A New Effect of Aluminum on Iron Metabolism in Mammalian Cells

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There is an increasing number of reports about new effects of aluminum on iron metabolism in mammalian cells. In this review, based on our recent work and that by others, we describe how aluminum disturbs iron homeostasis. The effects of aluminum may help to explain the pathogenesis of neurodegeneration in Alzheimer's disease.

Keywords: Aluminum, Iron, Transferrin, Iron regulatory protein, Alzheimer's disease

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List of Abbreviations

Al	aluminum
Fe	iron
Tf-IU	transferrin-independent iron uptake
IRP	iron regulatory protein
HO	heme oxygenase
ROS	reactive oxygen species
AD	Alzheimer's disease
β-AP	β-amyloid protein
Tf-R	transferrin receptor

1 Introduction

Aluminum (Al) is the most abundant metal in the environment. Ingestion from air, foods, water and medicine is unavoidable. Although no case has Al been shown to have a definite biological function, it may substitute for chemically similar elements in living cells. Chemically similar ions might have a similar metabolism. Hence, Al competes with a similar metal, iron which plays a critical role in mammalian cells. There are accumulating reports on new effects of Al on iron metabolism in mammalian cells. The present review includes recent topics on the relations between Al, iron metabolism-related protein and iron regulatory proteins as well as neuronal cell death by Al. Advances during the last five to ten years on these issues are described.

2 Transport Mechanism of Aluminum in Mammalian Cells

2.1 Aluminum Transport by Transferrin-Dependent and -Independent Uptake

Mammalian cells take up iron by three kinds of iron (Fe) uptake systems; the transferrin receptor (TfR)-mediated endocytosis, redox, and Tf-independent iron uptake (Tf-IU) systems [1–7] (Fig. 1).

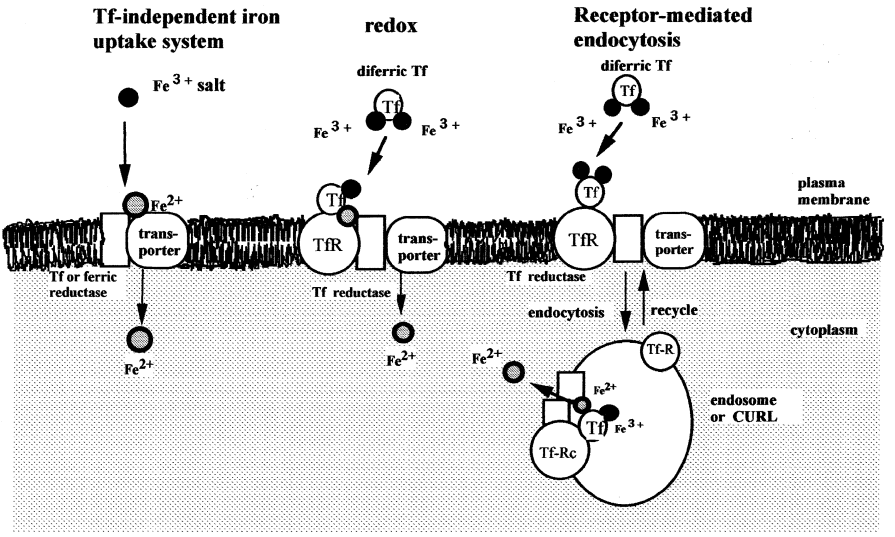

Fig. 1. The three kinds of iron uptake systems in mammalian cells

TfR-mediated endocytosis is a well-known uptake system; Tf binds one or two Fe atoms, but only diferric Tf (Fe_2Tf) has a high affinity for TfR to be taken up by the receptor-mediated endocytosis. This system uses a mobilization pathway that involves endosomal acidification, reduction of ferric Fe, and ferrous Fe transport [8]. Recently, it was clarified that divalent cation/metal ion transporter (DCT1) or Nramp2 involves iron transport from the endosome to the cytosol [9, 10]. Al resembles Fe in chemical characteristics; ionic radius, charge density, and coordination number [11]. Therefore, Al binds with Tf to form di—Al—Tf. Al bound to Tf even passes through the blood-brain barrier to enter the neuronal cells via Tf receptor-mediated endocytosis [12].

The redox system does not depend on endosomal acidification but needs TfR. Fe_2Tf first binds to TfR which is located in close proximity to the proton- and electron-pumping NADH:Tf oxidoreductase. The Fe—Tf bond is destabilized by proton efflux, making Fe^{3+} susceptible to reduction. Fe^{2+} is trapped by a plasma membrane binder and can be transported by a translocator [4]. As Al is a simple trivalent cation incapable of redox changes, it may be theoretically impossible that Al bound to Tf is taken up by a redox mechanism. Actually, no reports on a redox-mediated process of Al bound to Tf have been made.

Although non-Tf-bound Fe uptake occurs in a variety of the cells, detailed molecular mechanisms are unknown at present. However, we made a preliminary experiment on the basic mechanism and found that Tf-independent Fe is reduced at the plasma membrane prior to uptake, as the ferric reduction of Fe_2Tf taken up by the redox mechanism occurs at the same site [5, 13].

