Molecular characterization of β-trace protein in human serum and urine: a potential diagnostic marker for renal diseases

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Introduction

β-Trace protein (β-TP) is one of the most prominent polypeptide constituents of human cerebrospinal fluid (CSF) but its presumed biological function as a prostaglandin D synthase (prostaglandin H2 D isomerase; (5Z, 13E)-(15S)-9α-epidioxy-15-hydroxyprosta-5,13-dienoate D isomerase, EC 5.3.99.2) remains disputed. P-TP belongs to the lipocalin protein family (prostaglandin H2 isomerase; (5Z, 13E)-(15S)-9α-epidioxy-15-hydroxyprosta-5,13-dienoate D isomerase, EC 5.3.99.2) and comprises small, secretory proteins commonly involved in binding and transport of small hydrophobic ligands. A possible function as transporter of prostaglandins or other hydrophobic molecules has been suggested. Our protein preparations from different sources (CSF, serum, and produced by recombinant techniques) all have yellow-brownish colors suggesting presence of a colored ligand in these preparations, e.g., bound bilirubin (cf. discussion in Nagata et al., 1991).

β-TP is produced within the central nervous system (leptomeninges, choroid plexus epithelium, and oligodendrocytes) and male gonads (Urade et al., 1993; Olsson and Link, 1973; Hoffmann et al., in press, a) and secreted into adjacent body fluids such as the CSF, aqueous humor, and sperm fluid (Hartmann et al., 1983). Mean concentration values ranging from 10% of the total CSF protein (i.e., about 40 μg/ml; Pepe and Hochwald, 1967), 33 μg/ml (Felgenhauer et al., 1987), 26 μg/ml (Link, 1967; Thompson, 1988), and 23 μg/ml (Link and Olsson, 1972) to 8 μg/ml (Zahn et al., 1993) in CSF have been reported. Its concentration in normal human serum (Claußen, 1961; Link, 1967) and urine (Hochwald and Thorbecke, 1962; Ericsson et al., 1969) is supposed to be very low. For serum, 3.7 μg/ml (1/7 of the CSF-concentration) was measured (Olsson et al., 1973). The site of biosynthesis of the β-trace molecular forms in serum and urine has not yet been elucidated.

CSF-derived β-TP is a glycoprotein exhibiting significant microheterogeneity which is due to special truncated biantenary oligosaccharide chains that have the following characteristics (Hoffmann et al., 1994): high amounts of terminal galactose (Gal) residues, high amounts of terminal N-acetylgalactosamine (GlcNAc) (agalactoantennae as well as bisecting GlcNAc), proximal α1,6-fucosylation, and notable amounts of Lewisα-type peripheral fucosylation (fucose bound α1,3 to GlcNAc). Neuraminic acid (NeuAc) is present in α2,3- and α2,6-linkage in approximately equal amounts. We have recently raised the hypothesis that these features are characteristic of “brain-type” N-glycosylation (Hoffmann et al., 1994, 1995).

Especially asialo- and asialo-agalacto-antennae are not commonly seen with serum proteins which constitute some 80% of the CSF-protein (Wiederkehr, 1992) after transfer across the blood–brain and blood–CSF barriers. This is because they are not compatible with the known hepatic clearance mechanisms for proteins resulting in serum-type N-glycosylation features of the proteins in human serum which have been secreted by the liver, by blood cells, or which enter blood after resorption of CSF at the arachnoidal granulations or via nerve roots and the lymphatic system (Cser et al., 1990). At least five carbohydrate-specific receptor systems in the liver recognize nonreducing monosaccharides. The asialoglycoprotein receptor of hepatocytes (Ashwell and Harford, 1982) is specific for terminal galactose and N-acetylgalactosamine. There also are N-acetylgalactosamine- and mannose-receptors on Kupffer and endothelial liver cells, and a Lewisα-specific receptor on hepatocytes (Schauer, 1985). Only glycoproteins circulating in blood which do not bear such sugar residues can persist. Especially, glycoforms lacking N-acetyleneuraminic acid residues are rap-
Results

