Expression of melanotransferrin isoforms in human serum: relevance to Alzheimer's disease

Richard R. DESROSIERS*, Yanick BERTRAND*, Quynh-Tran NGUYEN*, Michel DEMEULE*, Reinhard GABATHULER†, Malcolm L. KENNARD‡, Serge GAUTHIER§ and Richard BÉLIVEAU*[1]

*Laboratoire de médecine moléculaire, Hôpital Sainte-Justine, Université du Québec à Montréal, C. P. 8888, Succursale Centre-ville, Montréal, Québec, Canada H3C 3P8, †Biomarin Pharmaceutical Inc., 371 Bel Marin Keys Blvd, Novato, CA 94949, U.S.A., ‡BioMarin Pharmaceutical (Canada) Inc., #300-2386 East Mall, Vancouver, British Columbia, Canada V6T 1Z3, and §McGill Center for Studies in Aging, Douglas Hospital, 6825 LaSalle Boulevard, Verdun, Quebec, Canada H4H 1R3

Levels of soluble melanotransferrin in serum have been reported to be higher in patients with Alzheimer's disease than in control subjects. The present study investigated melanotransferrin in human body fluids in the light of these findings. To clarify the correlation between melanotransferrin and Alzheimer's disease, the melanotransferrin content was determined by non-reducing, denaturing SDS/PAGE and Western blotting. Under these conditions, serum melanotransferrin migrated at 79 and 82 kDa. Melanotransferrin antigenicity and the relative proportions of the two forms were very sensitive to factors that altered its conformation, including disulphide bridges, pH and bivalent cations. Serum melanotransferrin levels were not significantly different between control subjects and patients with Alzheimer's disease using whole serum, EDTA-supplemented serum or serum immunoglobulin-depleted by Protein G–Sepharose and enriched by affinity precipitation with the lectin from Asparagus pea. Glycosylated forms of serum melanotransferrin bound to Asparagus lectin manifested similar patterns on two-dimensional gel electrophoresis in samples from controls and Alzheimer's disease subjects. Melanotransferrin was also present in saliva and at a high level in urine, but contents were similar in controls and patients with Alzheimer's disease. Together, these results demonstrate that serum melanotransferrin exists in various conformations depending on the binding of bivalent cations or following post-translational modification. These data also indicate that human serum melanotransferrin levels are unchanged in subjects with Alzheimer's disease.

Key words: Alzheimer's disease, immunodetection, immunoglobulin depletion, L235 antibody, melanotransferrin, p97.

INTRODUCTION

Mtf (melanotransferrin) or p97 was initially identified at high levels as a surface marker of malignant melanoma cells [1]. The p97 protein was later called Mtf because of its high sequence identity (37–39 %) with human lactoferrin and serum transferrin [2]. Thus Mtf belongs to the group of iron binding proteins. Mtf is a glycosylated protein that consists of two symmetrical domains, each of which is presumed to contain several disulphide bridges [3]. Two forms of Mtf have been reported. The first form is a membrane protein that is attached to the cell surface by a glycosylphosphatidylinositol anchor [4,5]. The second form is actively secreted and may originate from alternative processing of the mRNA [5]. Consequently, many post-translationally modified forms of Mtf occur that may have specific functions and the activities of which are likely to be tightly regulated.

An initial study showed that purified Mtf could bind iron (Fe) from Fe–citrate complexes [2], and this observation led to the assumption that Mtf may be involved in Fe transport. However, studies on human SK-Mel-28 melanoma cells, which show high levels of expression of Mtf, showed that the protein did not play a major role in Fe uptake [6–8]. In another report using CHO cell lines defective in the transferrin receptor and transfected with human Mtf, the Fe uptake doubled [9]. These transfected cells expressed $(1–1.2) \times 10^6$ Mtf molecules per cell [9], while an estimate of 0.34×10^6 Mtf molecules per cell was proposed for SK-Mel-28 cells [6]. Thus transfected CHO cells expressed about 4-fold higher levels of Mtf molecules than SK-Mel-28 cells, suggesting that the physiological relevance of results obtained with transfected cells must be considered cautiously. Subsequent studies demonstrated that depletion of Mtf from melanoma cells [10] and other cell types [11] did not have any marked effects on Fe uptake. More relevant to our present study examining serum Mtf, a recent study reported that soluble Mtf did not bind to the transferrin receptor and thus donate Fe to cells by this mechanism [12].

Besides Fe transport, other postulated functions of Mtf include protection against membrane-lipid peroxidation, metalloprotease activity, and it may act as an intercellular adhesion molecule [13]. However, further studies will be required to elucidate the functions of Mtf in these processes. Recently we reported that human Mtf accumulated in the mouse brain following intravenous injection and *in situ* brain perfusion [14]. In addition, Mtf underwent high levels of transcytosis across an *in vitro* model of the blood–brain barrier [14]. These data suggest that Mtf may represent a novel delivery system to target drugs to the brain.

Expression of Mtf mRNA is widespread in normal human tissues and is generally greater in adult than in fetal tissues, with highest expression in the salivary gland [10]. On the other hand, Mtf protein was reported to be expressed at a higher level in neoplastic cells and in fetal tissues than in normal tissues [1]. An explanation for this discrepancy between Mtf mRNA and protein expression is probably related to the fact that the techniques used are assessing different molecules, and that many reasons could account for their differential regulation in tissues.

Abbreviations used: AD, Alzheimer's disease; Caps, 3-(cyclohexylamino)-1-propanesulphonic acid; 2-D, two-dimensional; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase, IEF, isoelectric focusing; IPG, immobilized pH gradient; Mtf, melanotransferrin; NSIP, non-specific immunoreactive protein; rMtf, recombinant melanotransferrin; TBS, Tris-buffered saline.

[1] To whom correspondence should be addressed (e-mail oncomol@nobel.si.uqam.ca.).

In the human brain, Mtf was detected on the capillary endothelium [15]. Subsequently it was found that Mtf was localized in microglia cells associated with amyloid plaques found *post mortem* in the brains of patients with AD (Alzheimer's disease) [16,17]. These observations suggested that Mtf may be related to AD pathology. Interestingly, it has been proposed that the soluble form of Mtf could be released into the serum. Mtf levels have been reported to be elevated in the serum of AD patients compared with that of healthy controls when measured by sandwich fluorescent immunoassay [18], RIA [19,20] and dot-immunoblot assay [21]. These findings highlight the possibility that soluble Mtf could serve as a potential biochemical marker for AD.