Al is taken up not only by receptor-mediated endocytosis but also by the Tf-IU system (Fig. 2). Since Al has no suitable radionuclide, it has been

difficult to verify that Al is taken up by mammalian cells. However, it was shown using biochemical and molecular biology techniques that free Al salts can pass through the plasma membrane to change a function of iron metabolism-related protein in the cytosol of rat cortical cells after transport [14, 15]. Pretreatment of cells with μM levels of Al-nitrilotriacetate increased the Tf-IU system similarly as for Fe. Northern blot analysis showed that Al decreased the Tf receptor mRNA level. Al also increased the cytosol aconitase activity with increasing concentrations [14]. These results show that Al is transported by the Tf-IU system to change a function of iron metabolism-related protein in the cytosol of cortical cells. From the characterization of the Tf-IU system by our group and other investigators, among of three uptake systems, the Tf-IU system may contribute to metal accumulation in mammalian cells.

Gunshin et al. have cloned the divalent cation/metal ion transporter (DCT1 or DMT1). The translation product takes up free Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+} [9]. Since DCT1 mRNA have an IRE in the 3′ untranslated region, the regulation mechanism of DCT1 mRNA is considered to be analogous to the regulation of Tf-R mRNA. Although we can expect DCT1 to take up Al by a Tf-IU, this Tf-IU may be different from the above-mentioned Tf-IU because the former is down-regulated by accumulated metals and the latter up-regulated. As summarized in Fig. 2, so far, it has been reported that mammalian cells can taken up Al by Tf-dependent and -independent iron uptake systems.

Although the molecular mechanism of metal uptake via the Tf-IU system is unknown, there is a fair amount of experimental data on the characteristics of

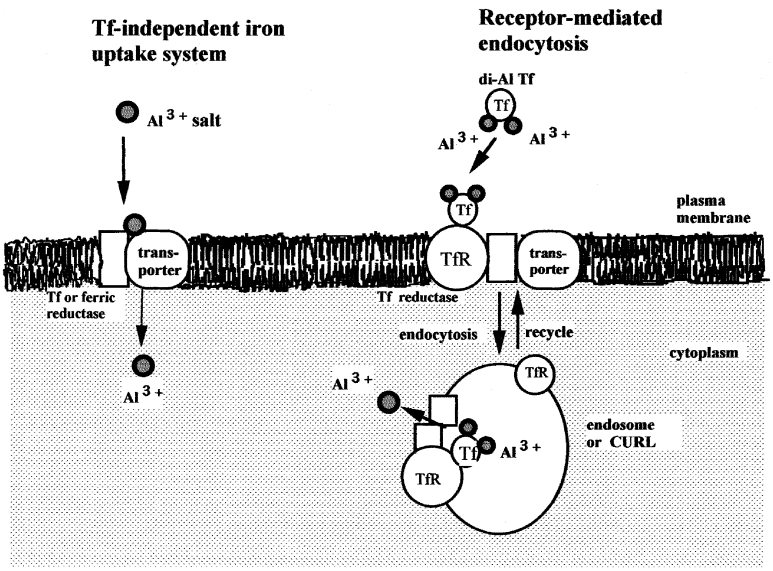

Fig. 2. Aluminum uptake by receptor-mediated endocytosis and transferrin-independent iron uptake systems in mammalian cells

multiple Tf-IU systems in various mammalian non-neuronal cells. From their characteristics, these transport systems can be classified into two types: a metal-induced and Ca^{2+}-dependent type, e.g., human skin fibroblasts and HeLa cells and a non-metal-induced and Ca^{2+}-independent type, e.g., K562 cells [2]. The Tf-IU system of human skin fibroblasts is up-regulated by iron salts. In HeLa cells and fibroblasts, divalent metal ions, such as Cu^{2+}, Zn^{2+}, and Fe^{2+} inhibit Fe^{3+} uptake. By contrast, the K562 cell transport mechanism is Ca^{2+}-independent and not up-regulated by extracellular Fe salts. Uptake of Fe^{3+}-NTA by K562 cells was specifically inhibited by Cd^{2+} and Cu^{2+}, whereas Mn^{2+}, Co^{2+}, and Ni^{2+} were ineffective. Quite recently, the presence of a non-Tf-mediated uptake for Fe and Mn in glial cells has been reported [16]. However, the Tf-IU of both neuronal and glial cells has not been characterized.

2.2 Characterization of Transferrin-Independent Iron Uptake System in Neuronal and Glial Cells

Therefore, we further examined the characteristics of the Tf-IU system in primary neuronal and glial predominant systems (NP and GP) from rat cerebral cortex thought to be involved in the accumulation of Al and Fe in the brain [17]. The Tf-IU system in cortical cells exhibited properties similar to those of HeLa cells and human skin fibroblasts in two regards: the dependence on Ca^{2+} and the up-regulation by transition metals. Comparing the characteristics in NP and GP cells, there are no differences between them. However, we found that GP cells are more remarkable than NP cells in terms of the degrees of up-regulation of Tf-IU by Al, suppression of Tf-R mRNA expression, and decrease of IRP/IRE complex by Al [15], suggesting that glial cells rather than neuronal cells contribute to the metal accumulation and are more resistant to oxidative stress caused by metals than neuronal cells. The present study may help to explain the pathogenesis of neurodegeneration in AD disorders caused by metal-generated oxidative stress [15].

