— **Minireview** —

Speciation of Aluminium in Human Serum Investigated by HPLC/High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS): Effects of Sialic Acid Residues of the Carbohydrate Chain on the Binding Affinity of Aluminium for Transferrin

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Aluminium (Al) in the blood is bound to transferrin (Tf), a glycoprotein of about 80 kDa that is characterized by its need for a synergistic anion. The binding affinity of both Al and iron (Fe) for Tf is surveyed in the context of our recent studies by on-line high-performance liquid chromatography/high-resolution inductively coupled plasma mass spectrometry (HPLC/HR-ICP-MS). First, Al in human serum without any *in vitro* Al-spikes was present in a form bound to the N-lobe site of human serum Tf (hTf). Next, the effects of sialic acid in the carbohydrate chain of hTf on the binding affinity of Al (or Fe) for hTf were studied by using asialo-hTf obtained by treating hTf with sialidase. The binding affinity of Fe for asialo-hTf and native-hTf was similar, but the binding affinity of Al for asialo-hTf was greater than that for native-hTf. These findings are discussed in relation to diseases in which the serum concentrations of carbohydrate-deficient Tf and oxalate are increased.

Key words —— speciation, aluminium, inductively coupled plasma mass spectrometry, transferrin, sialic acid, oxalate

INTRODUCTION

Aluminium (Al) is a component of soil, especially clay soils, and it is present in water. Al used to be considered non-toxic. However, the previously accepted view that Al is non-toxic is being questioned. Because Al was concluded to have a potential effect on the reproductive and developing nervous system at doses lower than those used in establishing the previous provisional tolerable weekly intake (PTWI, 7 mg Al/kg body weight per week), the sixty-seventh meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA, 2006) withdrew the previous PTWI for Al and proposed a lower PTWI of 1 mg/kg body weight per week.[1)] The Committee noted that the PTWI is likely to be greatly exceeded by some population groups, particularly children. Al has also been recognized as potentially neurotoxic for humans and animals, and it has been shown to inhibit the acetylcholinesterase activity in the brain[2)] and to cause increased free radical effects.[3)]

Al in human blood is bound to transferrin (Tf) and transferred to receptors. Tfs are a group of iron (Fe)-binding glycoproteins that require carbonate anions for metal binding,[4)] and they have two metal-binding sites: the N-lobe site and the C-lobe site.[5–7)] The lobes are homologous but distinct,[8)] and a striking difference between them is the presence of carbohydrate chain with sialic acid residues in the C-lobe. The carbohydrate chain can differ in its degree of branching and the differences are referred to microheterogeneity.[9, 10)] Carbohydrate-deficient Tfs (CDTs) have fewer sialic acid residues, and CDT levels are elevated in several diseases.[11–16)] It is unclear whether the an-

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ionic charge on sialic acid in the C-lobe affects the binding affinity of metals for the N-lobe site and the C-lobe site.

Many studies have examined the binding of Al to human serum Tf (hTf) from a thermodynamic standpoint.[17,18] Al forms stable complexes with citrate, and even more stable complexes with hTf.[19] Therefore, hTf is the predominant Al carrier in serum and passes through the blood-brain barrier.[20] To understand the mechanism of Al transport and Al accumulation, elucidation of the interactions between Al and hTf and the physiological factors that affect the interactions is essential.

This review surveys research on the binding affinity of Al for hTf in the context of our recent studies.

CHEMICAL FORMS OF METALS BOUND TO hTf

Hyphenated techniques have been used to speciate the chemical forms of Al in human blood in order to clarify the binding of Al to hTf. But few studies have been reported on the binding states of Al to hTf without any in vitro Al-spikes. Most studies have been conducted on Al-spiked serum[21–23] or serum from patients on continuous ambulatory peritoneal dialysis.[24,25] High-performance liquid chromatography/high-resolution inductively coupled plasma mass spectrometry (HPLC/HR-ICP-MS) is one such hyphenated technique, and it combines HPLC as a separation method with HR-ICP-MS as a metal detection method.[26] If the chemical forms of hTf can be separated into four chemical forms (for example, apo-, two monoferric and diferric hTfs), and if their Fe-content can be directly quantified, the preferential Fe-binding lobe and the need for synergistic anions can be clarified. In order to do so, we investigated the analytical method for metal (Fe and Al) binding to hTf with an HPLC instrument connected directly to an HR-ICP-MS instrument.[27]

The operating conditions for the hyphenated HPLC and HR-ICP-MS system have been reported previously.[27] Briefly, an HPLC apparatus (LC-10Ai; Shimadzu, Kyoto, Japan) equipped with an anion-exchange column (Mono-Q; Bio Sciences, Uppsala, Sweden) was connected directly to an ELEMENT 1 HR-ICP-MS instrument (Finnigan MAT, Bremen, Germany). A 0.1-ml sample was injected into the system, and the eluate was transferred to a UV detector and then introduced to the nebulizer of the HR-ICP-MS instrument. The levels of sulfur (S), Al and Fe were continuously monitored, and the ^{32}S level was used to monitor the protein levels in the HPLC eluate.

