced glycation end product (AGE) modification of β_2-microglobulin (β_2m) extracted from the amyloid tissue of a hemodialysis patient. Amyloid β_2m was composed of full-length β_2m, truncated β_2m and dimer β_2m. Truncated β_2m and dimer β_2m were modified with AGEs such as imidazolone and N^{ϵ}-(carboxymethyl)lysine, and showed fluorescence characteristic of AGE. Truncated β_2m species were formed by cleavage between amino acid residues of Pro^6/Ile^7, Gln^8/Val^9 and Val^9/Tyr^{10}. Heterogeneous dimer β_2m species showed the molecular masses of 22 591 and 22 675, which resulted from cross-linking between truncated β_2m.

Keywords: β_2-Microglobulin

1. Introduction

Dialysis-related amyloidosis (DRA) is a major complication in uremic patients on long-term dialysis, and presents with carpal tunnel syndrome (CTS), cystic bone lesions, destructive spondylarthropathy (DSA), arthritis and periarthritis, systemic organ involvement, and dialysis-related spinal canal stenosis [1–4]. The major component of amyloid was demonstrated to be β_2-microglobulin (β_2m). β_2m isolated from the amyloid deposits in patients with DRA is modified with advanced glycation end products (AGEs) [5–10].

3-Deoxyglucosone (3-DG) is a highly reactive carbonyl compound, and reacts again with free amino groups of proteins, leading to the formation of AGEs. Serum 3-DG levels are elevated not only in diabetic patients [11] but also in uremic patients [9,12]. 3-DG accumulating in uremic serum may be involved in the AGE modification of β_2m-amyloid [9,10]. Erythrocyte 3-DG levels are also increased in uremic patients [13,14], and its accumulation in erythrocytes may be related to cellular dysfunction because of AGE modification of intracellular enzymes such as glutathione peroxidase [15]. Imidazolone, a reaction product of the guanidino group of arginine with 3-DG, was found to be a common epitope of AGE-modified proteins [16,17]. Imidazolone is a reaction product most specific for 3-DG among AGE structures. Further, since N^{ϵ}-(carboxymethyl)lysine (CML) formation is closely linked to oxidative processes, CML represents a general marker of oxidative stress and long-term damage to proteins. We produced several clones of

*Corresponding author. Tel.: +81-52-744-1980; fax: +81-52-744-1954.

E-mail address: tniwa@med.nagoya-u.ac.jp (T. Niwa).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00139-6

monoclonal anti-AGE antibodies; subsequently, two of them were characterized as anti-imidazolone (AG-1) [10,16,17] and anti-CML (AG-10) [8] antibodies. Imidazolone and CML were localized in the amyloid deposits [8,10,18] and the aorta [19] of hemodialysis (HD) patients as demonstrated by immunohistochemistry [8,10,19] and immunoelectron microscopy [18] using these antibodies. The serum levels of imidazolone are increased in HD patients [20]. These results strongly suggest that imidazolone produced by 3-DG, and CML produced by glycoxidation may contribute to the progression of uremic complication.

This study aimed to characterize the molecular heterogeneity and AGE modification of β_2m extracted from the amyloid tissue of a hemodialysis patient. By using liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS), Western blotting and N-terminal amino acid sequence analysis, we determined the molecular masses of amyloid β_2m species, AGE modification of amyloid β_2m, and N-terminal amino acid sequence of truncated amyloid β_2m.

2. Experimental

2.1. Extraction of β_2m from amyloid tissue of a patient on HD

The β_2m fractions were extracted from DRA tissues as described in detail elsewhere [7]. In brief, an amyloid tissue was homogenized in 0.15 *M* NaCl and the homogenate was centrifuged at 12 000 *g* for 30 min. The supernatant was discarded and the pellet was again homogenized and recentrifuged. This extraction procedure was repeated until absorption (*A* 280 nm) of the supernatant decreased by less than 0.05. The lyophilized amyloid fibrils were dissolved in 80% formic acid for 8 h at room temperature, centrifuged at 12 000 *g* for 60 min and separated according to molecular size by Sephadex G-75 Superfine (Pharmacia) gel permeation chromatography at 4°C. The chromatogram monitored at UV 280 nm was composed of three peaks. β_2m levels in each fraction were quantified using enzyme-linked immunosorbent assay (ELISA), demonstrating that the second and the third peaks contained β_2m. The second peak was found to be β_2m dimer, while the third one was composed of full-length and truncated β_2m sub-fractions as confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. SDS–PAGE

