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Expression and localization of lactotransferrin mRNA in the cortex of Alzheimer's disease

Li An[a,b], **Haruhisha Sato**[c], **Yoshihiro Konishi**[d], **Douglas G. Walker**[e], **Thomas G. Beach**[e], **Joseph Rogers**[e], and **Ikuo Tooyama**[a,*]

a *Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu, 520-2192, Japan*

b *School of Public Health, China Medical University, Shenyang, 110001, China*

c *Department of Pathology, Marutamachi Hospital, Kyoto, 604-8045, Japan*

d *Department of Clinical Research, Nishi-tottori National Hospital, Tottori, 689-0202, Japan*

e *Sun Health Research Institute, Sun City, Arizona, 85372, USA*

Abstract

We and others have previously reported that lactotransferrin (LF), acting both as an iron-binding protein and inflammatory modulator, is greatly up-regulated in the brain of patients with Alzheimer's disease (AD). However, it remains unknown which type of cells express LF in the brain of AD. In this study, therefore, we investigated the expression and localization of LF messenger RNA (mRNA) in the cerebral cortex of AD and control cases using real-time polymerase chain reaction (PCR) and *in situ* hybridization histochemistry. Real-time PCR demonstrated that LF mRNA expression in the cortex of AD cases was significantly greater than that in control cases. LF mRNA-positive granules were observed in the cortex by *in situ* hybridization histochemistry, and the number of positive granules was increased in AD cases compared to controls. The double staining technique of LF mRNA *in situ* hybridization and D-related human leukocyte antigen (HLA-DR) immunohistochemistry revealed that positive granules were localized in a subpopulation of HLA-DR-positive reactive microglia. In addition, LF mRNA-positive granules were observed in some cells that were negative for HLA-DR. These cells were also negative for CD4 and CD8 but positive for leukocyte common antigen (CD45RB), suggesting they were monocytes/macrophages. These results indicate that reactive microglia in the cerebral cortex and monocytes/macrophages infiltrating from the circulation might be responsible for synthesizing LF in AD brain.

Keywords

Alzheimer's disease; Iron; Lactotransferrin; Microglia; Oxidative stress

Lactotransferrin (lactoferrin; LF) is an 80 kDa iron-binding glycoprotein considered to have a role in the binding, transport and storage of iron [1]. LF was first isolated from milk and is found in various secretions, such as tears, saliva, nasal secretions and intestinal mucus, and in plasma [7,11,20]. It is also stored in specific granules of neutrophilic leukocytes and released during the inflammatory process [24]. LF can bind iron tightly and in this way acts as a powerful

*Corresponding author: Tel.: +81-77-548-2328; fax: +81-77-548-2402, E-mail address: kinchan@belle.shiga-med.ac.jp (I. Tooyama).

iron chelator with antioxidant properties [5,6,33]. In addition, LF may serve as a regulator of the inflammatory-immune response [3,28], and reactive oxygen metabolism [15,19].

In the brain, excessive iron deposits occur in normal aging and in various neurodegenerative diseases [8,32,34]. LF is also associated with aging and particularly with neurodegenerative disorders including Alzheimer's disease (AD) [16,21,22,31]. In particular, LF has been detected in senile plaques and neurofibrillary tangles [16,22]. However, little information is available about LF messenger RNA (mRNA) in AD brain. In this study, therefore, we investigated the level of LF mRNA in the cortex of neurologically normal controls and AD patients using real-time polymerase chain reaction (PCR) and demonstrated its expression by *in situ* hybridization. In addition, we used *in situ* hybridization and immunohistochemistry to further examine the localization of LF mRNA.

Human brain tissues were obtained from the Brain Donation Program at the Sun Health Research Institute [4]. The Brain Donation Program has been approved by the Institutional Review Board of the Sun Health Corporation. Total RNA was purified from the temporal cortex of six sporadic AD cases (mean age ± S.D., 86.2 ± 6.6 years) and five control cases without neurological disease (mean age ± S.D., 80.0 ± 6.3 years). The average postmortem delay for the AD and control cases was 2.23 and 2.48 h, respectively.