New gradient conditions were established to separate all four chemical forms of the metals bound to hTf. Other gradient condition was established to analyze asialo-hTf solutions, because asialo-hTf contains a sialic acid-free sugar chain and behaves differently in anion-exchange experiments.[28] A thorough clean-up procedure was necessary to obtain a detection limit of 0.1 μg/l (S/N = 3) for Al-speciation.[29]

As an assignment of four chemical forms, typical chromatograms obtained by HPLC/HR-ICP-MS are shown in Fig. 1. Four UV-peaks were detected [Fig. 1(A), upper panel], and three ^{56}Fe-peaks were observed [Fig. 1(A), lower panel]. Iron in the form of iron citrate was added to the hTf solution. Since peak c did not show an Fe peak, it was assigned to apo-hTf. The ratios of ^{56}Fe-peak areas to UV-peak areas were compared. The peaks a and d had almost same ratio of 1. The peak b had 1.8-fold higher ratio than the peaks a and d. The peak b was therefore assigned to Fe_2-hTf (two Fe atoms bound to hTf). The peaks a and d were ascribed to monoferric hTfs, and DFO (desferrioxamine, desferal) was used to assign the two monoferric hTfs. DFO is a chelating agent that has been reported to preferentially remove Fe from the C-lobe site in hTf.[30] An appropriate amount of DFO (DFO : hTf ratio 1 : 4) was added to the diferric hTf solution. In the absence of DFO, the principal peak of Fe_2-hTf was detected [Fig. 1(B), upper panel], whereas after the addition of DFO, the height of peak d increased, and a large new peak representing Fe bound to DFO appeared at 1 min [Fig. 1(B), lower panel]. Fe-peak a (at 23 min) and Fe-peak d (at 39 min) were therefore assigned to Fe_C-hTf and Fe_N-hTf, respectively. Urea-polyacrylamide gel electrophoresis (PAGE)[31] was used to further ascertain the assignments of the two monoferric hTfs, and each of the peaks in the HPLC experiments was related to each of the bands in the urea-PAGE experiments: the peak at 23 min in the HPLC experiments was assigned to Fe_C-hTf, while the peak at 39 min was assigned to Fe_N-hTf.[27]

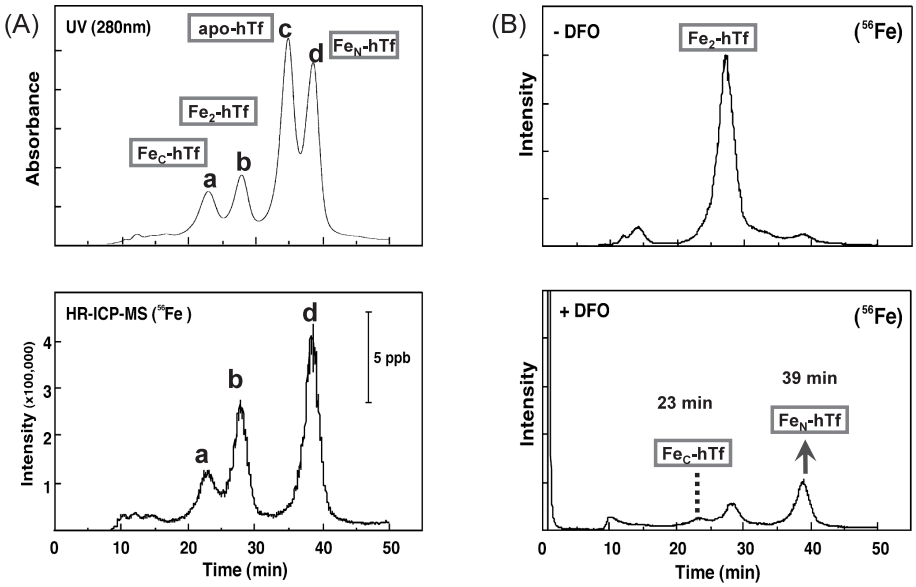

Fig. 1. Assignment of All Four Chemical Forms of Metals Bound to hTf (Modification of the Original Figure in Ref. 27)

(A) Typical HPLC/HR-ICP-MS chromatograms for apo-hTf partly saturated with Fe^{3+} (Fe : hTf = 0.5 : 1) in the presence of bicarbonate. (B) HPLC/HR-ICP-MS chromatograms (^{56}Fe level) for Fe-saturated hTf-solution before (top) and after (bottom) the addition of DFO.