All β_2m fractions were run on 22% gels prepared with a stock solution of acrylamide–*N*,*N*-methylene-bis-acrylamide (30:0.4). Separating gels were produced by mixing 6 ml of acrylamide stock solution, 2.5 ml of 1.5 *M* Tris–HCl, pH 8.8, 100 μl of 10% SDS, distilled water (to final volume of 10 ml), 10 μl of 10% ammonium persulfate (APS) and 10 μl of *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED). For preparation of stacking gels 1 ml acrylamide stock solution was added to 1.25 ml of 0.5 *M* Tris–HCl, pH 6.8, 50 μl of 10% SDS, distilled water (up to 5 ml), 50 μl of 10% APS and 5 μl of TEMED. Samples (1 to 4 μg of protein in 10 μl of Tris-buffered saline, TBS) were applied with 10 μl of 50 m*M* Tris–HCl (pH 6.8) containing 40% glycerol, 4% SDS, 2% β-mercaptoethanol and 0.01% bromophenol blue. Electrophoresis (Bio-Rad, Ready Gel Cell apparatus) was carried out at 50 mA constant current at ambient temperature in 1× Tris–glycine–SDS running buffer (Bio-Rad, 10× premix). Gels were stained in a solution of 0.2% coomassie blue R-250 in 10% acetic acid, 25% methanol and de-stained in the same solution devoid of the dye.

2.3. Western blotting

After SDS–PAGE the proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes Immobilon-P^{SQ} (Millipore, Bedford, MA, USA) presoaked for 30 min in a blotting buffer composed of 0.25 *M* Tris base, 0.19 *M* glycine and 5% methanol. The transfer was carried out in 1 l of blotting buffer in a Hoefer Sci Instr apparatus (2501 Midget Multiblot) for 40 min at 200 mA constant current at ambient temperature. After blotting the membranes were washed with roughly 150 ml of TBS (0.9% NaCl, 10 m*M* Tris–HCl, pH 7.4), and incubated overnight at 4°C in a blocking solution of

1% bovine serum albumin (BSA) in TBS. The next day, membranes were treated with either the monoclonal anti-AGE antibodies AG-1 and AG-10, specific for imidazolone [10,16,17] and CML [8], respectively, or a rabbit anti-human β_2m antibody (Dako Code No. A072). The reaction was performed by shaking the membranes at room temperature for 2 h in 5 ml of blocking buffer containing 5 μl of the respective antibodies. After reaction, membranes were washed five times with 10 ml TBS and then soaked by shaking in 5 ml of blocking buffer containing 5 μl of either anti-rabbit (Sigma product No. A0545) or anti-mouse (Miles Scientific) peroxidase-labeled IgGs. After washing five times as above, the membranes were stained with 10 ml of 25 m*M* Tris–HCl, pH 7.5 containing 1.6 mg 3.3′-diaminobenzidine and 10 μl of H_2O_2. The membrane with the blotted molecular mass standards (Bio-Rad, catalog No. 161-0317) was stained in a solution of 0.1% coomassie blue R-250, 7% glacial acetic acid and 50% methanol and destained in 7% acetic acid, 80% methanol.