To date, the search for a specific and sensitive biochemical marker of AD has been disappointing [22]. The identification of a serum biochemical marker of AD would be a significant advance to confirm the diagnosis, since only *post mortem* diagnosis is presently available. An AD marker will also be useful to predict the progression of the disease and responses to treatment. The purpose of the present study was to determine Mtf characteristics in human body fluids in light of the AD findings. To reach this objective, serum, saliva and urine proteins were fractionated by PAGE under denaturing conditions in the presence of SDS, in order to maximize antibody specificity. The effects of disulphide bridge cleavage, pH and bivalent cations on Mtf antigenicity were also examined. In addition, 2-D (two-dimensional) gel electrophoresis was performed to characterize the Mtf isoforms present in serum.

MATERIALS AND METHODS

Materials

Mouse monoclonal antibodies against Mtf (L235, 2C7, 9B6, Hyb C and Hyb F) and human recombinant Mtf (rMtf) expressed in BHK cell lines [23] were obtained from BioMarin Pharmaceutical (Canada) Inc. (Vancouver, British Columbia, Canada). Secondary antibodies, goat anti-(mouse IgG) and mouse anti-(goat IgG) conjugated to HRP (horseradish peroxidase), were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, U.S.A.). Lectins conjugated to agarose beads were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Protein A– and Protein G–Sepharose beads were from Pharmacia Biotech (Montreal, Quebec, Canada).

Diagnosis of AD and sample collection

Patients were diagnosed as probably having AD according to the NINCDS–ADRDA criteria [24]. All were in mild to moderate stages of AD, living at home with a carer. After obtaining informed consent from patients and their legal representatives, samples of blood, urine and saliva were collected. Blood was obtained through antecubital vein puncture into 2×10 ml serum separator tubes, allowed to clot for 30 min, and centrifuged at 1100 ***g*** for 15 min until clot and serum were separated by a well formed polymer barrier. Serum was divided into 1.0–1.5 ml fractions. Urine was collected and divided into 1.5 ml fractions. After 15 min without talking and swallowing to reduce evaporation, saliva was collected into a plastic container, then divided into aliquots. All samples were frozen immediately at − 80 °C.

Immunoglobulin depletion using Protein A– and Protein G–Sepharose beads, and lectin precipitation of Mtf

Human serum (50 μl) was incubated with Protein A– or Protein G–Sepharose beads (200 μl) in a buffer containing 20 mM Tris/HCl, pH 6.8, usually in the presence of 1 mM EDTA, in a final volume of 500 μl. Samples were agitated gently for 2 h at 4 °C. Then soluble proteins were separated from proteins bound to Protein A– or Protein G–Sepharose beads by centrifugation at 700 ***g*** for 3 min. Unbound proteins were solubilized in Laemmli sample buffer [25], usually without 2-mercaptoethanol, then separated by SDS/PAGE. Alternatively, to precipitate soluble Mtf, supernatants (300 μl) were incubated with various lectins conjugated to agarose beads (40 μl) in 20 mM Tris/HCl, pH 6.8, and 1 mM EDTA in a total volume of 500 μl with shaking for 2 h at 4 °C. Proteins bound to agarose beads were washed three times in 20 mM Tris/HCl, pH 6.8, and 1 mM EDTA. Proteins precipitated by Protein A– or Protein G–Sepharose beads and those bound to lectins conjugated to agarose beads were released by adding Laemmli sample buffer without 2-mercaptoethanol and incubating for 15 min at room temperature. For 2-D gel electrophoresis analysis, proteins were released from lectins conjugated to agarose beads by incubation in 20 mM Tris and 15 mM Caps [3-(cyclohexylamino)-1-propanesulphonic acid], pH 10. Solubilized proteins were separated from beads by centrifugation at 8000 ***g*** for 3 min.

One-dimensional and 2-D gel electrophoresis

SDS/PAGE was performed on 7.5 % (w/v) polyacrylamide gels in a discontinuous system [21] using a Mini-Protean II apparatus (Bio-Rad, Mississauga, Ontario, Canada). For 2-D gels, the first dimension was IEF (isoelectric focusing) and the second was SDS/PAGE. Proteins were added to reswelling buffer containing 8 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 2 % (v/v) carrier ampholyte (Bio-lyte; Bio-Rad), pH 3–10, a trace amount of Bromophenol Blue and protease inhibitors in a final volume of 125 μl. This mixture was incubated with 7-cm IPG (immobilized pH gradient) Drystrip (Pharmacia Biotech) at room temperature overnight. The proteins were focused using a horizontal electrophoresis Multiphor II apparatus (Pharmacia Biotech). Voltage was increased linearly from 0 to 200 V during 1 min followed by 29 min at 200 V, then it was increased linearly from 200 to 3500 V during 90 min and finally it was kept constant at 3500 V for 65 min. Following IEF, strips were equilibrated for 15 min in 50 mM Tris/HCl, pH 6.8, 6 M urea, 30 % (v/v) glycerol and 2 % (w/v) SDS and a trace of Bromoplenol Blue under non-reducing conditions. After equilibration, IPG gel strips were loaded on to SDS/7.5 %-PAGE and run as described above.

Immunoblotting

For immunoblotting experiments, proteins were electroblotted on to 0.45-μm-pore-diameter PVDF membranes (Immun-Blot; Bio-Rad). The blots were blocked overnight at 4 °C in TBS (Tris-buffered saline; 137 mM NaCl, 20 mM Tris/HCl, pH 7.5) containing 0.3 % (v/v) Tween 20 and 5 % (w/v) non-fat dried milk (Carnation). Usually, blots were then incubated with a 1:500 dilution of different primary antibodies in TBS containing 0.3 % (v/v) Tween and 3 % BSA for 1 h at room temperature, followed by a 1 h incubation with a 1:5000 dilution of goat anti-(mouse IgG) conjugated to HRP in TBS containing 0.3 % (v/v) Tween. Following 2-D gel electrophoresis, blots were treated similarly except that primary and secondary antibodies were diluted 1: 250 and 1:2000 respectively. Immunoreactive proteins were detected using the ECL® Western blotting kit as described in the manufacturer's instructions (Amersham Biotech, Oakville, Ontario, Canada) with preflashed Fuji films. To quantify the immunodetected Mtf, autoradiograms were scanned with a