Identification, purification, and quantitation of β-TP from human sera and plasma

In order to characterize β-TP from human blood we initially screened whole serum and plasma samples from normal volunteers for β-TP by Western blotting using the polyclonal antiserum against the CSF-protein. No significant amounts of β-TP could be identified by this procedure. The β-TP from five nonpathological plasma and three serum samples as well as from one serum obtained from a hemodialysis patient was purified by immunoaffinity chromatography as described in the Materials and methods section. The Western blot of Figure 1 shows the results for the serum samples. The amount of isolated β-TP was found to be low for the normal sera (lanes 2–7). By comparison with a defined amount of standard-β-TP from CSF, values of about 40 ng/ml β-TP for all samples from healthy individuals were obtained. However, Figure 1 demonstrated an intriguingly high amount of β-TP in case of the dialysis patient’s serum (lanes 8 and 9), estimated to be about 5 μg/ml, i.e., ca. 100-fold elevated.

In order to confirm the elevated concentration of β-TP in the sera of dialysis patients, β-TP was quantitatively purified from sera of five further patients by immunoaffinity chromatography. Figure 2 compares isolated protein from these patients and two normal plasma samples. Lanes 3–7 represent β-TP from the patients (2.5% of the eluted protein from about 20 ml samples), lanes 8 and 9 from plasma of normal individuals (12.5% from 50 ml samples). The samples obtained from the dialysis patients contain significantly higher levels of β-TP. Based on comparison with a standard, calculated concentrations of 1.7, 3.6, 3.6, 4.2, and 5.7 μg/ml, respectively, were determined. These values are roughly 100x higher than those found for the healthy specimens, suggesting a specific accumulation of β-TP in serum of dialysis patients.

Detection of β-TP in hemofiltrate of dialysis patients

During the process of dialysis β-TP should penetrate into the filtrate due to its rather low molecular weight whereas higher molecular weight proteins are retained by the cut-off of the membranes thus making hemofiltrate (HF) a more convenient source for purification of β-TP than serum and being available in unlimited supply. Western blot analysis of hemofiltrates from seven different patients (Figure 3A) revealed similar amounts of β-TP, with concentrations of about 200–300 ng/ml. The molecular weight of β-TP was identical in all cases. As in the case of β-TP from samples of normal individuals SDS-electrophoresis revealed a higher molecular weight compared to the CSF-protein (Figure 3B) but identical to the one of β-TP from nonpathological sera (Figure 2). Since major structural differences between β-TP in blood samples from healthy volunteers and patients were not detected, β-TP from human HF was used for subsequent analysis of posttranslational modifications.

Comparison of blood-derived and urinary β-TP with CSF-β-TP

HF-β-TP. Using immunoaffinity chromatography 14 mg of purified HF-β-TP were obtained from 72 l of pooled hemofiltrate.

Fig. 1. Western blot analysis of β-TP isolated from sera of three normal volunteers (lanes 2–7) and one dialysis patient (lanes 8 and 9) by immunoaffinity chromatography. For detection of β-TP, a rabbit antiserum against CSF-β-TP was used. Lanes 1 and 10: purified CSF-β-TP, 200 and 50 ng, respectively; lanes 2, 4, 6, 8: 2.5% of the isolated protein preparations; lanes 3, 5, 7, 9: 5% of the isolated protein preparations. Migration positions of standard proteins are indicated on the left side.

Fig. 2. Western blot analysis of β-TP isolated from sera of 5 dialysis patients (lanes 3–7) and 2 normal plasma (lanes 8 and 9) by immunoaffinity chromatography. For detection of β-TP, a monoclonal antibody raised against denatured CSF-β-TP was used. Lanes 1 and 2: purified HF-β-TP, 50 and 250 ng, respectively; lanes 3–7: 2.5% of the isolated protein from 20 ml sera of dialysis patients; lanes 8 and 9: 12.5% of the isolated protein from 50 ml plasma of normal volunteers. The molecular weight of serum-derived and HF-β-TP is identical. Migration positions of standard proteins are indicated on the left side.
Human P-trace in serum and urine

Fig. 3. Analysis of β-TP from HF and CSF by SDS-PAGE. (A) shows a Western blot of hemofiltrates; 150 μl samples obtained from seven different dialysis patients were applied to the gel after ethanol-precipitation (lanes 1–7); lane 8: 140 ng purified HF-β-TP. (B) compares the Coomassie-stained purified CSF (left) and HF-(right)-β-TP preparations (5 μg each). The higher molecular weight of HF-β-TP is obvious. The faint bands below the broad bands represent monoglycosylated protein species (about 2%). Migration positions of standard proteins are indicated on the left side.