2.4. High-performance liquid chromatography (HPLC) and fluorescence measurements

The chromatograph assembly (Japan Spectroscopic, Tokyo, Japan) was equipped with a Model 880-PU pump, a Model 880-50 three-line degasser, a Model 875-UV detector and a Model 820-FP fluorescent detector, a Model 880-02 ternary gradient unit, a Model 851-AS sampler, a Model Chromatocorder 12 and a reversed-phase column (TSKgel Phenyl-5PW, 75×8 mm; Nomura, Aichi, Japan). A mobile phase consisting of solution A [0.1% trifluoroacetic acid (TFA) in distilled water] and solution B (0.08% TFA, 90% acetonitrile) was delivered at a flow-rate of 0.5 ml/min at ambient temperature. Protein samples (20–50 μg in 20 μl of solution A) were automatically injected and elution was carried out isocratically for 5 min with 15% solution B and then with a linear gradient of solution B up to 100% for 30 min. The eluate was monitored for UV absorbance at 280 nm and fluorescence at 445 nm upon excitation at 370 nm. The relative fluorescence was expressed as a ratio of the fluorescence peak area to the UV peak area.

2.5. Electrospray ionization mass spectrometry coupled to liquid chromatography (LC–ESI-MS)

The mass spectra were obtained on a Finnigan MAT triple stage quadrupole TSQ 7000 (San Jose, CA, USA) with an atmospheric pressure ionization (API) ion source operated in the ESI mode. Mass spectral data were recorded in the positive ion mode. The electrospray was operated at a potential differential of 4.5 kV. The nitrogen sheath gas was set to 70 p.s.i. (1 p.s.i.=6894.76 Pa). Heated nitrogen at 275°C was used as a drying gas and introduced into the capillary region at a flow-rate of 25 l/min. The system was run in an automated LC–MS mode. The mass spectrometer was equipped with a Hewlett-Packard HPLC system Series 1050. Approximately 10 μg of sample was applied to the column (TSKgel Phenyl-5PW, 75×8 mm; Nomura) in a volume of 40 μl at an injection speed of 4 μl/s. The automated analysis used a mobile phase consisting of solution A (2% acetonitrile in 0.2% acetic acid) and solution B (90% acetonitrile in 0.2% acetic acid) delivered at a flow-rate of 0.5 ml/min. The mobile phase was linearly programmed from 15 to 40% solution B in 30 min. The mass spectra were acquired in the m/z range 600–1400 by scanning the magnetic field in 2 s with a mass resolution of 1000 u. Deconvoluted mass spectra were recorded in the Bioworks ICIS window (Version 8.2).

In all experiments human β_2m from urine of healthy subjects (Sigma product No. M 4890) was used as a reference sample.

2.6. N-Terminal amino acid sequencing

The N-terminal sequence of β_2m was determined on a HPG1000A protein sequenator (Hewlett-Packard) with the routine 3.5 chemistry method and the PTH_4 M HPLC method.

3. Results

The β_2m was extracted from DRA tissues of a patient on HD and separated according to molecular size by gel permeation chromatography. The fractions containing β_2m were detected by ELISA and found to contain a number of molecular size species

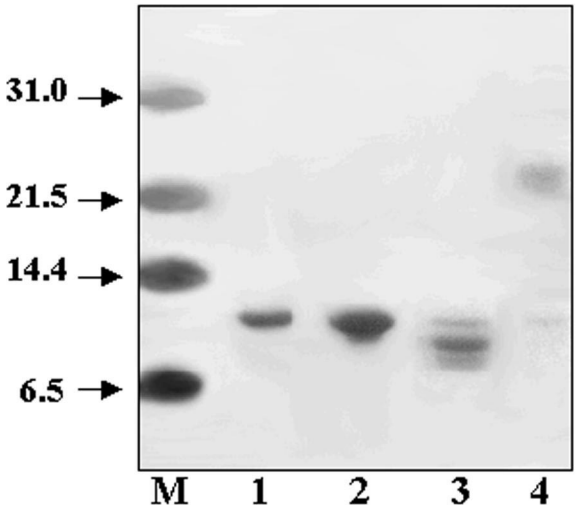

Fig. 1. SDS–PAGE of reference β_2m (lane 1), amyloid full-length β_2m (lane 2), amyloid truncated β_2m (lane 3) and amyloid dimer β_2m (lane 4). M, molecular mass standards in k mass.

by SDS–PAGE. According to their electrophoretic pattern the isolated fractions were designated as (i) full-length β_2m, (ii) truncated β_2m and (iii) dimer β_2m (Fig. 1). The latter two fractions appeared as broad bands on the SDS gel, indicating the presence of several truncated and dimer species in the amyloid β_2m.