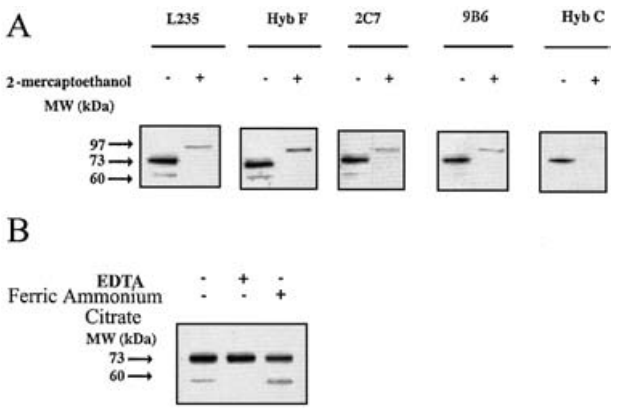

Figure 1 Immunodetection of rMtf following SDS/PAGE under non-reducing and reducing conditions, and effects of EDTA and iron on the proportions of the rMtf forms

(**A**) Human rMtf (5 ng of protein) was dissolved in Laemmli sample buffer in the presence (+) or the absence (−) of 5% (v/v) 2-mercaptoethanol and analysed by SDS/PAGE. Following electrotransfer of proteins on to PVDF membranes, Mtf was immunodetected with a battery of monoclonal antibodies as described in the Materials and methods section. (**B**) The effects of EDTA (2 mM) and ferric ammonium citrate (2 mM) added to rMtf were also examined by immunoblotting after SDS/PAGE under non-reducing conditions. The molecular masses (MW) of immunodetected rMtf froms are indicated by arrows. Results are representative of two experiments performed in duplicate.

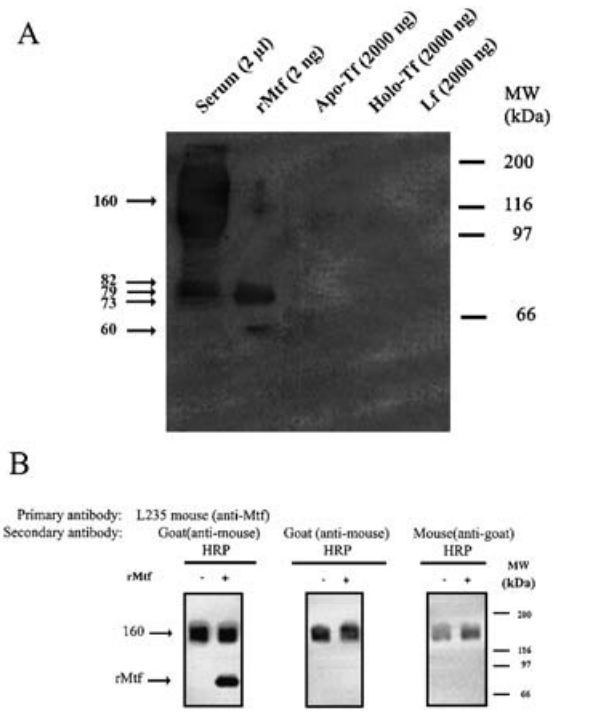

Figure 2 Specificity of the L235 antibody for Mtf

(**A**) Immunodetection of Mtf by the L235 monoclonal antibody after non-reducing SDS/PAGE analysis of human serum (2 μl) was compared with that of other human proteins belonging to the transferrin family: rMtf, apo-transferrin (Apo-Tf), holo-transferrin (Holo-Tf) and lactoferrin (Lf). (**B**) The recognition of Mtf in human serum (0.2 μl) by secondary antibodies with or without added rMtf (1 ng) was analysed by SDS/PAGE under non-reducing conditions. To discriminate between chemiluminescent signals due to immunodetection by the first and the second antibodies, PVDF membranes were probed as usual by the primary (L235) and secondary (goat anti-mouse conjugated to HRP) antibodies, or by the secondary antibody only or by an unrelated secondary antibody (mouse anti-goat conjugated to HRP), as described in the Materials and methods section. The molecular masses (MW) of standard proteins are indicated on the right. These results are representative of two experiments done in duplicate.

Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) or with the cooled CCD imaging system of Alpha Innotech Corporation (Canberra Packard, Montreal, Quebec, Canada).

Statistical analysis

The results are expressed as means ± S.E.M., and were analysed using Student's t test. Only significant differences ($P < 0.05$) are indicated in the Figures.

RESULTS

Monoclonal antibodies against rMtf recognize conformational epitopes

Several antibodies are available to characterize human Mtf expression. In order to quantify serum Mtf levels, the best antibody for immunodetection of rMtf was determined following SDS/PAGE under non-reducing and reducing conditions and Western blot analysis. Under non-reducing conditions, rMtf migrated at 73 kDa, and a faint immunoreactive protein was also detected at 60 kDa (Figure 1A). The L235 and Hyb F antibodies were those showing the strongest immunodetection of rMtf (Figure 1A). Under reducing conditions, rMtf migrated at 97 kDa and its immunodetection was virtually abolished for all antibodies tested (Figure 1A). Since Mtf is an iron binding protein, the effect of this metal on its immunodetection was also tested. When EDTA was added to rMtf, only the protein at 73 kDa was immunodetected, and the signal was slightly weaker (by 12%) than in absence of chelating agent (Figure 1B). In the presence of ferric ammonium citrate, the total signal was the same as in the absence of chelating agent, but the amount of protein at 73 kDa decreased, while that of protein at 60 kDa increased simultaneously (Figure 1B). These results suggested that the rMtf at 60 kDa could bind iron. Together, these results indicated that monoclonal antibodies against rMtf recognized conformation-dependent epitopes that were modulated by reducing and chelating agents. Consequently, Mtf characterization was performed by electrophoresis under non-reducing conditions in the presence of EDTA with the L235 antibody to obtain the strongest signal during immunoblot analysis.