In relation to our findings that Al up-regulates Tf-IU system in both neuronal glial cells, Abreo et al. observed similar phenomena [18].

They describe the uptake and toxicity of Al, the effect of Al on Fe uptake, and the expression of NFT protein in murine neuroblastoma cells (Neuro 2A). Significant cell Al uptake and inhibition of cell growth were seen in Neuro 2A cells at 1, 2, 3, and 4 days after plating in medium containing Al-Tf and Al citrate. Al-loaded Neuro 2A cells showed increased rates of ^{59}Fe and ^{125}I-Tf uptake and total cellular Fe content at each day after plating compared with control cultures. Significant increases in NFT protein staining were detected in Al-exposed cells at 3 and 4 days in culture compared with controls. The intensity of NFT staining in Al-loaded cells was directly proportional to the time in culture. These results suggest that the accumulation of Al in Neuro 2A cells resulted in increased uptake of Fe, inhibition of cell growth, and expression of NFT protein, partially mimicking the pathological hallmarks of Alzheimer's disease.

3
New Effect of Aluminum on Iron Metabolism in Mammalian Cells

3.1
Effect of Al on Iron Regulatory Protein-1 and -2 in Mammalian Cells

Iron regulatory proteins (IRPs) regulate the cellular iron level in mammalian cells. IRPs are known as cytosol mRNA binding proteins which control the stability or the translation rate of mRNAs of iron metabolism-related proteins such as TfR, ferritin, and 5-aminolevulinic acid synthetase in response to the availability of cellular iron [19–21] after uptake [5]. The regulatory mechanism involves the interaction between the iron-responsive element (IRE) in the 3′ or 5′ untranslated regions of the transcripts and cytosolic IRPs (IRP-1 and -2). IRP-1 is an iron-sulfur (Fe-S) protein with aconitase activity containing a cubane 4Fe-4S cluster. When Fe is replete, IRP-1 prevails in a 4Fe-4S form as a holo-form and is an active cytoplasmic aconitase. As shown in Fig. 3, when Fe is deplete, it readily loses one Fe from the fourth labile Fe in the Fe-S cluster to become a 3Fe-4S cluster and in this state has little enzymatic activity [22, 23].

As it is less known whether IRP-1 modulates various non-Fe metals, we examined whether treatment of homogeneously purified IRP-1 with non-Fe

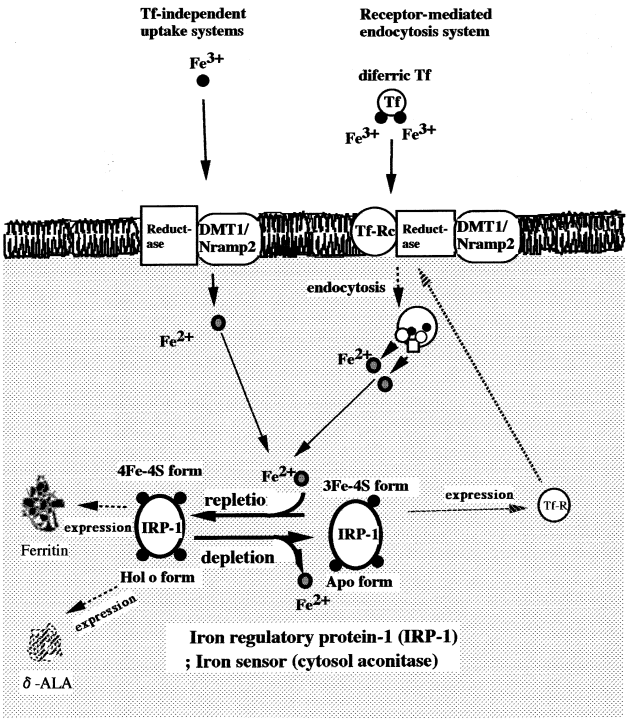

Fig. 3. Effect of aluminum on post-transcriptional regulation by iron regulatory protein-1

metals decreased the affinity to IRE in RNA-band shift assays and increased the aconitase activity. As results of these experiments, we showed that the purified IRP-1 directly bind not only Fe, but Mn, Ni, Cu, Cd, and Hg. Also, in competition experiments, non-Fe metals inhibited ^{55}Fe incorporation into the fourth labile position of the Fe-S cluster of IRP-1. In PLC hepatoma cells, metal loading inactivated the binding activity and activated the enzyme activity. Non-Fe metals also suppressed TfR mRNA expression in the cells. These results suggest that various non-Fe metals modulate IRP-1 by conversion of a 3Fe-4S apo-form to a [1 non-Fe metal + 3Fe]-4S holo-form [24]. Therefore, as shown in Fig. 4, we present a hypothesis that IRP-1 binds Al to form a [1 Al + 3Fe]-4S holo-form. It is an interesting question whether Al also binds IRP-1 *in vivo* and *in vitro* as Fe does. Actually, we have observed that Al binds to the purified IRP-1 to increase its aconitase activity and decrease its mRNA binding activity (Oshiro S, et al., unpublished data).

In contrast, IRP-2 possesses neither the Fe-S cluster nor aconitase activity [25]. The binding activity of IRP-1 to IRE is modulated by intracellular Fe levels.