Urinary β-TP. In order to demonstrate that serum-derived β-TP is physiologically eliminated via the kidneys we also investigated the glycoforms of urinary β-TP which should be identical to serum-β-TP. Several urine samples from normal volunteers were screened for β-TP by Western blotting (Figure 4). As expected, the molecular weights of both, urinary and blood-derived β-TP, as well as NH₂-termini were identical. Some individual urines only differed by the amount of monoglycosylated protein species present. Figures 2–4 show that upon SDS-gel electrophoresis the serum-derived and urinary proteins exhibit higher molecular weights than their CSF counterpart (22–28 kDa). The broad major (23–30 kDa) and one or two minor bands (22 and 20 kDa, respectively) in the preparations which are reactive with antisera or monoclonal antibodies against CSF-β-TP represent the di- or mono- and unglycosylated protein species thereof as discussed previously (Hoffmann et al., 1994).

Analysis of N-linked glycans. Seven milligrams HF-β-TP, 1 mg urinary β-TP, and 700 μg CSF-β-TP were digested with trypsin as described previously (Hoffmann et al., 1993). The peptides were separated by reversed-phase HPLC, and oligosaccharides were liberated from the glycopeptides by enzymatic digestion with polypeptide-N⁴-(N-acetyl-glucosaminyl)-asparagine amidase F. The total free glycans of each preparation were isolated by reversed-phase HPLC, desalted, and subjected to methylation analysis (data not shown), to matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-MS, Figure 5), and to high-pH anion exchange chromatography (HPAEC, Figures 6, 7).

These analyses revealed differences of HF- and urinary β-TP oligosaccharides to those from CSF although all N-glycans were of the complex type, proximally fucosylated, with predominantly biantennary carbohydrate chains as in the CSF-protein. MALDI-MS data (Figure 5A) obtained for CSF-βTP oligosaccharides were in agreement with our previously reported data on the primary structural analysis of the individual carbohydrate chains of this protein (cf. Hoffmann et al., 1994) with large amounts of asialo and asialoagalacto chains. The HF- and urinary β-TP oligosaccharides were preponderantly mono and disialylated and were very similar in the higher mass range. Five major clusters of signals were obtained (num-
ferred 9, 12–15): the signal m/z 2622 Da (no. 9) results from a biantennary monosialylated oligosaccharide, the most prominent signal obtained at 2984 Da (no. 13) is caused by the corresponding disialylated derivative; the signals no. 12 and 14 (2868 and 3229 Da, respectively) are close relatives of these oligosaccharides, bearing an additional bisecting GlcNAc residue each (the presence of which was inferred from the methylation data). Thus, MS data demonstrate prevalence of fully sialylated (and hence, galactosylated) oligosaccharides over mono- and unsialylated ones whereas in the CSF-protein, asialo-oligosaccharides were predominant (cf. Table I and Figure 5A). In the urine-derived protein, the amount of monoversus disialylated glycans is slightly higher than in HF-β-TP.

Figure 6 shows fingerprinting of the native oligosaccharides by HPAEC for HF- and urinary β-TP in comparison to three serum proteins synthesized by the liver. Panels A and B demonstrate that the glycan pattern for both β-TP preparations is essentially identical. Moreover, the peaks with elution times around 20–30 min which represent the monosialylated glycans are virtually absent from the oligosaccharides of the three liver-derived serum proteins shown in panels C–E. Here, disialylated glycans (elution times 30–40 min) largely prevail (higher sialylation status). Mono- and disialylated glycans from the β-TPs each show two major peaks resulting from α2,6-linked (lower retention time) or α2,3-linked neuraminic acid (higher retention time), respectively; their identity as confirmed by comparison of their retention times with authentic reference oligosaccharides in the gradient system applied (see Grabenhorst et al., 1995). In contrast, the serum proteins yielded only one prominent peak derived from biantennary chains with two α2,6-linked NeuAc as commonly found for glycoproteins secreted by the liver.