Specificity and sensitivity of the L235 antibody against Mtf

The specificity of the L235 antibody for Mtf was assessed using other members of the transferrin family. Human rMtf was readily immunodetected at 2 ng, whereas approx. 1000 times that amount (2000 ng) of human apo-transferrin, holo-transferrin or lactoferrin was undetectable (Figure 2A). These observations demonstrated that the L235 antibody is highly specific for Mtf. In addition, the L235 antibody was very sensitive, since it was able to immunodetect rMtf concentrations as low as 50 pg (results not shown). Analysis of serum proteins under the same conditions revealed two proteins migrating at 79 and 82 kDa close to rMtf at 73 kDa (Figure 2A), suggesting that they were Mtf isoforms. In serum, the antibody also cross-reacted strongly with proteins migrating at approx. 160 kDa.

Serum contains several abundant proteins, such as albumin and immunoglobulins, that could react non-specifically with the

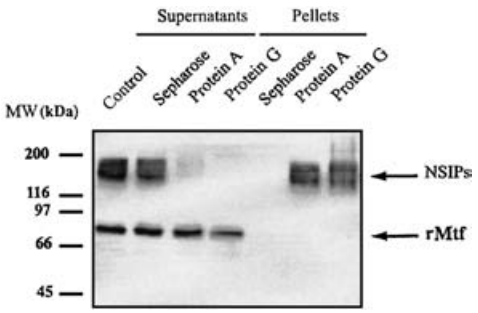

Figure 3 Immunodepletion of NSIPs in human serum by Protein A– and Protein G–Sepharose beads

rMtf (1 ng of protein) mixed with human serum (0.2 μl) was incubated with Sepharose beads or with Protein A– or Protein G–Sepharose beads (25 μl) for 1 h at room temperature. The proteins bound to the beads were pelleted by centrifugation. The soluble proteins in supernatants and those bound to beads were dissolved in Laemmli sample buffer and then were separated by SDS/PAGE under non-reducing conditions. The immunodetection of rMtf was performed as described in the Materials and methods section. The molecular masses (MW) of standard proteins are indicated on the left. These results are representative of two experiments performed in duplicate.

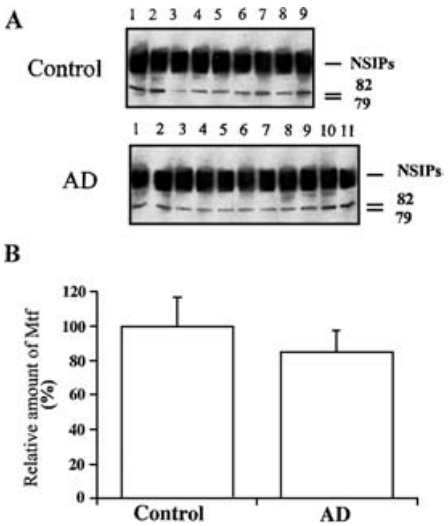

Figure 4 Determination of Mtf levels in serum samples from control and AD subjects by Western blotting

Equal volumes (2 μl) of human serum from control subjects and AD patients were added to Laemmli sample buffer containing 1 mM EDTA. (**A**) Solubilized proteins were analysed by SDS/PAGE under non-reducing conditions and immunodetected with L235 monoclonal antibody as described in the Materials and methods section. (**B**) Quantitative analysis of Mtf levels in serum samples from controls and AD patients were assessed by densitometry. Results represent means $\pm$ S.E.M. of nine (control) and 11 (AD) samples.

primary (L235) or the secondary antibody. To test these possibilities, immunoblots of serum proteins were probed with L235 antibody and the secondary antibody [goat anti-(mouse IgG)–HRP] and compared with detection by either this secondary antibody only or an unrelated secondary antibody [mouse anti-(goat IgG)–HRP] (Figure 2B). The relative intensities of immunodetected proteins were directly comparable, since the detection was performed at the same time. Both secondary antibodies recognized the proteins at 160 kDa, but not serum Mtf or rMtf added to serum. On this occasion Mtf was not detected by L235 (Figure 2B) because a smaller volume of serum (0.2 μl) was used than previously (2 μl) (Figure 2A). This smaller volume of serum was chosen to facilitate the detection of very immunoreactive proteins at 160 kDa. Interestingly, the signal for proteins at 160 kDa recognized by L235 plus the secondary antibody was stronger than that with the secondary antibody alone, indicating that L235 also reacted with proteins at 160 kDa (Figure 2B). Together, these results demonstrated that both primary and secondary antibodies recognized proteins at 160 kDa, but that only the L235 primary antibody detected Mtf.

Protein A and Protein G remove NSIPs (non-specific immunoreactive proteins) or immunoglobulins from human serum

The serum proteins at 160 kDa that were recognized by the L235 antibody could potentially be oligomerized Mtf or NSIPs. To assess the levels of serum Mtf accurately, it was necessary to clarify the identity of the proteins at 160 kDa. Since the size of these proteins corresponded to immunoglobulins, Protein A and Protein G were used as a tool to determine whether this was indeed the case. Treatment of human serum with Protein A– or Protein G–Sepharose beads resulted in efficient removal of the proteins at 160 kDa from the serum compared with controls performed with Sepharose beads (Figure 3). rMtf added to serum was not recovered in pellets of Sepharose beads or Protein A– or Protein G–Sepharose beads (Figure 3). Together, these results demonstrated that the removal of immunoglobulins from serum with Protein A– or Protein G–Sepharose beads was specific. More importantly, these data indicated that proteins at 160 kDa recognized by the L235 antibody under non-reducing conditions were not oligomerized Mtf.

Comparison of serum Mtf levels in control and AD subjects

Since only serum proteins migrating at 79 and 82 kDa were Mtf, their levels were compared in healthy control subjects and patients with AD. Immunoblot analysis of human serum proteins following non-reducing SDS/PAGE in the presence of EDTA showed that Mtf levels were quite variable in both controls ($n = 9$) and AD subjects ($n = 11$) (Figure 4A). Densitometric quantification of immunodetected Mtf indicated that the relative amounts were 1.00 ± 0.17 and 0.84 ± 0.12 in control and AD subjects respectively (Figure 4B). However, no significant differences were observed in serum Mtf levels between controls and patients with AD.