With this objective in mind, we examined the effects of Al accumulated in brain cells on the iron metabolism using primary cultures from the fetal rat cerebral cortex. To examine the influence of Al on the iron metabolism, the

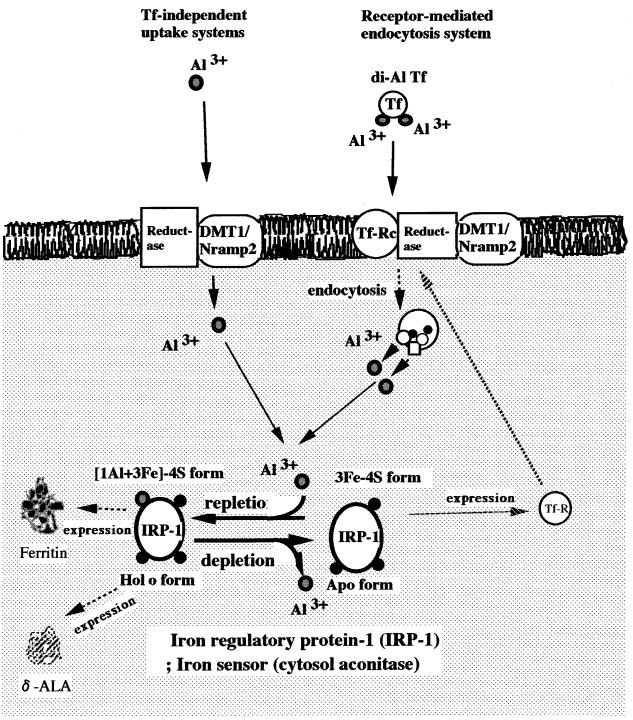

Fig. 4. Effect of aluminum on post-transcriptional regulation by iron regulatory protein-1

interaction between Al accumulated in the cells and IRP-1 known as a cellular iron regulator was examined by Northern blot analysis and assay of its aconitase activity; Al decreased the Tf receptor mRNA level and increased the aconitase activity of IRP with increasing concentrations. The increase of the enzyme activities by Al was also observed in an *in vitro* experiment. These results suggest that Al taken up by mammalian cells affects the iron metabolism in the cells. Al seems to interact with IRP to regulate TfR expression.

IRP-2 is also one of the central regulators of iron homeostasis. IRP-2 regulates the expression of molecules involved in iron metabolism by binding to IREs in the transcripts of those molecules in Fe depletion. IRP-2 is degraded efficiently by the proteasome in Fe-replete cells. IRP-2 is oxidized and ubiquitinated in cells before degradation. Moreover, Fe-dependent oxidation converts IRP-2 into a substrate for ubiquitination *in vitro*. Excess Fe is sensed by its ability to catalyze site-specific oxidations in IRP-2, oxidized IRP-2 is ubiquitinated, and ubiquitinated IRP-2 subsequently is degraded by the proteasome [26]. Yamanaka et al. reported that Al antagonizes the Fe-induced decrease in IRE binding activity of IRP-2. Al also inhibits Fe-induced oxidation of IRP2 *in vitro*. These results suggest that Al stabilizes IRP-2 by interfering with the iron-catalyzed oxidation, which results in perturbation of iron metabolism. The action of Al may involve selective targeting and removal of oxidatively modified proteins that may contribute to the turnover of many proteins degraded by the proteasome [27].

The IRP-1 is present at similar levels in both AD and control brains. In contrast, IRP-2 co-localizes with redox-active Fe in NFT, senile plaque neurites, and neuropil threads. Hence, alterations in IRP-2 might be directly linked to impaired iron homeostasis in AD [28].

4
Implication of the Aluminum Effect in Alzheimer's Diseases

4.1
Contribution of Paired Helical Filaments Tau to Plaque and Tangle Formation in Alzheimer's Diseases by Al Modulation

The histopathological characteristics of the brain in Alzheimer's diseases (AD) are the presence of intraneuronal neurofibrillary tangles (NFTs), extraneuronal amyloid-rich senile plaques, and a massive loss of neurons of the telencephalon. Although amyloid proteins are unique to senile plaques, several components are common to both senile plaques and NFTs: hyperphosphorylated tau proteins, ubiquitin, α1-antichymotrypsin, apolipoprotein E, heparan sulfate proteoglycans, and Al and Fe [29]. Recent reports on Al and Fe transport mechanisms and the relation between them in AD are reviewed below.