The HPAEC profile (Figure 7) of the oligosaccharides from HF-, urinary, and CSF-β-TP after desialylation—which reduces heterogeneity—allows for a more precise structural assignment. Retention times are shifted towards higher values for both, HF and urinary β-TP compared to CSF-β-TP. Since addition of sugar residues (except for fucose) delays elution in this chromatography system, this argues for a more complete glycosylation of HF and urinary β-TP, as already evidenced by MALDI-MS. In conjunction with the MS data the most prominent—desialylated—oligosaccharide (about 80% as calculated from the respective peak areas, peak 2) is identified as a biantennary structure with three 

![Graph](image-url)
Fig. 6. High-pH anion exchange profiles of native oligosaccharides from different human glycoproteins. β-TP oligosaccharides were obtained as described under Materials and methods. The other oligosaccharides were isolated by hydrazinolysis using the Glycophrep as described (Hoffmann et al., in press, b). (A) Oligosaccharides from HF-β-TP, (B) urinary β-TP, (C) hemopexin, (D) α₁-antitrypsin, (E) α₁-microglobulin. The glycan patterns for both β-TP preparations (A, B) are essentially identical. The peaks with elution times around 20–30 min represent monosialylated glycans, disialylated oligosaccharides have elution times between 30–40 min. The serum proteins hemopexin, α₁-antitrypsin, and α₁-microglobulin possess only one prominent peak derived from disialylated glycans bearing almost exclusively α2,6-linked NeuAc.

Fig. 7. High-pH anion exchange profile of desialylated complex-type oligosaccharides with proximal fucose. (A) Oligosaccharides from HF-β-TP, (B) urinary β-TP, (C) CSF-β-TP. Numbered peaks: 1, NeuAc; 2, diantennary; 3, triantennary 1,4 isomer; 4, triantennary 1,6 isomer; 5, tetraantennary; 6, tetraantennary with one lactosamine repeat; 7, diantennary with bisecting GlcNAc lacking two Gal; 8, diantennary lacking one Gal; 9, diantennary with bisecting GlcNAc lacking one Gal. Peak 10 corresponds to a fully galactosylated biantennary oligosaccharide with a bisecting GlcNAc-residue which is also found in CSF-β-TP. The minor peaks 11–13 represent triantennary glycans (11), a biantennary glycan with an intact lactosamine repeat (12), and oligosaccharides the structures of which could not be conclusively elucidated (13) due to the low amounts of material.

tennary, completely galactosylated and proximally fucosylated oligosaccharide without bisecting GlcNAc. In contrast, CSF-oligosaccharide structures causing peaks 7 and 8 (carbohydrates with two agalactoantennae plus bisecting GlcNAc or one agalactoantenna only but lacking bisecting GlcNAc) are almost completely absent from the HF protein.

Finally, the glycan structures in HF- and urinary β-TP were found to be essentially identical containing almost fully sialylated oligosaccharide chains and differ from the CSF-β-TP which contains large amounts of asialo-oligosaccharides. These data are summarized in Table I.