Human serum Mtf is highly glycosylated

The two Mtf forms migrating at 79 and 82 kDa were sometimes difficult to immunodetect (Figure 4A). A subsequent step was performed to enrich Mtf from serum in order to improve quantification by densitometry following immunodetection. Since Mtf is a glycosylated protein, various lectins conjugated to agarose beads were used for its precipitation. The results demonstrated that human serum Mtf is composed of a mixture of glycosylated forms, since different lectins specifically precipitated certain forms (Figure 5). For example, the lectin from Asparagus pea (*Tetragonolobus purpureas*) gave the strongest signal, while that from *Pisum sativum* showed a weak capacity to precipitate Mtf (Figure 5). The lectin from Asparagus pea was selected to enrich serum Mtf, since it gave the strongest immunodetection signal and it possessed a poor capacity to precipitate NSIPs (Figure 5).

Sequential NSIP removal and lectin treatment of human serum greatly increase the specificity of immunodetection of Mtf

The efficiency of the strategy to improve the signal/noise ratio of immunodetected Mtf was assessed using a serum volume

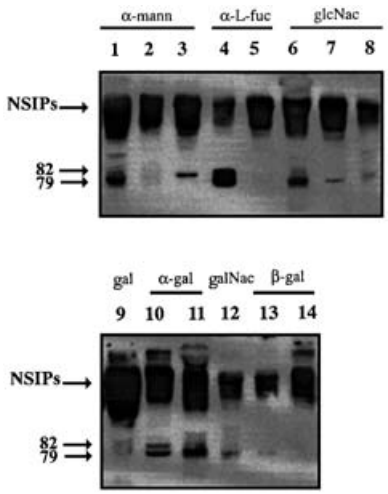

Figure 5 Precipitation of Mtf from human serum using various lectins

To measure the ability of lectins to precipitate Mtf, human serum samples (50 μl) were treated with different lectins conjugated to agarose beads (20 μl), as described in the Materials and methods section. Proteins bound to lectins were analysed by SDS/PAGE under non-reducing conditions. Mtf precipitated by lectins from *Canavalia ensiformis* (concanavalin A; lane 1), *Pisum sativum* (lane 2), *Galanthus nivalis* (lane 3), *Tetragonolobus purpureas* (Asparagus pea; lane 4), *Ulex europaeus* (lane 5), *Bandeiraea simplicifolia* (lane 6), *Phytolacca americana* (lane 7), *Triticum vulgaris* (lane 8), *Abrus precatorius* (lane 9), *Artocarpus integrifolia* (lane 10), *Dolichos biflorus* (lane 11), *Glycine max* (lane 12), *Arachis hypogaea* (lane 13) and *Ricinus communis* (lane 14) were immunodetected by the L235 monoclonal antibody. The sugars moieties recognized by the different lectins are identified: α-mannose (α-mann), α-L-fucose (α-L-fuc), *N*-acetyl-D-glucosamine (glcNac), galactose (gal), α-galactose (α-gal), *N*-acetyl-D-galactosamine (galNac) and β-galactose (β-gal).

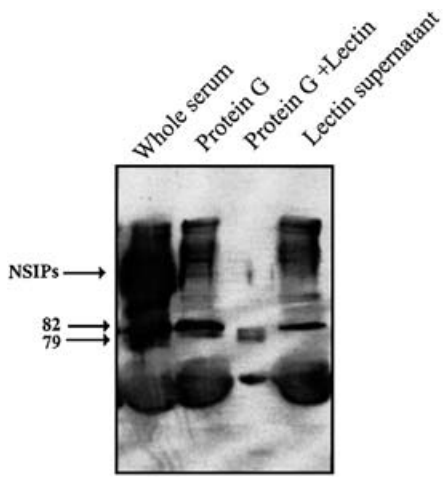

Figure 6 Immunodetection of Mtf in human serum following NSIP depletion and lectin-mediated precipitation

Following SDS/PAGE under non-reducing conditions, Mtf was immunodetected with L235 monoclonal antibody in whole serum (2 μl), and in equivalent volumes of serum following sequential treatment for NSIP depletion with Protein G–Sepharose beads and precipitation of glycosylated Mtf with the lectin Asparagus pea agglutinin conjugated to agarose beads. The supernatant, containing Mtf forms that did not bind to Asparagus pea lectin, is also shown. These results are representative of two experiments done in duplicate.

10-fold greater (2 μl) than that usually analysed, in order to facilitate Mtf detection. Figure 6 shows immunodetection of Mtf in whole serum and in equivalent volumes of serum following sequential treatments for NSIP depletion with Protein G–Sepharose beads, Mtf precipitation with the lectin Asparagus pea agglutinin conjugated to agarose beads and in the lectin supernatant containing unbound Mtf. The Mtf that bound to the lectin (40 %) was mostly the form at 79 kDa, whereas the unbound Mtf (60 %) was mainly at 82 kDa (Figure 6). These data also demonstrated that NSIPs were recovered in the unbound Mtf fraction (Figure 6).

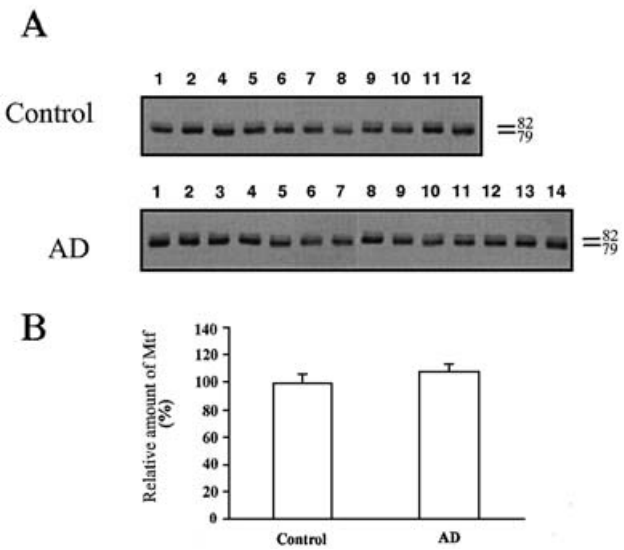

Figure 7 Determination by Western blotting of Mtf content in serum samples from control and AD subjects following NSIP depletion and treatment with Asparagus pea lectin

Equal volumes (15 μl) of human serum from control subjects and AD patients were treated sequentially to remove NSIPs or immunoglobulins with Protein G–Sepharose beads, then incubated with the lectin Asparagus pea agglutinin conjugated to agarose beads. Glycosylated Mtf bound to the lectin was solubilized in Laemmli sample buffer, separated by SDS/PAGE under non-reducing conditions and immunodetected with L235 monoclonal antibody as described in the Materials and methods section (**A**). Mtf levels bound to lectin in serum samples from control and AD subjects were assessed by densitometry (**B**). Results represent means $\pm$ S.E.M. for 12 (control) and 14 (AD) samples.