Shin et al. probed interactions between paired helical filaments (PHF) tau, Al salts, and other plaque and tangle components. They investigated the *in vivo* interactions of PHF tau and $AlCl_3$ with other plaque and tangle

components by injecting PHF tau with and without $AlCl_3$ into the rodent brain. PHF tau co-injected with $AlCl_3$ formed aggregates that persisted much longer in the rat brain, and induced longer-lived co-deposits of A beta, ubiquitin, α1-antichymotrypsin, and ApoE than did PHF tau alone. Injections of PHF tau with $AlCl_3$ also induced neurons near the injection site to acquire PHF tau-like properties as monitored with antibodies that recognize defined PHF tau epitopes containing phosphoserine residues. Injections of $AlCl_3$ alone as well as injections of normal adult and fetal CNS tau, several different synthetic peptides, neurofilament proteins, α1-antichymotrypsin, haparan sulfate proteoglycans, or ApoE with and without $AlCl_3$ failed to induce co-deposits of A beta or alter the immunoreactivity of tau in rodent neurons. To determine if Al salts interact directly and specifically with PHF tau *in situ*, they pretreated sections of AD hippocampus with 10 mM $AlCl_3$ and then probed these sections by immunohistochemistry with antibodies to PHF tau as well as to a number of other plaque and tangle components. Preincubation of these sections with $AlCl_3$ diminished PHF tau immunoreactivity in NFTs and senile plaques using the PHF tau-specific antibodies, and PHF1, while the immunoreactivity of other plaque and tangle proteins was not abolished. They also examined the effects of $AlCl_3$ on PHF tau and normal adult human CNS tau *in vitro*. $AlCl_3$ had no effect on normal adult human CNS tau, while increasing concentrations of $AlCl_3$ (from 0.1 to 1.0 mM) induced PHF tau to aggregate at the top of the stacking gel, and at high concentrations (0.3 and 1.0 mM) of $AlCl_3$, PHF tau completely failed to enter the gel. These studies suggest that Al binds to PHF tau, induces these proteins to aggregate, and retards their proteolysis. Further, since intracerebral injections of PHF tau with and without $AlCl_3$ in rats appear uniquely capable of inducing co-deposits of a number of proteins found in authentic AD senile plaques and NFTs, they speculate that the contributions of PHF tau to plaque and tangle formation in AD may be modulated by Al [30].

4.2
Effect of Al on Fe Uptake in Neuronal Cells

The uptake and toxicity of Al, the effect of Al on Fe uptake, and the expression of NFT protein in murine neuroblastoma cells (Neuro 2A) were examined in the following study. Significant cell Al uptake and inhibition of cell growth were seen in Neuro 2A cells at 1, 2, 3, and 4 days after plating in medium containing Al-Tf and Al citrate. Al-loaded Neuro 2A cells showed increased rates of ^{59}Fe and ^{125}I-Tf uptake and total cellular Fe content at each day after plating compared with control cultures. Significant increases in NFT protein staining were detected in Al-exposed cells at 3 and 4 days in culture compared with controls. The intensity of NFT staining in Al-loaded cells was directly proportional to the time in culture. There was no difference in malonodialdehyde levels measured in control versus Al-loaded Neuro 2A cells. These results suggest that the accumulation of Al in Neuro 2A cells resulted in increased uptake of Fe, inhibition of cell growth, and expression of NFT protein, partially mimicking the pathological hallmarks of AD. This model system may also be applicable for Al-induced dialysis dementia, because the Al

concentrations at which cell toxicity occurred can be found in dialysis patients [29]. This report supports our hypothesis [14, 15, 17].

4.3
Effect of Al on Fe Metabolism in AD Brain

As mentioned above, there are the profound relationships between Al and Fe metabolism in mammalian cells; Al can bind proteins bound to Fe. Apo-Tf binds to Al to form di-Al-Tf (Al_2Tf). Al_2Tf is recognized by TfR to be taken up by brain cells. Al binds Fe storage protein, ferritin and also influences the expression of ferritin mRNA. If this is a reliable phenomenon, Al is required to interact with IRPs which post-trascriptionally regulate the expression of ferritin or TfR mRNAs.

4.4
Amount of Al and Fe in Ferritin in AD Brain

One of two groups of rats was fed $AlCl_3$ (100 μM) for 1 year in the drinking water. Brain tissue contained about one-third of the amount of ferritin found in the liver. While brain ferritin from normal rats contained 42.1 ± 14.3 mol of Al, that from the Al-fed group contained 115.4 ± 48.3 mol of Al per mol of ferritin. Liver ferritin from both groups contained similar amounts of both Al and Fe, and the amounts were less than that found associated with brain ferritin. Ferritin isolated from AD brains contained more Al and more Fe than that from age-matched controls [31, 32].

4.5
Post-Transcriptional Regulation of Ferritin Heavy Chain in AD Brain

In the aging human brain, the concentrations of iron and its major storage protein, ferritin, rise but the distribution of metal and protein remains non-uniform. More ferritin could be isolated from the brains of humans who died of AD than from age- and sex-matched controls. Also, brain ferritin of rats chronically exposed to $AlCl_3$ in their drinking water contained more Al and Fe. Based on the above earlier observations, a more detailed study of human brain ferritin was initiated. Ferritin was a component of senile plaques in AD. Ferritin obtained from normal or AD brains was composed of 24 subunits [70% heavy (H) chain; 30% light (L) chain]. The techniques of molecular biology revealed the presence of an additional ferritin mRNA species for the H subunit which was more abundant in the brain than in other human tissues. It contained not only the entire sequence of 919 nucleotides of H chain mRNA from liver but also an additional segment of 279 nucleotides in the 3′-untranslated region. The two mRNA seemed to arise by the use of an alternate polyadenylation site of the same primary transcript. Ribonuclease protection assays revealed that the concentrations of the longer mRNA in the normal hippocampus and the hippocampus of patients with AD brains were similar [32]. These results show that expression of ferritin H chain in AD brain

does not regulate at the transcriptional level but rather the post-transcriptional level.