To study the AGE-modifications of the different β_2m fractions, we performed the Western blot analysis presented in Fig. 2. As visible, all the β_2m fractions proved to be modified with CML and imidazolone, with the full-length protein showing lower reactivity to both anti-AGE antibodies. The levels of AGE-modifications in the amyloid β_2m fractions were demonstrated by comparative studies on the relative fluorescence (Fig. 3). The different fractions were subjected to reversed-phase (RP) HPLC coupled to fluorescence detection. Total fluorescence was recorded at 445 nm upon excitation at 370 nm. Under these conditions, the fluorescence of the reference β_2m was negligible as compared to that of all the amyloid β_2m fractions studied (Fig. 3). Among the amyloid β_2m fractions, the full-length protein showed the lowest relative fluorescence (Table 1). The truncated β_2m species produced 1.6 times higher relative fluorescence than the full-length protein, whereas the β_2m dimer demonstrated a remarkable fluorescence, an order of magnitude higher than that of the full-length β_2m. By the RP-HPLC analysis both, the reference and the full-length amyloid β_2m, eluted earlier [retention time (t_R), 15.0 min] than the truncated and dimer β_2m fractions (t_R, 18.8 min).

LC–ESI-MS analysis was performed, in order to more precisely analyze the molecular heterogeneity

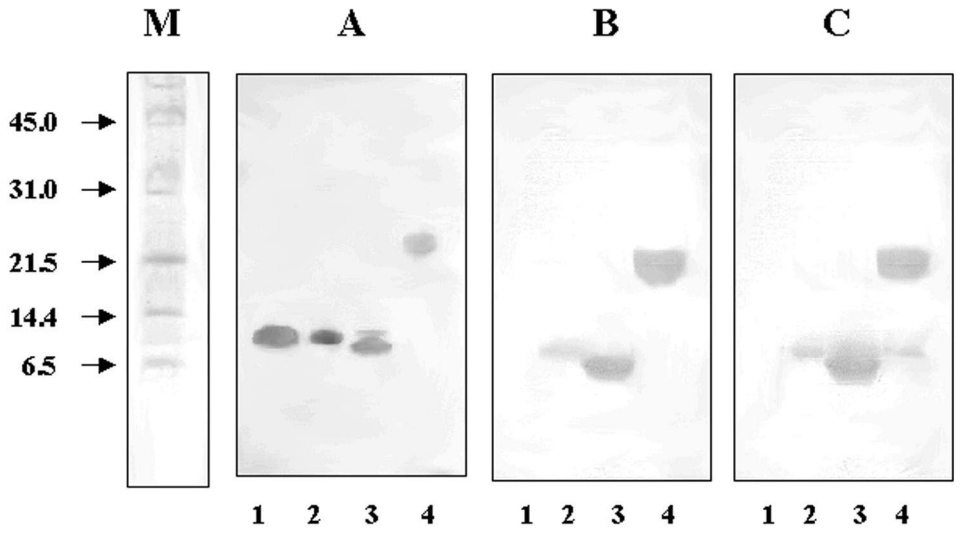

Fig. 2. Western blots of reference β_2m (lane 1), amyloid full-length β_2m (lane 2), amyloid truncated β_2m (lane 3) and amyloid dimer β_2m (lane 4) treated with an anti-β_2m (A), anti-imidazolone (B) or anti-CML antibody (C). M, molecular mass standards in k mass.