Comparison of serum Mtf levels in control and AD subjects following NSIP depletion and Mtf precipitation with Asparagus pea lectin

Human serum samples from control subjects ($n = 12$) and AD patients ($n = 14$) were treated with Protein G–Sepharose beads to remove NSIPs. Then the depleted serum was incubated with the Asparagus pea lectin, and lectin-bound and -unbound Mtf forms were separated by centrifugation. Quantitative analysis of immunoblots (Figure 7A) by densitometry of lectin-bound Mtf forms showed that their relative levels were 1.00 ± 0.06 and 1.09 ± 0.05 in serum from control and AD subjects respectively (Figure 7B). These differences were not significant.

Analysis of the effects of different buffers and bivalent cations on rMtf immunodetection

Mtf immunodetection was very sensitive to conformational changes (Figure 1). Thus Mtf levels in serum samples from control subjects and AD patients were measured after SDS/PAGE under non-reducing conditions using sera preincubated with EDTA to standardize its detection. We further investigated the effects of

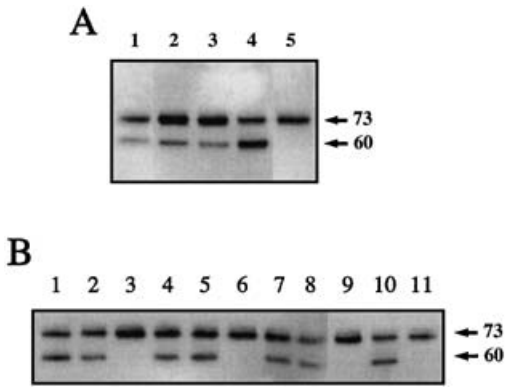

Figure 8 Effects of buffers and bivalent cations on immunodetection of rMtf

The effects of different buffers on the generation of rMtf forms (**A**) were evaluated by dilution of the protein in Tris, pH 7.0 (lane 1), TBS, pH 7.5 (lane 2), PBS, pH 7.5 (lane 3), Ringer/Hepes, pH 7.4 (lane 4), or sodium citrate, pH 5.0 (lane 5). The impact of bivalent cations on the proportions of rMtf forms was also examined (**B**). rMtf was diluted in Ringer/Hepes buffer, pH 7.4 (lane 1), or in Ringer/Hepes, pH 7.4, without calcium and magnesium salts (lanes 2–11), then 1 mM EGTA and 1 mM EDTA (lane 3) or 1 mM $CaCl_2$ (lane 4), $MgCl_2$ (lane 5), $ZnCl_2$ (lane 6), $MnCl_2$ (lane 7), $CoCl_2$ (lane 8), $CuCl_2$ (lane 9), ferric ammonium citrate (lane 10) or molybdate ammonium (lane 11) were added. Following incubation for 15 min at room temperature, proteins were analysed by SDS/PAGE under non-reducing conditions and immunodetected with L235 antibody to evaluate the proportions of the rMtf forms at 60 and 73 kDa. These results are representative of two experiments.

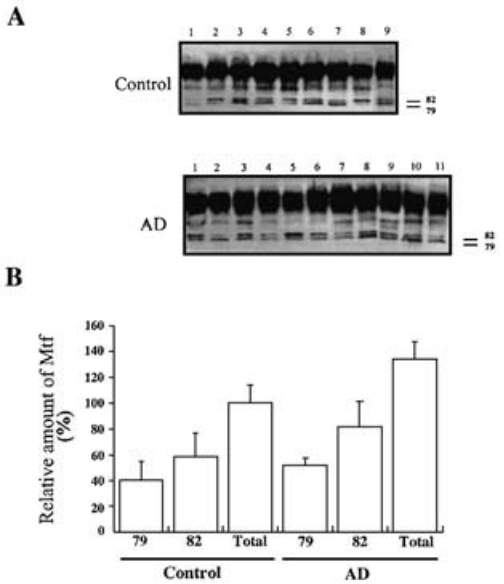

Figure 9 Quantification of Mtf forms in untreated serum samples from control subjects and patients withAD

Equal volumes (2 μl) of human serum from control subjects and AD patients were added directly to Laemmli sample buffer. (**A**) Proteins were analysed by SDS/PAGE under non-reducing conditions to separate the two Mtf forms and immunodetected with L235 monoclonal antibody as described in the Materials and methods section. (**B**) Histograms show quantitative analysis of 79 and 82 kDa Mtf forms and the sum of the two forms (Total) in serum from control and AD subjects, as assessed by densitometry. Results represent means $\pm$ S.E.M. of nine (control) and 11 (AD) samples.

buffers and bivalent cations, since serum samples were diluted in immunological assays [18–21], which might modulate Mtf conformation and consequently its quantification. When rMtf was diluted in various buffers and then analysed by SDS/PAGE under non-reducing conditions, the ratio between the form at 60 kDa and that at 73 kDa was quite variable (Figure 8A). rMtf migrated mostly at 73 kDa when diluted in PBS (90 %), TBS (89 %), Tris (75 %) and Ringer/Hepes (68 %) buffers at neutral pH. All rMtf was recovered at 73 kDa in an acidic buffer (citrate at pH 5.0). Thus the proportions of rMtf forms at 60 and 73 kDa varied with pH, and even at neutral pH the ratio was affected by buffer type.

Experiments with ferric ammonium citrate and EDTA suggested that the rMtf form at 60 kDa bound exogenous iron (Figure 1B). Furthermore, in Ringer/Hepes buffer, which contains calcium and magnesium ions, the proportion of rMtf at 60 kDa was higher than in other buffers at neutral pH (Figure 8A, lane 4). Thus the effects of bivalent cations on the generation of various rMtf forms was examined. When calcium and magnesium salts were omitted from Ringer/Hepes buffer, the amount of the immunodetected 60 kDa form decreased, and it disappeared altogether in the presence of EDTA and EGTA (Figure 8B, lanes 1–3). The subsequent addition of bivalent cations such as calcium, magnesium, manganese, cobalt and iron restored the detection of rMtf at 60 kDa, but zinc, copper and molybdate did not (Figure 8B, lanes 4–11). These data indicated that rMtf could bind several bivalent cations in addition to iron.