4.6
Acceleration of β-AP Generation by Al in AD Brain

Clauberg and Joshi presented an interesting hypothesis that Al accumulated in AD brain accelerates the generation of β-AP [33]. The plaques from AD brain contain β-AP, α1-antichymotrypsin, and Al. They examined the relation of all three components of plaques to each other. The β-AP is derived by proteolytic processing from β-AP and some of these proteins contain a domain that is highly homologous to the bovine pancreatic trypsin inhibitor. Bovine pancreatic trypsin inhibitor also inhibits α-chymotrypsin and they showed that Al affects both the activity and the inhibition of this enzyme. At pH 6.5, in the presence of Al, the enzyme activity is doubled, and the inhibitor is only 1% as effective as in the absence of the metal ion. The inhibition by BX-9, a protease inhibitor prepared from protein components of amyloid plaques, is also reduced by Al; so too is that by α1-antichymotrypsin but to a lesser degree. In the Alzheimer brain, they propose that Al may accelerate proteolytic processing of the α-amyloid precursor protein by suppression of the inhibitor domain. Thus, the β-amyloid protein (β-AP) may accumulate and initiate plaque formation.

4.7
Aggregation and the Deposition of β-AP by Al and Senile Plaques Formation

β-AP, which is the major component of senile plaques in the brains of AD, has an intrinsic tendency to form insoluble aggregates. The aggregation of β-AP enhances its neurotoxicity and is assumed to play a key role in the amyloid deposition. The plaques are predominantly composed of human β-AP β A4, a 40-mer whose neurotoxicity is related to its aggregation. In physiological buffers, Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, or K^{+} at 10 mM had no effect on the rate of β A4 aggregation. In sharp contrast, Al, Fe, and Zn under the same conditions strongly promoted aggregation (rate enhancement of 100- to 1000-fold). The aggregation of β A4 induced by Al and Fe is distinguishable from that induced by Zn in terms of rate, extent, pH-, and temperature-dependence. High concentrations of certain metals may play a role in the pathogenesis of AD by promoting aggregation of beta A4. The aggregation of β-AP (beta 1–40) is promoted by Al [34, 35]. Al concentrations of 15–80 ppm (0.5–3.0 mM) were detected in tangle NFTs of AD [36]. Therefore, it is possible that Al directly influences the process of aggregation and the deposition of senile plaques.

Beta-amyloid precursor proteins (β-APPs) are normal components of the human brain and some other tissues. Proteolysis of these, presumably by serine proteases, generates a 39 to 42 amino acid-long peptide, α-AP. In AD brains, β-AP aggregates into plaque, the hallmark of AD brains. Some of the

α-APPs also contain a 56 amino acid-long segment which inhibits serine proteases. *In vitro*, at pH 6.5, Al activates β-chymotrypsin 2-fold and makes it dramatically resistant to protease inhibitors such as bovine pancreatic trypsin inhibitor (bPTI) or its mimic present in the β-APPs [30].

4.8 Generation of Reactive Oxygen Species by Al

We observed active oxygen species in rat glial and neuronal cells after treatment of Al- and Fe-NTA using an *in vitro* model of AD brain. Reactive oxygen species (ROS) were measured by using a non-permeant chemiluminescence probe, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]-pyrazin-3-one hydrochloride (MCLA) specific for superoxide (O_2^-) and singlet oxygen ($^1O_2$) [37]. Rat neuronal predominant (NP) or glial predominant (GP) cells were incubated with 50 μM Al- or Fe-NTA at 37 °C for 24 h, then washed three times with PBS. While the cells were being incubated in PBS containing 25 μM MCLA at 37 °C, chemiluminescence was measured for 5 min. In addition, to examine whether O_2^- is produced by NP or GP cells after Al- or Fe-loading, 0.5 μM Cu-Zn superoxide dismutase (SOD) was added to the reaction mixture to examine the production of O_2^- in these cells. Although $FeCl_3$ forms O_2^- in cerebral cortex after intracortical injection it is not known which of the cortical cells generates O_2^-. To investigate the mechanism by which the metal toxicity is expressed in these cells, ROS were measured using a chemiluminescence analysis specific for O_2^- and $^1O_2$. In this experiment, we assayed ROS extracellularly released from the cells after metal-loading using MCLA reacting specifically with both ROSs.

For the generation of ROS by Fe-loading, the MCLA-dependent intensity in GP or NP cells was 2.0- or 2.4-times as high as than that in each of the control cells. The intensity in the NP cells with or without Fe treatment increased 3.8- and 1.5- times over that in GP cells without treatment, respectively. The ROS level in Fe-loaded NP cells was 2.0-times that of Fe-loaded GP cells.

For the generation of ROS by Al-loading, the amount of ROS generated by GP or NP cells was 1.5- or 1.8-times than that by each of the control cells. The intensity in the NP cells with or without Al-treatment increased 2.5- and 1.4-times over that in GP cells without treatment, respectively. ROS level in Al-loaded NP cells was 2.5-times higher than that of Al-loaded GP cells. Al-loading generated less ROS than Fe-loading in both cells. In addition, to examine whether O_2^- is produced in these cells, SOD was added to the reaction mixtures containing ROS. In Fe-loaded GP and NP cells, there was a 40 and a 54% decrease of chemiluminescence generated in Fe-loaded cells, respectively. In Al-loaded GP and NP cells, there was a 27 and a 43% decrease compared to GP or NP cells with Al-treatment.