LF mRNA was detected by real-time PCR using a LightCycler System (Roche Applied Science, Mannheim, Germany). The sense primer was 5′–AGGCCACAAAATGCTTCCAATGGCAAA–3′, and the antisense primer was 5′–GGCTGTCTTTCGGTCCCGTAGACTTCC–3′. Real-time PCR analysis for β-actin mRNA was also employed to assess the variability of mRNA content. In order to correct for the variability of mRNA, the amount of LF mRNA from each sample was divided by the amount of β-actin mRNA. The relative value was calculated by the following formula: (LF mRNA)/(β-actin mRNA) × 10^5. A one-sample Kolmogorov-Smirnov test showed that the distribution of data was exponential. Thus, the original data were converted to logarithmic form (Log 2) to show a Gaussian distribution. The difference in LF mRNA between two groups was analyzed by Student *t* test for independent samples. Two-tailed *p* values of less than 0.05 were considered statistically significant.

The location of expression of LF mRNA was examined by *in situ* hybridization using samples of temporal cortex from three AD cases and three control cases obtained from the Brain Donation Program at the Sun Health Research Institute [4]. Digoxigenin-labeled riboprobes were used. Frozen, fixed sections were mounted on RNase-free silane-coated glass slides (Dako Japan Co. Ltd., Tokyo, Japan) and air-dried. The sections were treated for 10 min at room temperature with 10 μg/ml proteinase K in 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. For pre-hybridization, the sections were reacted for 1 h at 37° C in hybridization buffer containing 50% formamide, 5 × Denhardt's solution, 0.5 × saline/sodium citrate (SSC; 1 × SSC = 150 mM NaCl and 15 mM sodium citrate), 0.5 mg/ml yeast tRNA (Invitrogen) and 0.5 mg/ml heat-denatured salmon sperm DNA (Wako Pure Chemicals, Co., Osaka, Japan). The probes were diluted in hybridization buffer to a final concentration of 3 μg/ml, and hybridization was performed overnight at 60° C.

After hybridization, the sections were washed for 2 h in pre-warmed 0.1 × SSC buffer at 60° C, followed by a 5 min rinse in 0.1 M Tris HCl (pH 7.5) containing 150 mM NaCl (NT buffer) at room temperature. Subsequently, the sections were treated for 60 min with 1% skim milk in NT buffer to block nonspecific protein binding, and then reacted overnight at 4° C with alkaline phosphatase-labeled anti-digoxigenin antibody (1:200 dilution; Roche Diagnostics, Mannheim, Germany) in NT buffer containing 1% skim milk. After washing with NT buffer, positive signals were detected by incubating in 0.1 M Tris HCl buffer (pH 9.5) containing 100

mM NaCl, 50 mM $MgCl_2$, 500 μg/ml nitroblue tetrazolium chloride and 187 μg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt.

After *in situ* hybridization, some sections from both AD and control cases were double-stained by immunohistochemistry. The sections were incubated with 0.5% hydrogen peroxide in 0.1 M PBS (pH 7.4) containing 0.3% triton-X100 (PBST) for 30 min at room temperature to eliminate endogenous peroxidase. After washing with PBST, the sections were incubated for 30 min with PBST containing 1% bovine serum albumin to block nonspecific protein binding. Then, sections were incubated overnight at 4° C with mouse monoclonal antibody directed against D-related human leukocyte antigen (HLA-DR, clone TAL.1B5; 1:100 dilution; Dako, Glostrup, Denmark), CD4 (Nichirei Co., Tokyo, Japan), CD8 leukocyte common antigen (LCA, clone PD7/26 and 2B11; 1:100 dilution; Dako). After washing with PBST, the sections were incubated for 1 h with biotinylated anti-mouse IgG (1:1000 dilution; Vector Laboratories, Burlingame, CA) and for 1 h with avidin-biotinylated peroxidase complex (1:2000 dilution; Vector Laboratories), both at room temperature. Sections were then incubated with 0.02% 3,3′-diaminobenzidine in 50 mM Tris–HCl buffer (pH 7.6) to precipitate brown chromogen.

As shown in Figure 1, LF mRNA expression, determined by real-time PCR, in the normal human cortex was very low, but expression in the cortex of AD patients was significantly increased compared to that of control cases (AD/Control = 5.50, $p = 0.02$).

In the cerebral cortex, granular signals of LF mRNA were detected in both control (Fig. 2A and B) and AD (Fig. 2D and E) by *in situ* hybridization using the antisense probe. The number of LF-positive granules was increased in AD cases compared to controls (Fig. 2). The sense probe did not show any signal (Fig. 2C and F).