Quantification of Mtf forms in untreated serum from control and AD subjects

rMtf conformation was severely affected by buffer, pH and bivalent cations. These data suggested that Mtf antigenicity in buffered serum could be altered, explaining the variation in measured levels according to immunological assays. To investigate this possibility, both Mtf forms were assessed in serum in the absence of the chelating agent EDTA. Once again, densitometric analysis of immunodetected Mtf indicated that the amounts of the 79 and 82 kDa forms, and the sum of both proteins, were not significantly different between control and AD subjects (Figure 9).

2-D gel electrophoresis of Mtf forms in serum from controls and AD patients following immunoglobulin depletion and lectin-mediated precipitation

During experiments to enrich Mtf by affinity chromatography using various lectins, we observed that certain lectins preferentially bound specific Mtf forms present in serum (Figure 5). These observations suggested the possibility that certain glycosylated forms, rather than the whole Mtf content in serum, might be a marker for AD. Consequently, human serum samples from controls and AD patients were immunoglobulin-depleted. Then serum was treated with the lectin from Asparagus pea to provide lectin-bound Mtf forms prior to analysis by 2-D gel electrophoresis (IEF/SDS) and immunodetection. In serum samples from both control subjects and AD patients, lectin-bound Mtf presented three forms at 82 kDa and three forms at 79 kDa that migrated between pI 7.0 and 7.4, and one form at 79 kDa found at pI 7.6 (Figure 10). However, no specific and reliable difference in glycosylated Mtf pattern typical of AD compared with control subjects was apparent following 2-D gel electrophoresis.

Comparison of Mtf levels in urine and saliva from control subjects and AD patients

To further characterize Mtf forms and evaluate possibilities to measure differences in Mtf levels between control and AD

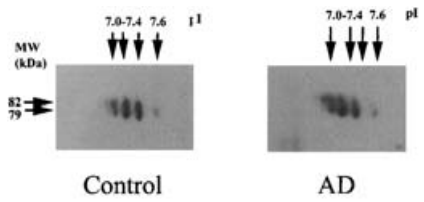

Figure 10 2-D gel electrophoresis and Western blot analysis of Mtf forms in serum from control and AD subjects following immunoglobulin depletion and precipitation by Asparagus pea lectin

Equal volumes (15 μl) of human serum samples from control subjects and AD patients were treated sequentially with Protein G–Sepharose beads to remove immunoglobulins, then with the lectin Asparagus pea agglutinin conjugated to agarose beads to precipitate glycosylated Mtf carrying α-L-fucose moieties. Mtf precipitated by the lectin was subjected to IEF electrophoresis as the first dimension in IPG (pH 3–10) gel strips. After separation by IEF, proteins in gel strips were subjected to SDS/PAGE as the second dimension. Proteins were run under non-reducing conditions in both dimensions. Following electrotransfer, Mtf forms were immunodetected with L235 monoclonal antibody as described in the Materials and methods section. Only regions where Mtf isoforms migrated are shown.

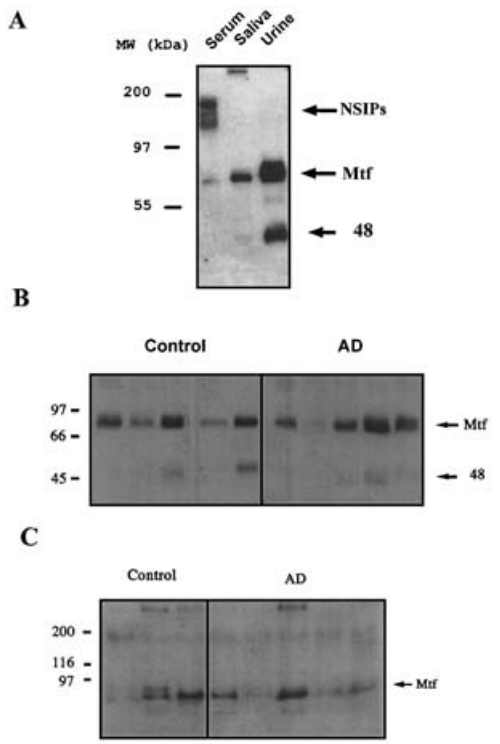

Figure 11 Western blot analysis of Mtf content in saliva and urine from control subjects and patients with AD

(**A**) To determine whether Mtf is present in body fluids other than serum, samples of serum, saliva and urine containing the same amount of protein (7.5 μg) were analysed by SDS/PAGE under non-reducing conditions. To compare Mtf content in controls and AD patients, urine (**B**) and saliva (**C**) (7.5 μg of protein each) were solubilized in Laemmli sample buffer containing 1 mM EDTA. Proteins were analysed by SDS/PAGE under non-reducing conditions and immunodetected with the L235 monoclonal antibody. These results are representative of two experiments.

subjects, the presence of Mtf was investigated in urine and saliva. When equal amounts of protein from healthy people were analysed by SDS/PAGE, Mtf levels were higher in urine (25-fold) and in saliva (10-fold) than in serum (Figure 11A). In urine, a protein at 48 kDa was also immunodetected, possibly a proteolytic fragment of Mtf (Figure 11A). These results suggested that urine and/or saliva could be used as alternative body fluids to serum in order to compare Mtf levels in control and AD subjects. Thus urine and saliva samples from control subjects and AD patients were analysed by SDS/PAGE and Western blot. The levels of immunodetectable Mtf varied considerably in both urine and saliva from each group (Figures 11B and 11C). Quantitative analysis by densitometry confirmed that there were no significant differences in Mtf levels between controls and AD patients in either urine or saliva (results not shown).