DETECTION OF METAL-hTf IN BLOOD FROM HEALTHY VOLUNTEERS WITH NO Al-SPIKES

HR-ICP-MS is extremely sensitive and is the preferred method of analyzing biological samples, including serum.[26,29,32–34] The polyatomic ions that may be produced from protein samples in ICP-MS experiments are $^{12}C^{15}N^+$ (m/z 27.000), $^{13}C^{14}N^+$ (m/z 27.006) and $^{12}C^{14}N^1H^+$ (m/z 27.011), and they interfere with the detection of ^{27}Al (m/z 26.982, natural abundance, 100%).[35] Moreover, polyatomic ion interference by $^{40}Ar^{16}O^+$ with the detection of ^{56}Fe (m/z 55.935; natural abundance, 91.7%) is inevitable.[36] Although $^{40}Ar^{16}O^+$ (m/z 55.957) can be reduced by orders of magnitude by using modern quadrupole-based instruments under cool plasma conditions or with a hexapole collision cell, HR-ICP-MS is still preferable, if available. Production of $^{40}Ca^{16}O^+$ (m/z 55.958) may also interfere with the detection of ^{56}Fe in serum samples, since serum contains a Ca level of 100 mg/l. Detection of ^{32}S (m/z 31.972; natural abundance, 95.0%) by HR-ICP-MS is a promising means of monitoring proteins, since proteins contain S atoms and the HR-ICP-MS instrument can separate ^{32}S from $^{16}O_2^+$ (m/z 31.990). Thus, HPLC/HR-ICP-MS is a powerful method for simultaneously elucidating Al and Fe distribution patterns in serum.

To eliminate any Al or Fe adsorbed onto the column and lower the background levels of Al and Fe, the column was cleaned with a sodium citrate solution prior to the start of each analysis. This step lowered the detection limit for Al to 0.1 μg/l (S/N = 3), and the detection of limit for Fe to 0.02 μg/l. In the absence of any Al-spikes, the concentration of Al in the serum of healthy volunteers is about 3 μg/l.[37]

Figure 2 shows chromatograms depicting the ^{32}S, ^{56}Fe and ^{27}Al levels in blood samples with no Al-spikes obtained from two healthy volunteers. It was possible to reproduce the chemical states of Al and Fe in fresh serum.[29] Therefore, as shown in Fig. 2, Al in the blood was present in the forms of Al_N-hTf and Al_N,Fe_C-hTf. The height of the Al peak was almost as low as the detection limit level of Al, because the Al level in the blood was very low (3 μg/l).

EFFECTS OF C-LOBE SIALIC ACID RESIDUES OF hTf ON METAL BINDING

Many types of plasma proteins in the blood are glycosylated with *N*-linked oligosaccharides. hTf has carbohydrate chains in the C-lobe and has two potential glycosylation sites that are normally occupied by *N*-linked oligosaccharide chains, and 90% or more of the hTfs purified from the blood of healthy humans by affinity chromatogra-