Using the double staining technique of LF mRNA *in situ* hybridization and HLA-DR immunohistochemistry, LF mRNA-positive granules were localized in cell bodies of a subpopulation of HLA-DR-positive reactive microglia in both AD (black arrows in Fig. 3A and B) and control cases (data not shown). Approximately 4 % and 8% of HLA-DR-positive microglia showed LF-positive staining, in control and AD cases, respectively. In AD cases, some LF mRNA-positive granules were negative for HLA-DR (red arrows in Fig. 3B and C), and these granules were often seen in the vicinity of microvessels (red arrow in Fig. 3C). These cells were negative for CD4 (Fig. 4A) and CD8 (Fig. 4B) but positive for LCA (Fig. 4C), suggesting they were monocytes/macrophages.

In the present study, using real-time PCR, we found LF mRNA expression was up-regulated in the cortex of AD patients. These results are in agreement with previous immunohistochemical studies [16,22] showing up-regulation of LF at the protein level. We also demonstrated that LF mRNA was detected in granular structures in the cortex of both control and AD cases using *in situ* hybridization. Histochemical double staining for LF mRNA and HLA-DR confirmed that LF mRNA was expressed by a subpopulation of HLA-DR-positive microglia. These results are supported by a previous report showing that LF is produced by activated microglia in the substantia nigra in Parkinson's disease [14]. In neurodegenerative disorders such as AD, a subtle and chronic inflammation reaction takes place leading to a marked activation of glia indicated by HLA-DR-positive staining [25,26, 27].

We also observed LF mRNA in some cells that were negative for HLA-DR and which were often observed in the vicinity of microvessels. Since T cells and monocytes/macrophages are known to infiltrate the brain parenchyma in AD, we employed double staining techniques for the T cell markers, CD4 and CD8, as well as LCA (CD45RB). These cells were negative for the T cell markers, CD4 and CD8, but positive for LCA (CD45RB), suggesting they were monocytes or macrophages. Thus, LF protein is synthesized in the brain not only by reactive

microglia, but also by infiltrating monocytes/macrophages. However, it cannot be ruled out that some LF protein is transported from the blood into the brain [12,13].

The precise role of LF in AD still remains unknown, however this and previous studies raise several possible explanations. LF markedly inhibits classical C3 convertase [17,18] and thus suppresses the complement classical pathway that occurs in the brain of AD [10]. LF also suppresses the production of inflammatory cytokines such as interleukin 1 and tumor necrosis factor [9,23]. Therefore, it is possible that the up-regulation of LF in areas affected by AD may be a defensive response of the AD brain. Another possibility is that LF may have a role in anti-oxidative stress [5,6,33], because oxidative stress is involved in the pathology of AD [29, see 35 for review] and increased levels of oxidative damage occur prior to the onset of amyloid beta deposition [30]. Redox-active iron, a source of redox-generated free radicals which contribute to the oxidative damage, is associated with the senile plaques and neurofibrillary tangles in AD [32,34]. It has been reported that transferrin and ferritin are responsible for iron regulation and play an important role in the disruption of brain iron homeostasis in AD [8]. Like transferrin, LF consists of two lobes, each possessing an iron-binding site capable of reversibly binding one ferric ion and LF binds iron more tightly than transferring [2]. Therefore, LF acts as a powerful iron chelator with antioxidant properties [2,5,6,33]. Both inflammatory processes and oxidative stress contribute to an increased production of LF by microglia, or to an increased number of activated cells leading to enhanced LF production which might explain its accumulation. So it is likely that LF may prevent cell injury and tissue damage, and therefore protect brain integrity, through both its anti-inflammatory and antioxidant functions.

In conclusion, this present study has shown that the expression of LF mRNA is up-regulated in the cortex of AD patients. We have also demonstrated that LF mRNA is expressed by activated microglial cells or leukocytes that have infiltrated the cortex of the AD brain.