Discussion

Evaluation of the studies on the glycosylation features of β-TP, transferrin, hemopexin, and α₁-acid glycoprotein from human CSF and hemofiltrate or serum, respectively, which we present here and elsewhere (Hoffmann et al., 1994, 1995, in press, b) clearly shows that the site of biosynthesis of glycoproteins can often be revealed due to their characteristic tissue-specific posttranslational modification. For example, CSF-proteins like asialo-transferrin and β-TP, which are produced within the central nervous system and do not originate from serum via
passage across the blood–brain and/or blood–CSF barriers, were found to be “brain-type” N-glycosylated. The characteristics of this glycosylation were discussed in the introduction; they differ from the glycosylation seen with the majority of serum proteins which are synthesized by the liver. Since the liver parenchymal cells lack the respective glycosyltransferases, oligosaccharides bearing bisecting GlcNAc, proximal fucose, and α2,3-linked NeuAc are virtually absent in glycoproteins secreted by this tissue. Because of their “serum-type” glycosylation features including fully sialylated oligosaccharide antennae the sialylated CSF-transferrin variant, hemopexin, and α1-acid glycoprotein found in human CSF can be clearly identified as being derived from serum. These proteins enter CSF via passage across the blood–brain and blood–CSF barriers and presumably are not additionally synthesized intrathecally (Thomas et al., 1987).

Body fluids like blood and CSF represent dynamic systems the components of which are subject to a steady turnover and elimination. The daily production of CSF amounts to about 500 ml. However, the total CSF volume of 100–150 ml in humans remains constant and therefore can be calculated to be exchanged about 4–5 times per day (Segal, 1993). After bulk resorption at the arachnoidal granulations or via nerve roots into the lymphatic system, all CSF-proteins, whether originally serum-derived or intrathecally synthesized, are cleared into the blood. The novel data obtained in the present study show that those intrathecally synthesized protein species with “non-serum compatible” carbohydrate structures are rapidly eliminated from the circulation by the hepatic glycoprotein clearance receptor systems. Consequently, β-TP molecules bearing asialo-oligosaccharides are almost completely absent from HF-β-TP/serum β-TP, in contrast to molecules with mono- and disialylated oligosaccharides. However, those “brain-type” glycosylation features that escape the hepatic clearance mechanisms, i.e., bisecting GlcNAc, proximal fucose plus α2,3-linked NeuAc, and monosialylated carbohydrate antennae, persist in the circulation. Such sugar residues are not commonly found with the majority of serum proteins. This is impressively demonstrated by Figure 6, which shows the presence of large amounts of monosialylated glycans and oligosaccharides bearing α2,3-linked NeuAc for the brain-derived β-TP glycoforms in human serum whereas almost no such oligosaccharides can be detected with hemopexin, α2-antitrypsin, and α1-microglobulin which are synthesized by the liver. Thus, intrathecally synthesized glycoproteins in the blood can be identified based on their glycosylation characteristics differing from those, e.g., synthesized by the liver. The uncleared β-trace molecules with serum-compatible glycosylation persisting in blood constitute the “HF-” or serum-β-TP, respectively, investigated in our study.

Our study also shows that this sialylated β-TP pool is physiologically eliminated via the kidney. Indicative for this is that, first, β-TP accumulates in the serum of patients with impaired renal function, and second, the carbohydrate patterns of urinary and serum-derived β-TP are identical. This further implies that an additional secretion of β-TP into the serum or the urine by the liver, the kidney, or other cells and tissues that have their own specific glycosylation patterns does not occur or in negligible amounts only corroborating results obtained by in situ expression analyses of β-TP mRNA (Hoffmann et al., in press, a).

The differing glycosylation forms of β-TP isolated from several human body fluids as demonstrated by the present study suggest this protein to be used as valuable marker to follow communication pathways between different body fluids, for studying the clearance mechanisms of proteins and also to be a valuable tool for detection of pathological alterations of normal conditions.

The second most interesting finding reported in our study concerns the highly elevated β-TP concentrations in serum of dialysis patients. The glycoforms of urinary and blood-derived β-TP are almost identical and again are identical to the small amount of sialylated glycoforms found in CSF (Hoffmann et al., 1994). In renal diseases, elimination and catabolism of proteins through the kidney is disturbed resulting either in elevated concentrations of high-molecular weight (glomerular proteinuria) or low-molecular weight proteins (tubular proteinuria) in the urine. In cases of terminal renal failure, seen with dialysis patients, also elevated serum concentrations of proteins are found (β2-microglobulin, cystatin C: 5- to 8-fold, personal communication Dr. Merle, Behringwerke AG, Germany). Here, the excretion pathway of CSF-derived residual β-TP from serum into the urine is functionally disturbed, with β-TP—and other proteins—concurrently accumulating in the serum.