DISCUSSION

Mtf was identified initially as a surface marker of malignant melanoma cells that is present at high levels [1]. Since Mtf is a homologue of transferrin, it has been postulated that Mtf might be involved in iron transport. Subsequently, however, several studies concluded that Mtf plays only a minor role in Fe uptake [6–8,10–12]. The functions of membrane-bound and soluble Mtf thus remain to be established. In human brain Mtf is associated with the capillary endothelium [15], and it is also present in serum [18–21]. From these observations, we suggested that soluble Mtf can cross the blood–brain barrier. Indeed, we demonstrated recently that Mtf accumulated in mouse brain following intravenous injection or during *in situ* brain perfusion [14]. Furthermore, we showed that Mtf underwent high transcytosis across an *in vitro* model of the blood–brain barrier [14]. These results suggest that one possible crucial use of soluble Mtf could be as a novel delivery system to target drugs to the brain.

Using SDS/PAGE and Western blotting analysis, we found that the L235 antibody was among the best antibodies for immunodetection of rMtf and serum Mtf. Of members of the human transferrin family, only rMtf was immunodetected by the L235 antibody, i.e. it did not detect apo-transferrin, holo-transferrin or lactoferrin. In addition, while serum Mtf forms migrated at 79 and 82 kDa, apo-transferrin, holo-transferrin and lactoferrin migrated at approx. 70 kDa under non-reducing conditions (results not shown). These data clearly demonstrate that the L235 antibody is specific for Mtf compared with other members of the transferrin family. This monoclonal antibody is also very sensitive, since it can detect rMtf at concentrations as low as 50 pg. However, when rMtf was immunodetected following SDS/PAGE under reducing conditions, the signal intensities decreased markedly for all antibodies. This observation shows that the cleavage of disulphide bridges and the resulting modifications of the conformational epitope recognized by L235 antibody could alter its sensitivity.

Human serum Mtf is a highly glycosylated protein, since it seems to carry many different sugar moieties. This is supported by the affinity precipitation of Mtf using various lectins that interact with α-mannose, α-galactose, β-galactose, α-L-fucose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. Furthermore, 2-D gel electrophoresis showed that Mtf forms migrating at 79 and 82 kDa present shapes that are not completely focused and which are observed frequently for glycosylated proteins. SDS/PAGE and Western blot analysis of Mtf in serum diluted in various buffers or in serum incubated in the presence of EDTA or with different bivalent cations shows that the proportions of the two forms at 82 kDa and 79 kDa are highly variable. Together, these results demonstrate that serum Mtf might exist in several conformations, the proportions of which are regulated by the binding of bivalent cations or by post-translational modification due to various types of glycosylation.

Soluble Mtf was also immunodetected in urine and saliva. Its amount is higher in urine (25-fold) and in saliva (10-fold) than

in serum. It has been reported that Mtf mRNA shows highest expression in the salivary gland, and that it is also expressed in kidney and bladder [10]. This could explain the high levels of immunodetectable Mtf in saliva and urine. However, the molecular mechanisms involved in Mtf secretion are still unclear. Interestingly, a protein at 48 kDa was strongly immunodetected in urine. This protein could be a proteolytic fragment originating from Mtf. Alternatively, this protein at 48 kDa might be the product of the short *Mtf1* gene that is postulated to encode a soluble protein consisting of 302 amino acids [26].

Previous studies have reported that the serum Mtf concentration is elevated in patients with AD, and it has been proposed that Mtf could be a possible biochemical marker for this condition [18–21]. Thus, in the present study, we further investigated the possibility of using serum Mtf as a biochemical marker for AD. Mtf levels were not significantly different in serum from control subjects and from patients with mild to moderate stages of AD when analysed by SDS/PAGE under non-reducing conditions. Results were similar using whole serum, EDTA-supplemented serum or immunoglobulin-depleted serum (using Protein G–Sepharose) enriched in glycosylated Mtf by affinity chromatography with the lectin from Asparagus pea. Additionally, these glycosylated forms of serum Mtf presented similar patterns in samples from both AD and control subjects following 2-D gel electrophoresis. These data call into question the findings of previous reports that serum Mtf levels are elevated in AD subjects [18–21].

A possible explanation for the differences between our results and those published elsewhere could be related to the immunological techniques used to measure serum Mtf levels. In our assays, serum Mtf was quantified under denaturing conditions following SDS/PAGE and Western blotting analysis. These techniques allow an unambiguous identification of antigens due to the separation of proteins according to molecular mass prior to their immunodetection. These characteristics improve the specificity and the reliability of results obtained for serum Mtf. In addition to the specificity of antibody L235, we also clearly demonstrate that Mtf antigenicity and the proportions of the 79 and 82 kDa forms are very sensitive to factors that alter its conformation, such as the number of disulphide bridges, buffer components (pH and bivalent cations) and possibly glycosylation moieties. Together, our experimental approaches to improve L235 antibody specificity for Mtf and the recognition of Mtf isoforms strongly support our conclusion that serum Mtf levels are unchanged in AD patients compared with healthy control subjects.

In view of our findings, there is another reason why it is essential that SDS/PAGE analysis must be performed to obtain a clear distinction between Mtf and non-specific antigens in serum prior to immunodetection. This is based on our observation that the major serum antigens recognized by the L235 antibody were immunoglobulins and not Mtf. In other studies, amounts of serum Mtf were assessed directly in whole serum [18–21]. In one study, Mtf levels were measured by a sandwich immunoassay, in which the Hyb C antibody was immobilized on beads and detection was carried out with a fluoresceinated L235 antibody using FITC [18]; for studies using RIA, the FITC was replaced with ^{125}I [19,20]. In another report, Mtf levels were quantified by dot-immunoblot assays using L235 as the primary antibody, followed by incubation of the nitrocellulose membrane with the secondary antibody conjugated to HRP [21]. Since all of these immunological assays to quantify Mtf levels were done on serum samples, this could lead to non-specific binding of L235 antibody to various non-specific antigens, including immunoglobulins. Thus the specificity of the L235 antibody for serum Mtf could be lower in these assays, and affect assessment of Mtf levels in normal or AD subjects.

Another observation in agreement with our conclusion that serum Mtf levels are unaltered in patients with AD relates to tissue Mtf expression. Mtf mRNA expression is widespread in human tissues, being in several cases higher than that in brain [10]. These data suggest that several tissues in addition to brain produce Mtf and possibly release soluble Mtf into the serum. Thus the contribution of secreted brain Mtf to total serum Mtf might be too small to account for a significant increase in Mtf levels in the serum of AD patients. The consequence of this would be that serum Mtf levels are unchanged in patients with AD.

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