Acknowledgements

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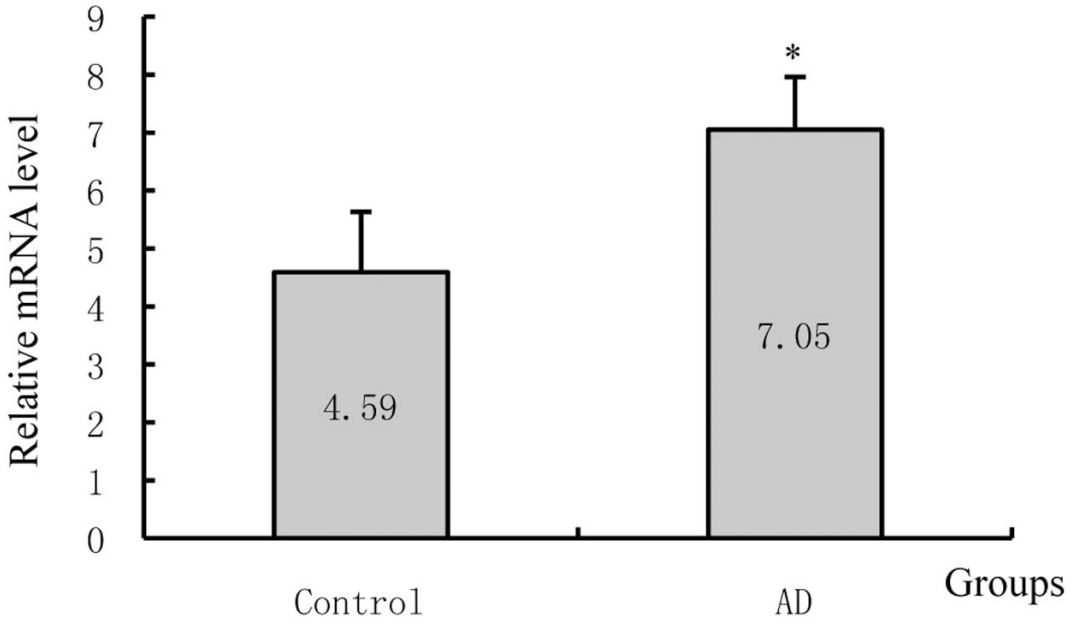

Figure 1.
Lactotransferrin (LF) mRNA expression levels in the cerebral cortex of control and Alzheimer's disease (AD) patients. Asterisk indicates significantly different to control ($p < 0.05$).

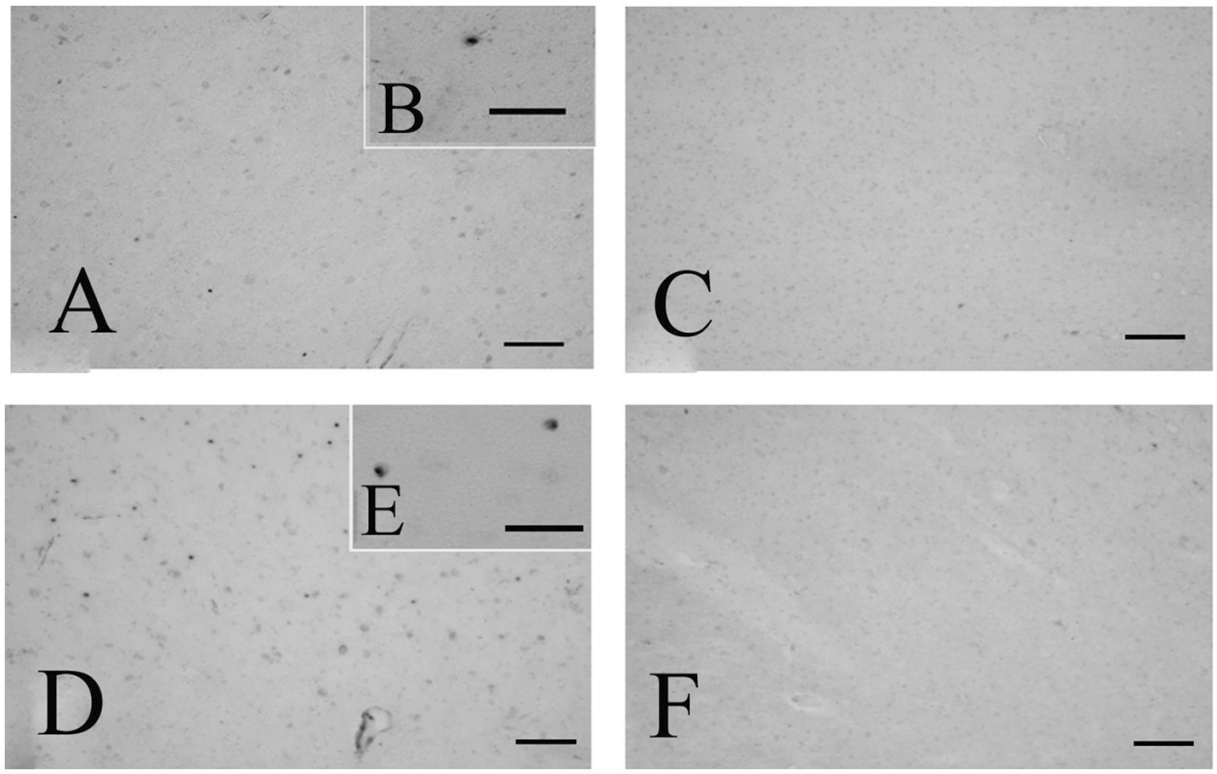

Figure 2.