Elevated β-TP concentrations in the serum of patients with renal diseases had already been suggested in a previous study (Felgenhauer et al., 1987). However, to our knowledge the present study provides for the first time reliable concentration values for this protein in different body fluids as obtained by quantitative immunoaffinity chromatography in conjunction with amino acid sequencing and SDS gel electrophoresis and reveals a broad range of concentrations. In contrast, earlier studies used polyclonal antisera against β-TP which later were shown to crossreact with other proteins and less sensitive methods like immunoelectrophoresis and/or radial immunodiffusion (cf. the concentration values cited in the Introduction). Our study revealed that in normal CSF, β-TP concentration amounts to about 8000 ng/ml, i.e., about 1/50 (2%) of the total CSF-protein thus being lower than reported in the majority of previous studies. In a single case of hydrocephalus cerebri with highly elevated total protein (at least 5×) we found an extremely low CSF-concentration of β-TP (about 1/10 from normal). In contrast, normal serum β-TP concentrations range from 20–40 ng/ml and are highly elevated (ca. 100-fold) in the serum of patients with terminal renal failure. Functional disturbances of the kidneys in this case result in abnormal handling of plasma proteins due to loss of functionally active nephrons. However, usually in such cases only a moderate elevation of protein concentrations is found.

Alterations in concentration or amount of β-TP have also been discussed to be implicated in a variety of other diseases, e.g., cerebral infarction, multiple sclerosis, schizophrenia,

| Table I. Comparison of structural features of β-TP oligosaccharides from HF, urine, and CSF |
|-----------------|-----------------|-----------------|
|                  | HF-β-TP         | Urinary β-TP    | CSF-β-TP |
| Asialo-agalacto-antennae | <2%             | <2%             | 30%      |
| Asialo-oligosaccharides | 10%             | 10%             | 40%      |
| Monosialo-oligosaccharides | 40%             | 42.5%           | 40%      |
| Disialo-oligosaccharides | 50%             | 47.5%           | 20%      |
| NeuAc α2,3 and α2,6 | ++              | ++              | ++       |
| Bisecting GlcNAc | 20%             | 20%             | 70%      |
| Peripheral fucose (Lewis') | <5%             | <5%             | 20%      |

alterations in concentration or amount of β-TP have also been discussed to be implicated in a variety of other diseases, e.g., cerebral infarction, multiple sclerosis, schizophrenia,
paraproteinemia, and lymphoma (Ericsson et al., 1983; Felgenhauer et al., 1987; Harrington et al., 1993; Hiraoa et al., 1993). Due to the availability of mono- and polyclonal antibodies against β-TP and establishment of a nephelometric latex agglutination test for rapid measurement of concentrations, it will now be possible to reliably investigate a putative context between altered β-TP-concentrations and diseases. Using the nephelometric test, β-TP in CSF, serum, and urine samples of healthy individuals and patients suffering from a variety of different diseases has already been quantified. These measurements have exactly confirmed the results presented in our study (Dr. Merle, Behringwerke AG, Marburg, Germany, unpublished data).

Since β-TP accumulates more significantly in serum (about 100-fold) in pathological conditions than other proteins in current use, we suppose that especially in early diagnosis of renal diseases or in following therapeutic treatments, e.g., renal transplantation, it may become a much more reliable and sensitive parameter, and this may have a substantial impact not only on studies and diagnosis of renal but also on other kinds of diseases.

Materials and methods

Materials

Media. Hybridoma standard medium: Optimum I (Gibco-BRL, Eggenstein, Germany) containing 5% FCS, 2 mM glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin sulfate; hybridoma selective medium: standard medium supplemented with 1 mg/ml asparagine plus 13.6 mg/ml hypoxanthine (Sigma, Deisenhofen, Germany).