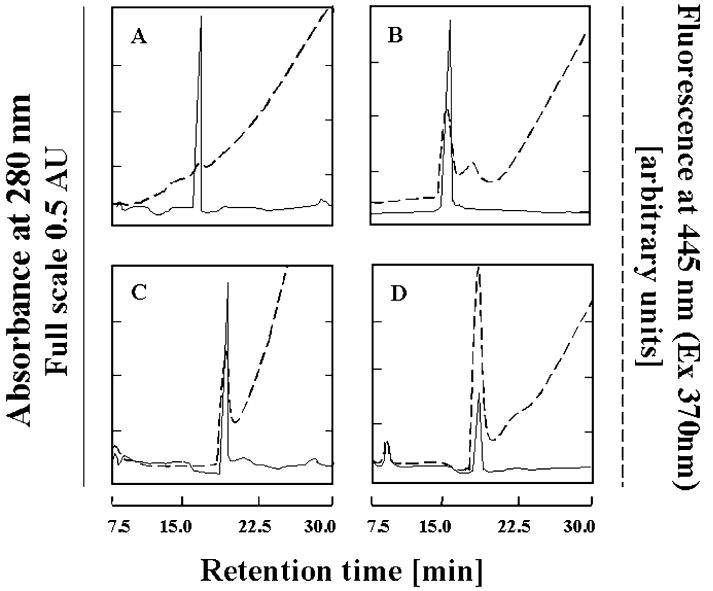

Fig. 3. RP-HPLC and total fluorescence of reference β_2m (A), amyloid full-length β_2m (B), amyloid truncated β_2m (C) and amyloid dimer β_2m (D).

of the amyloid β_2m. We determined a molecular mass of 11 730 for both, the reference and the amyloid full-length β_2m (Fig. 4A and B). In addition, the reference β_2m showed the presence of a higher molecular mass derivative (11 746), most probably the result of oxidation of the only methionine residue in β_2m (Met^{99}) to its sulfoxide (+16). The truncated amyloid β_2m fraction was composed of several species with molecular masses of 11 004 (species I), 10 762 (species II) and 10 663 (species III), respectively (Fig. 4C). This fraction contained also a species with a molecular mass of 11 729, which evidently corresponded to the full-length β_2m. As one can see on the SDS–polyacrylamide gel in Fig. 1, the truncated β_2m fraction contained also a trace amount of the full-length β_2m. In the deconvoluted ESI mass spectrum of the β_2m dimer sample there was no pronounced peak registered (Fig. 4D). This fraction obviously represented a heterogeneous population of a number of dimer species with close molecular masses. The most abundant dimer species were those with molecular masses of 22 591 and 22 675. These mass values indicated that dimers resulted predominantly from cross-linking between truncated β_2m.

The N-terminal sequencing of the truncated β_2m species indicated that they resulted from cleavage between amino acid residues Pro^6/Ile^7, Gln^8/Val^9 and Val^9/Tyr^{10}. These data well coincided with the molecular masses of the corresponding β_2m truncated species as determined by ESI-MS (Table 2).

Table 1
Relative fluorescence of different β_2m fractions

β_2m fraction	Relative fluorescence[a]
Reference	0.02±0.02
Full-length	0.5±0.1
Truncated	0.8±0.1
Dimer	5.5±1.2

[a] Values are expressed as mean±SE (n=3). The relative fluorescence (arbitrary unit) was expressed as a ratio of the fluorescence peak area to the UV peak area.

4. Discussion

We demonstrated that amyloid β_2m species are heterogeneous, and consist of full-length β_2m, truncated β_2m, and dimer β_2m. Dimer β_2m showed the most intensive fluorescence characteristic of AGE, followed by truncated β_2m and full-length β_2m. Dimer β_2m and truncated β_2m are modified with