In situ hybridization histochemistry of the cerebral cortex of control (A, B, C) and Alzheimer's disease (AD) cases (D, E, F) using antisense (A, B, D, E) and sense (C, F) probes. Granules imply the localization of lactotransferrin mRNA. The number of positive granules is increased in AD cases (D) compared to controls (A). Using sense probes (C and F) as the control for the antisense probe, no granule of blue color is detected in the cortex. Bars = 100 μm in A, C, D, F, and 50 μm in B, E.

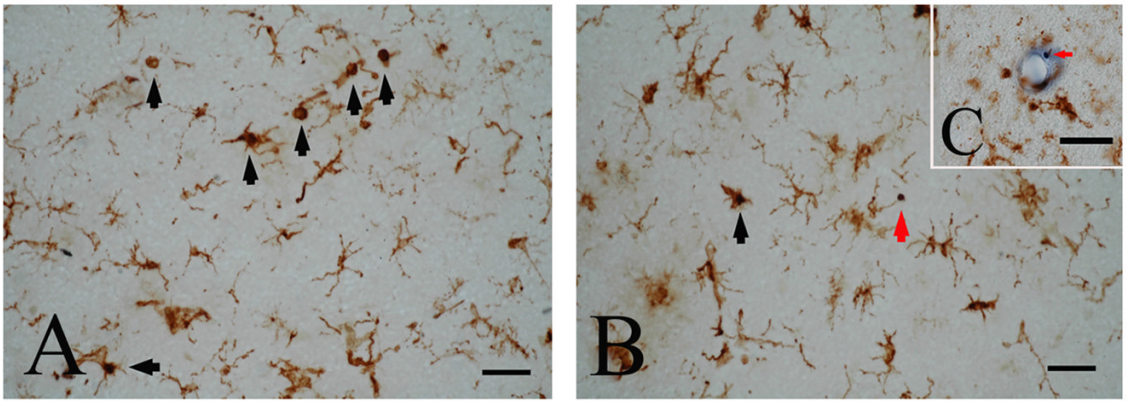

Figure 3.
Double staining of lactotransferrin (LF) mRNA *in situ* hybridization and D-related human leukocyte antigen (HLA-DR) immunohistochemistry in the cerebral cortex of an Alzheimer's disease case. LF mRNA is expressed in a subpopulation of HLA-DR-positive reactive microglia (black arrows in A and B). Some LF mRNA-positive cells which are negative for HLA-DR are seen in the parenchyma of the cerebral cortex (red arrow in B) and in the vicinity of microvessels (red arrow in C). Bars = 50 μm.

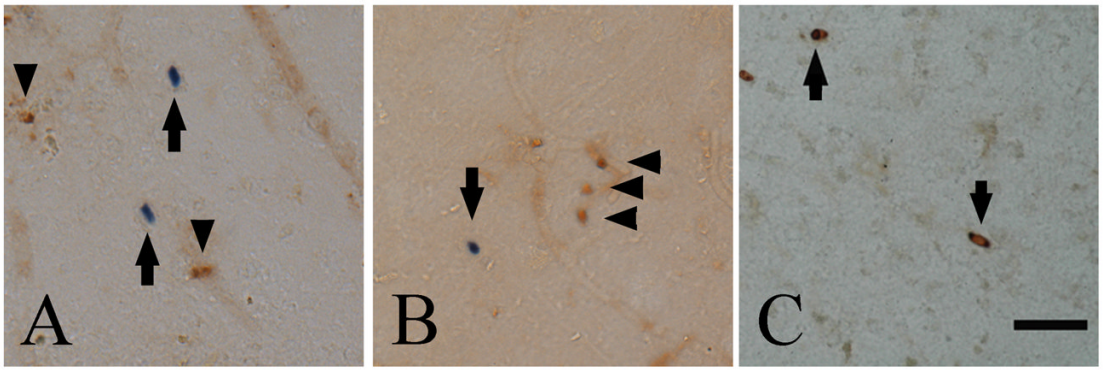

Figure 4.

Double staining of lactotransferrin (LF) mRNA *in situ* hybridization and CD4 (A), CD8 (B) or leukocyte common antigen (LCA) immunohistochemistry (C) in the cerebral cortex of an Alzheimer's disease case. LF-positive signals (arrows in A and B) were negative for CD4 (arrowheads in A) and CD8 (arrowheads in B). Granular signals for LF mRNA are seen in the cytoplasm of LCA-positive cells (arrows in C). Bar = 50 μm.