For the generation of $^1O_2$, it is known that $^1O_2$ is involved in oxidative damage to membrane lipids *in vitro*, so it should be detectable [38]. Although we could not clearly show the generation of $^1O_2$ in this experiment, a part of the neuronal cell death we observed might be due to the cell toxicity of ROS. Comparing the sensitivities of the cells to oxidative stress, GP cells were more

resistant to Al and Fe toxicities than NP cells. Neurons are more vulnerable than glia to insults such as ischemia, hypoxia, and brain trauma [39]. It was also reported that the antioxidant capacity of neurons in culture is lower than that of glial cells in culture [40]. Our findings are compatible with these reports. The relation between the amount of ROS in both cells and the viabilities of these cells may reflect the difference between the antioxidant defense systems of both cells [15].

4.9
Activation of Transcription Factor by Oxidative Stress Generated by Fe in Neuronal Cells

In *in vivo* and *in vitro* studies, Al can potentiate the oxidative stress produced by Fe [41, 42]. Transcription factor, nuclear factor (NF)-κB is activated by oxidative stress. The relevance of NF-κB to neurodegeneration is suggested by a correlation between the amount of activated transcription factor NF-κB and a key inflammatory enzyme, COX-2, in both aging and AD temporal lobe neocortex [43].

4.10
Effect of Al and/or Fe on Neuronal and Glial Cell Death

Savory et al. proposed that aging is an important factor in the susceptibility of neurons to oxidative stress and to subsequent apoptosis [44]. They demonstrated that aged rabbits treated intracisternally with Al maltolate exhibit intense intraneuronal silver positivity indicative of the formation of neurofilamentous aggregates, together with oxidative stress. These changes occur in the CA1 region of the hippocampus as well as in cerebral cortical areas. Apoptosis, measured by the TUNEL *in situ* technique, co-localizes with oxidative stress. Young animals treated with Al show few of these alterations, while age-matched controls are essentially negative. Further studies on the time course of these and related changes demonstrate that oxidative stress and redox-active Fe accumulation in hippocampal neurons occur very rapidly, within a period of 3 hours, and increased in intensity at 72 hours. Changes suggestive of apoptosis are seen by 24 hours and are pronounced at 72 hours. In aged animals there is an initially intense immunopositivity at 3 hours for Bcl-2, with negative staining for Bax. By 72 hours, when apoptosis is strongly evident, Bcl-2 is negative and Bax strongly positive. In contrast to the aged rabbits, young animals treated similarly with Al exhibit much less oxidative stress with no apoptosis, and maintain Bcl-2 immunopositivity and negative Bax staining. These findings strongly support the key role that oxidative damage plays in the process of neurodegeneration and in the increased vulnerability to Al-induced injury in the aged animal. These are novel observations which may have important implications for aiding our understanding of the pathogenesis of the neurodegeneration occurring in AD [44].

4.11
Effect of Al on Glial Cell Death

Al exposure and apoptotic cell death have been implicated in several neurodegenerative conditions including AD. For the apoptosis of astrocytes by Al, the proportion of apoptotic cells and cell cycle distribution were determined by flow cytometric analysis. Exposure to Al at low levels of 100 and 200 μM for up to 6 days did not result in apoptosis of the astrocytes. This result is in agreement with our previous result [15]. A dramatic blockage of apoptotic cells was found at 200 μM Al. However, at 400 μM, Al markedly induced the apoptosis of astrocytes, which was associated with a significant change in cell cycle distribution characterized by increase of G2/M phase cells (128%). Measurements of the intracellular Ca^{2+} concentration using the fluorescent calcium indicator dye Fluo-3 demonstrated a significant increase in the levels of intracellular Ca^{2+} after Al treatment. However, no differences were observed among Al-treated groups. These findings suggest that Al induces and blocks selectively the apoptosis of astrocytes, which depends upon the concentrations of Al. Increased intracellular Ca^{2+} may not be the primary mechanism of Al-mediated apoptotic cell death [45].

4.12
Apoptosis Pathway Induced by Al in Brain Cells

Neurodegenerative diseases, including AD are characterized by a progressive and selective loss of neurons. Apoptosis under mitochondrial control has been implicated in this neuronal death process, involving the release of cytochrome c into the cytoplasm and initiation of the apoptosis cascade. However, a growing body of evidence suggests an active role for the endoplasmic reticulum in regulating apoptosis, either independent of mitochondrial, or in concert with mitochondrial-initiated pathways. Members of the Bcl-2 family of proteins have been shown to either inhibit apoptosis, as is the case with Bcl-2, or to promote it, as in the case of Bax. Intracisternal administration of Al-maltolate to New Zealand white rabbits, an animal system relevant to a study of human disease in that it reflects many of the histological and biochemical changes associated with AD, showed that Al-maltolate induces both cytochrome c translocation into brain cytosol and caspase-3 activation. Furthermore, these effects are accompanied by a decrease in Bcl-2 and an increase in Bax reactivity in the endoplasmic reticulum [46].