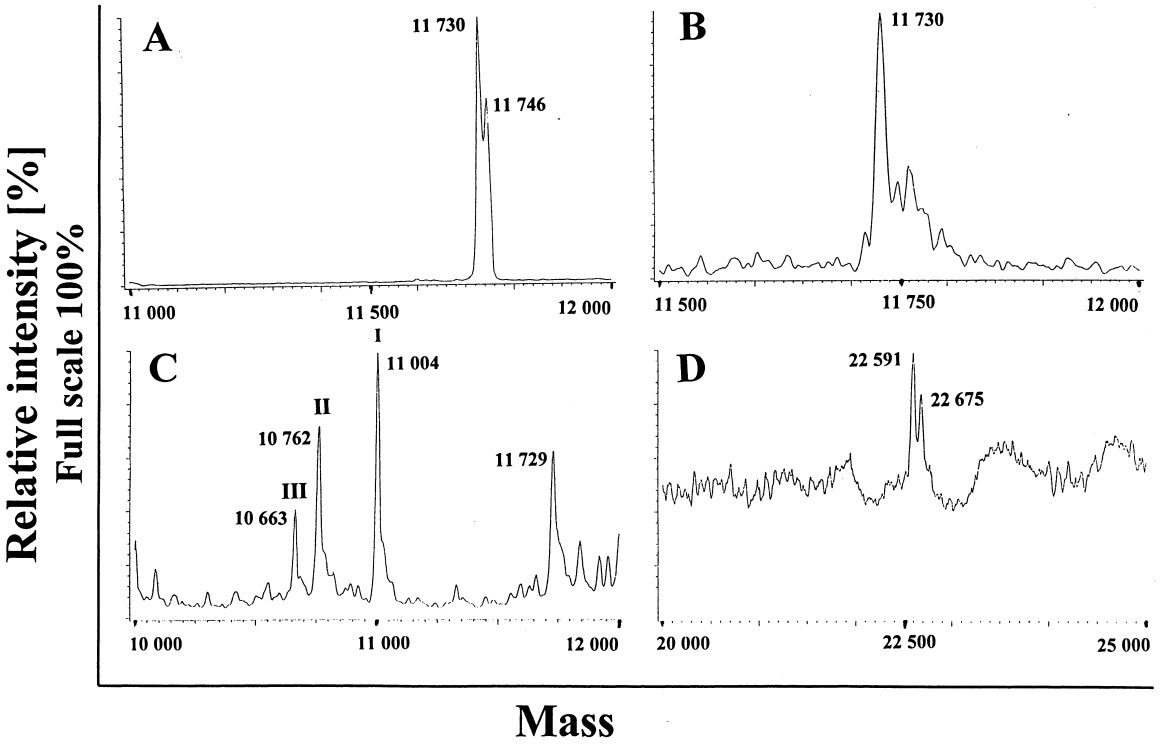

Fig. 4. Deconvoluted ESI mass spectra of reference β_2m (A), amyloid full-length β_2m (B), amyloid truncated β_2m (C) and amyloid dimer β_2m (D).

AGEs such as imidazolone and CML. Truncated β_2m species are formed by cleavage between amino acid residues Pro^6/Ile^7, Gln^8/Val^9 and Val^9/Tyr^{10}. Dimer β_2m species may be formed by cross-linking between the truncated β_2m species.

The dimeric form of β_2m was also demonstrated to exist in the dialysate and urine of uremic patients, and is more susceptible to imidazolone modification than the monomer [21]. Recently, oligomers of β_2m from patients with dialysis-associated amyloidosis were reported to induce complement activation via the classical pathway [22]. Soluble β_2m purified from peritoneal dialysates was found to be a potent complement activator while β_2m purified from urine exhibited lower activity, a difference which may be explained by differences observed in oligomers and isoforms.

The major component of dialysis amyloid is a full-length form of β_2m, while AGE-modified β_2m, truncated β_2m, and dimer β_2m are the minor com-

Table 2
N-Terminal sequence and molecular mass of truncated β_2m species

Truncated β_2m species	N-Terminal sequence	Molecular mass	
		Determined	Predicted[a]
I	Ile^7–Gln^8–Val^9–Tyr^{10}–Ser^{11}	11 004	11 004.6
II	Val^9–Tyr^{10}–Ser^{11}–Arg^{12}–His^{13}	10 762	10 762.4
III	Tyr^{10}–Ser^{11}–Arg^{12}–His^{13}–Pro^{14}	10 663	10 663.4

[a] Mass was calculated by subtracting the theoretical monoisotopic mass of the N-terminal missing peptides from the mass of 11 729 determined for the full-length β_2m in the mass spectrum in Fig. 4C.

ponents. Thus, 3-DG and the other dicarbonyl compounds accumulating in uremic serum may promote the modification of β_2m with AGEs mainly after deposition of β_2m as amyloid.