Antibodies. Goat anti mouse IgG + IgM (H+L), adsorbed with human, bovine, and horse serum proteins, peroxidase-conjugated (Dianova, Hamburg, Germany)/goat anti-mouse IgG, IgG(H+M), IgG1, IgG2a, IgG2b, IgG3, peroxidase-conjugated (Medac, Hamburg, Germany).

Methods

Generation of monoclonal antibodies. Three female Balb/c mice were immunized six times (16, 13, 9, 6, 3, and 1 d prior to fusion) with the native unbound protein fraction from DEAE MemSep-chromatography containing about 40% β-TP or denatured purified CSF-β-TP (Hoffmann et al., 1993). For the first injection, 100 μl of complete Freund’s adjuvant (Sigma, Deisenhofen, Germany) were mixed with 10 μl antigen preparation in 90 μl PBS (about 20 μg protein). The first booster injection was done with incomplete adjuvant, all following booster injections without adjuvant. Injections of about 20 μl were performed subcutaneously into both hind legs of the mice. After the last injection, the mice were sacrificed, lymphocytes were isolated from the popliteal lymph nodes and fused to X63Ag8 myeloma cells. Supernatants of wells with growing clones of hybridoma cells were tested by enzyme-linked immunosorbent assay and Western blotting for immunoglobulin production. Selected clones were subcloned twice by limiting dilution and frozen with 8% DMSO/92% FCS. Specificity analysis was performed by enzyme-linked immunosorbent assay and immunoblotting.

SDS-PAGE and immunoblotting. Proteins were separated by SDS-PAGE (Laemmli, 1970) using 12.5% acrylamide gels with 3% stacking gels under reducing or nonreducing conditions, respectively. Following transfer of proteins to nitrocellulose membranes, immunodetection of P-TP was performed with growing clones of hybridoma cells and purified urinary P-trace protein and commercial antibodies against human, bovine, and horse serum proteins, peroxidase-conjugated (Dianova, Hamburg, Germany). The first booster injection was done with incomplete Freund’s adjuvant, all following booster injections without adjuvant. Injections of about 20 μl were performed subcutaneously into both hind legs of the mice. After the last injection, the mice were sacrificed, lymphocytes were isolated from the popliteal lymph nodes and fused to X63Ag8 myeloma cells. Supernatants of wells with growing clones of hybridoma cells were tested by enzyme-linked immunosorbent assay and Western blotting for immunoglobulin production. Selected clones were subcloned twice by limiting dilution and frozen with 8% DMSO/92% FCS. Specificity analysis was performed by enzyme-linked immunosorbent assay and immunoblotting.

Production of monoclonal antibodies. Three petri dishes (9 cm diameter) were inoculated with growing clones of hybridoma cells or 12F5-29 cells producing IgG, “roller”-flasks in 200 ml hybridoma standard medium. After 2 and 4 days, respectively, another 200 ml of medium was added. The cell supernatants were tested for IgG bodies against P-TP and establishment of a nephelometric latex agglutination test for rapid measurement of concentrations, it will now be possible to reliably investigate a putative context between altered β-TP-concentrations and diseases. Using the nephelometric test, β-TP in CSF, serum, and urine samples of healthy individuals and patients suffering from a variety of different diseases has already been quantified. These measurements have exactly confirmed the results presented in our study (Dr. Merle, Behringwerke AG, Marburg, Germany, unpublished data).

Since β-TP accumulates more significantly in serum (about 100-fold) in pathological conditions than other proteins in current use, we suppose that especially in early diagnosis of renal diseases or in following therapeutic treatments, e.g., renal transplantation, it may become a much more reliable and sensitive parameter, and this may have a substantial impact not only on studies and diagnosis of renal but also on other kinds of diseases.

Materials and methods

Human β-trace in serum and urine

Acknowledgments

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**Abbreviations**

\(\beta\)-TP, \(\beta\)-trace protein; CSF, cerebrospinal fluid; Gal, galactose; GlcNAc, N-acetylglucosamine; HP, hemofiltrate; HPAC, high-pH anion exchange chromatography; MALDI-MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; MS, mass spectrometry; NeuAc, neuraminic acid (NeuAc).

**References**