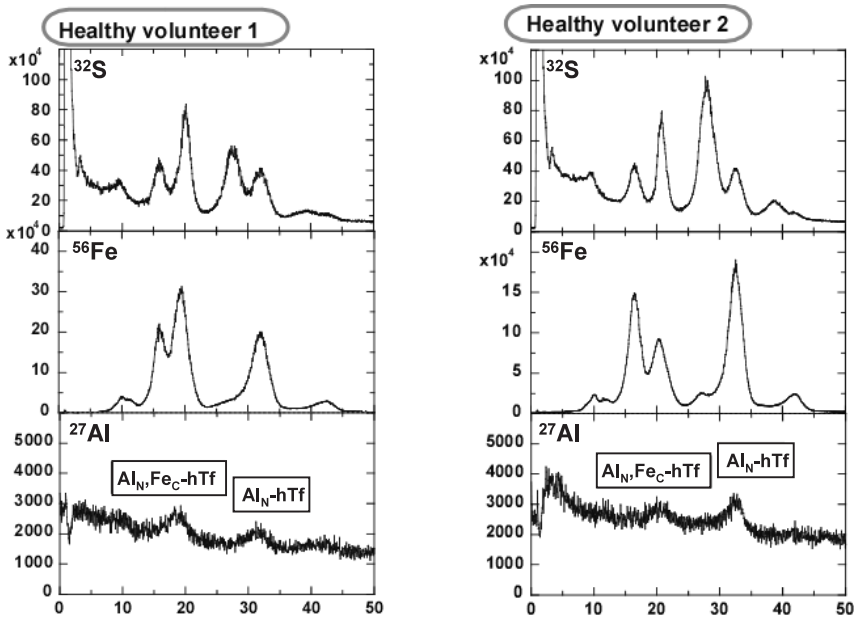

Fig. 2. HPLC/HR-ICP-MS (^{32}S, ^{56}Fe and ^{27}Al) Chromatograms for Sera from Two Healthy Volunteers without Any Al-spikes (Modification of the Original Figures in Ref. 29)

phy with anti-Tf-Sepharose contain four sialic acid molecules.[38] On the other hand, the amounts of CDTs, which contain a smaller number of sialic acid residues, are reportedly elevated in several diseases. Carbohydrate-deficient glycoprotein (CDG) syndrome is a hereditary autosomal recessive disorder first reported by Jaeken *et al.* in 1984,[39] and is characterized by severe nervous system symptoms, growth retardation, and hepatopathy during infancy.[40] A decrease in tetrasialo-hTf followed by increases in disialo- and asialo-hTf isoforms has been observed in patients with CDG syndrome.[41,42]

The increase of CDTs in the blood is observed in alcohol abuse or heavy drinking, and it disappears with abstinence.[11,43–45] hTfs containing smaller numbers of sialic acid residues in chronic alcohol abusers have been found to exhibit higher sialidase activity.[46] The free sialic acid level in blood is elevated by high alcohol consumption.[47]

The carbohydrate chain in the C-lobe site can differ both in degree of branching and number of sialic acid residues. hTfs exist as disialo, trisialo, tetrasialo, pentasialo, and asialo forms of hTf.[48] The existence of carboxylic acid residues in sialic acid molecules probably influences protein conformation and the principal function of hTf, thereby changing the binding affinity of hTf for metals. Moreover, if the presence of sialic acid residues in hTf affects metal binding to hTf, the metal-binding behavior of patients with increased CDT levels and of healthy persons may be different. To determine whether that is true, we compared the binding affinity of Al for native-hTf and for asialo-hTf.[28]

To compare the binding affinity of Fe for native-hTf and asialo-hTf, we used sialidase (neuraminidase) to prepare asialo-hTf and supplemented native- and asialo-hTfs with an Fe-citrate mixture (Fe : citrate : hTf ratio, 1 : 1 : 1) in the presence of bicarbonate. Fe added in the form of Fe-citrate exhibited a preference for the N-lobe site, and its binding affinity for native- and asialo-hTfs was similar, *i.e.*, the heights of the Fe-peaks were comparable. Next, the binding affinity of Al for native-hTf and asialo-hTf was compared (Fig. 3), and the results showed that Al added as an Al-citrate complex had selectively bound to the N-lobe site and that its binding affinity for asialo-hTf was greater than for native-hTf.

Oxalate is present in serum, and its concentration enhanced in several diseases such as primary hyperoxaluria and nephrocalcalcinosis.[49] We then examined the effect of oxalate on the binding of Al to hTf in the presence of bicarbonate (Fig. 4). Al was added in the form of Al-oxalate complex in a ratio of 1 : 4. The binding affinity of Al for the N-lobe site of asialo-hTf increased further after the addition of the Al-oxalate, and the area of the metal$_2$-hTf peak also increased. There was a considerable difference between the Al-peaks for native-hTf and