References

[1] T. Niwa, Nephron 76 (1997) 373.
[2] T. Niwa, Semin. Dial. (2001) in press.
[3] K. Nokura, H. Koga, H. Yamamoto, A. Kimura, H. Tamai, S. Yazaki, N. Suzuki, S. Miyazaki, T. Niwa, J. Neurol. Sci. 178 (2000) 114.
[4] F. Takayama, S. Miyazaki, T. Morita, Y. Hirasawa, T. Niwa, Kidney Int. 59 (Suppl. 78) (2001) S172.
[5] T. Miyata, O. Oda, R. Inagi, Y. Iida, N. Araki, N. Yamada, S. Horiuchi, N. Taniguchi, K. Maeda, T. Kinoshita, J. Clin. Invest. 92 (1993) 1243.
[6] T. Niwa, S. Miyazaki, T. Katsuzaki, N. Tatemichi, Y. Takei, T. Miyazaki, T. Morita, Y. Hirasawa, Kidney Int. 48 (1995) 771.
[7] T. Niwa, T. Katsuzaki, T. Momoi, T. Miyazak, H. Ogawa, A. Saito, S. Miyazaki, K. Maeda, N. Tatemichi, Y. Takei, Kidney Int. 49 (1996) 861.
[8] T. Niwa, M. Sato, T. Katsuzaki, T. Tomoo, T. Miyazaki, N. Tatemichi, Y. Takei, T. Kondo, Kidney Int. 50 (1996) 1303.
[9] T. Niwa, T. Katsuzaki, T. Momoi, T. Miyazaki, H. Ogawa, A. Saito, S. Miyazaki, K. Maeda, N. Tatemichi, Y. Takei, Kidney Int. 49 (1996) 861.
[10] T. Niwa, T. Katsuzaki, S. Miyazaki, T. Momoi, T. Akiba, T. Miyazaki, K. Nokura, F. Hayase, N. Tatemichi, Y. Takei, Kidney Int. 51 (1997) 187.
[11] T. Niwa, N. Takeda, H. Yoshizumi, A. Tatematsu, M. Ohara, S. Tomiyama, K. Niimura, Biochem. Biophys. Res. Commun. 196 (1993) 837.
[12] T. Niwa, N. Takeda, T. Miyazaki, H. Yoshizumi, A. Tatematsu, K. Maeda, M. Ohara, S. Tomiyama, K. Niimura, Nephron 69 (1995) 438.
[13] S. Tsukushi, C. Naito, I. Aoyama, F. Takayama, T. Miyazaki, T. Katsuzaki, K. Shimokata, T. Niwa, Kidney Int. 55 (1999) 1970.
[14] S. Tsukushi, K. Shimokata, T. Niwa, J. Chromatogr. 731 (1999) 37.
[15] T. Niwa, S. Tsukushi, Kidney Int. 59 (Suppl. 78) (2001) S37.
[16] T. Niwa, T. Katsuzaki, S. Miyazaki, T. Miyazaki, Y. Ishizaki, F. Hayase, N. Tatemichi, Y. Takei, J. Clin. Invest. 99 (1997) 1272.
[17] T. Niwa, T. Katsuzaki, Y. Ishizaki, F. Hayase, T. Miyazaki, T. Uematsu, N. Tatemichi, Y. Takei, FEBS Lett. 407 (1997) 297.
[18] D. Brancaccio, M. Gallieni, P. Braidotti, T. Niwa, G. Coggi, J. Nephrol. 13 (2000) 126.
[19] F. Takayama, I. Aoyama, S. Tsukushi, T. Miyazaki, S. Miyazaki, T. Morita, Y. Hirasawa, K. Shimokata, T. Niwa, Cell. Mol. Biol. 44 (1998) 1101.
[20] G. Münch, R. Keis, A. Weβels, P. Riederer, U. Bahner, A. Heidland, T. Niwa, H.-D. Lemke, R. Schinzel, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 669.
[21] S. Franke, T. Niwa, W. Deuther-Conrad, M. Sommer, G. Hein, G. Stein, Clin. Chim. Acta 300 (2000) 29.
[22] M. Nybo, E.H. Nielsen, S.E. Svehag, Amyloid 6 (1999) 265.