4.13
Apoptosis of Astrocytes by Al

When the concentration of 1 mM of Al was incubated with cultured astrocytes and neurons, Al accumulated both in neurons and astrocytes, and

Al caused strong changes in the morphology of astrocytes, including shrinkage of cell bodies and retraction of processes after 8–12 days exposure. Exposures over 15–18 days reduced the astrocytes' viability by 50%. The Al-induced degeneration of astrocytes involved the DNA fragmentation characteristic of apoptosis, and staining of Al-treated astrocytes with the DNA-binding fluorochrome Hoechst 33258 revealed the typical apoptotic condensation and fragmentation of chromatin. Al was also found to be neurotoxic, causing first (4–6 days) abnormal clustering and aggregation, and later (8–12 days) neuronal death. Interestingly, Al neurotoxicity occurred in neuroglial cultures containing approximately 10% astrocytes but not in near-pure neuronal cultures containing only 1% astrocytes. Staining of co-cultured cells with Hoechst 33258 showed apoptotic condensation and fragmentation of chromatin in Al-treated astrocytes but not in co-cultured neurons. This study demonstrates that Al can induce the apoptotic degeneration of astrocytes, and that this toxicity is critical in determining neuronal degeneration and death. Al-mediated apoptosis of cultured astrocytes may be also a valuable model system to study the mechanisms underlying apoptosis in glial cells [47].

4.14
Protection of Oligodendrocytes by Heat Shock Proteins (HSP) or Stress Proteins from Oxidative Stress

Heat shock proteins (HSP) or stress proteins serve as biomarkers to identify the contribution of stress situations underlying the pathogenesis of degenerative diseases of the CNS. Goldbaum et al. have analyzed by an immunoblot technique the constitutive and inducible occurrence of stress proteins in cultured rat brain oligodendrocytes subjected to heat shock or oxidative stress exerted by hydrogen peroxide, or a combination of both [48]. The data demonstrate that oligodendrocytes constitutively express HSP32, HSP60, and the cognate form of the HSP70 family of proteins, HSC70. After heat shock, HSP25, alpha B-crystallin, and HSP70 were up-regulated, while after oxidative stress the specific induction of HSP32 and alpha B-crystallin was observed. HSP32 represents heme oxygenase 1 (HO-1), a small stress protein with enzymatic activity involved in the oxidative degradation of heme which participates in iron metabolism [49, 50]. The presence of the iron chelators phenanthroline or desferroxiamine (DFO), which previously has been shown to protect oligodendrocytes from oxidative stress-induced onset of apoptosis, caused a marked stimulation of HSP32 without affecting HSP70. This indicates that DFO possibly exerts its protective role by directly influencing the antioxidant capacity of HO-1. In summary, HSP in oligodendrocytes are differentially stimulated by heat stress and oxidative stress. HO-1 has been linked to inflammatory processes and oxidative stress [51], its specific up-regulation after oxidative stress in oligodendrocytes suggests that it is an ideal candidate to investigate the involvement of oxidative stress in demyelinating diseases.

5 Conclusion

Mammalian cells take up iron (Fe) via three kinds of iron uptake systems: transferrin (Tf) receptor-mediated endocytosis, redox, and Tf-independent Fe uptake systems. As Al has similar chemical properties to Fe but is a simple trivalent cation incapable of redox changes, the cells can take up Al by Tf receptor-mediated endocytosis and Tf-independent iron uptake systems. Mammalian cells modulate the intracellular levels of various metals by interaction between iron regulatory proteins (IRP-1 and -2) and Fe-responsive elements in untranslated regions of the mRNAs of Fe metabolism related proteins. Al also directly binds a cellular iron sensor, IRP-1, to alter the function of its aconitase and mRNA binding proteins in the same way as Fe does. Al also antagonizes the Fe-induced decrease in IRE binding activity of IRP-2 and inhibits Fe-induced oxidation of IRP-2 *in vitro*. Therefore, Al stabilizes IRP-2 by interfering with the iron-catalyzed oxidation, which results in perturbation of iron metabolism. Al may involve selective targeting and removal of oxidatively modified proteins that may contribute to the turnover of many proteins degraded by the proteasome [27]. The IRP-1 is present at similar levels in both AD and control brains. In contrast, IRP-2 co-localizes with redox-active Fe in NFT, senile plaque neurites, and neuropil threads. Hence, alteration in IRP-2 might be directly linked to impaired iron homeostasis in AD [28]. These new effects of Al in brain cells may be concerned with a cause of Alzheimer's disease. Apoptosis pathways in brain cells are induced by Al and/or Fe to result in neuronal and/or glial cell death. On the other hand, heat shock and stress proteins are induced for cell protection. The apoptosis process by oxidative stress is inhibited by Fe chelator or suppressed by some of stress proteins (e.g., heme oxygenase-1) concerned with Fe metabolism. Therefore, it is considered to be important to clarify at, the level of molecular biology, the relations between apoptosis process, oxidative stress, and Fe metabolism in neuronal and glial cells. The more information we have on Fe metabolism, the more will we be able to deeply understand a new effect of Al in mammalian cells.

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