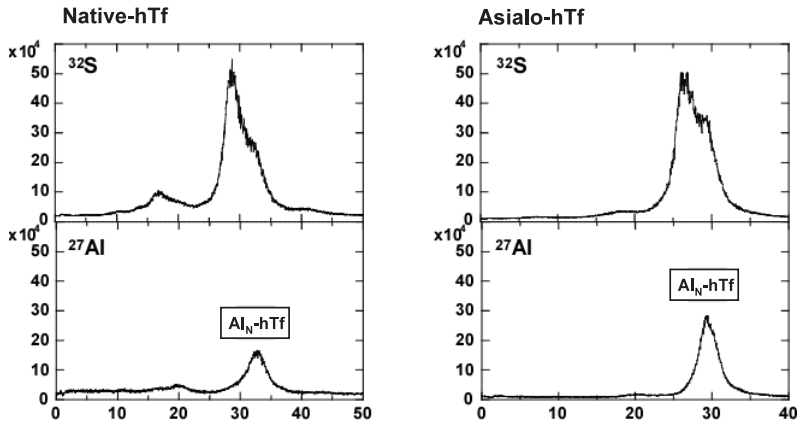

Fig. 3. HPLC/HR-ICP-MS (^{32}S and ^{27}Al) Chromatograms for Native-apo-hTf Solution and Asialo-apo-hTf Solution plus Al-citrate (Al : citrate = 1 : 1) in the Presence of Bicarbonate
The Al : hTf ratio was 1 : 1 (modification of the original figure in Ref. 28).

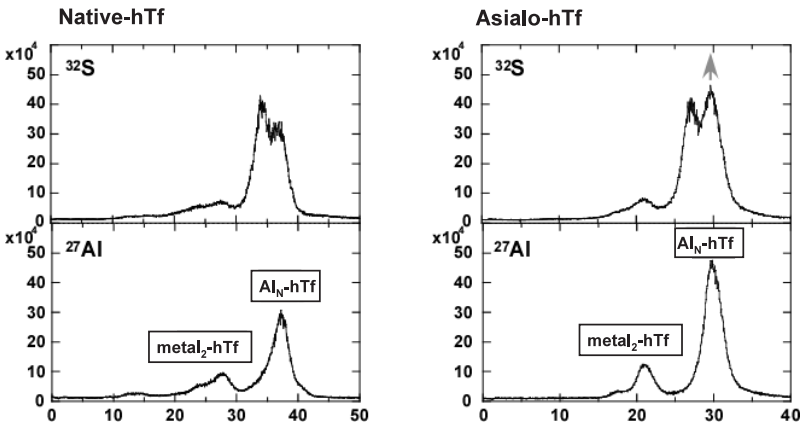

Fig. 4. HPLC/HR-ICP-MS (^{32}S and ^{27}Al) Chromatograms for Native-apo-hTf Solution and Asialo-apo-hTf Solution plus Al-oxalate (Al : oxalate = 1 : 4) in the Presence of Bicarbonate
The Al : hTf ratio was 1 : 1 (modification of the original figure in Ref. 28).

asialo-hTf. Surprisingly, in the absence of bicarbonate, Al-oxalate showed a preference for the C-lobe site in native-hTf and comparable affinity for both lobes of asialo-hTf.[28] Thus, the lack of sialic acid in the glycans and the presence of oxalate enhanced the binding affinity of Al for hTf.

In Fig. 4, the height of the $metal_2$-asialo-hTf peak was larger than that of the $metal_2$-native-hTf peak. Since according to a previous report[50] $metal_2$-hTf is more easily transferred to the hTf receptor than monoferric-hTf or apo-hTf, Al_2-hTf or Al,Fe-hTf may be easily incorporated into brain cells. Al may also easily pass through the blood-brain barrier and cause toxicity. However, the affinity of hTf receptor 1 for Al bound to native-hTf was too weak to be detected in an *in vitro* experiment.[51] Furthermore, asialoglycoproteins bind to receptors in the liver, are taken up by the liver, and quickly disappear from the circulatory system.[52,53] Thus, despite several studies on this topic, the binding affinity of Al-native-hTf and Al-asialo-hTf for the Tf receptor and the actual degree of binding in the human body remain to be resolved.

The findings in this study suggest that the binding affinity of Al for hTf may be increased in patients whose blood contains CDTs. The oxalate concentration in the blood of healthy persons is approximately 2 μmol/l,[54] but it is elevated in the serum of dialysis patients to 48.5 μmol/l before dialysis and 17.7 μmol/l after dialysis.[55] Under these conditions, Al toxicity may increase as a result of the increase in Al_2-hTf or Al,Fe-hTf. Thus, the role of oxalate in Al-hTf binding and Al toxicity should be carefully considered in some diseases.

CONCLUSION

This review has surveyed research on the binding affinity of Al for human Tf in the context of our recent studies.

The detection limit (S/N = 3) of ^{27}Al by HPLC/HR-ICP-MS was lowered to 0.1 μg/l by clean-up procedure.

Al in human serum with no *in vitro* Al-spikes was detected as Al_N-hTf and $metal_2$-hTf (Al_N,Fe_C-hTf).

The absence of sialic acid residues in the carbohydrate chain of the C-lobe site of hTf and the presence of oxalate increased the binding affinity of Al for hTf, thereby possibly increasing Al toxicity.

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