Deposition of lactoferrin in fibrillar-type senile plaques in the brains of transgenic mouse models of Alzheimer's disease

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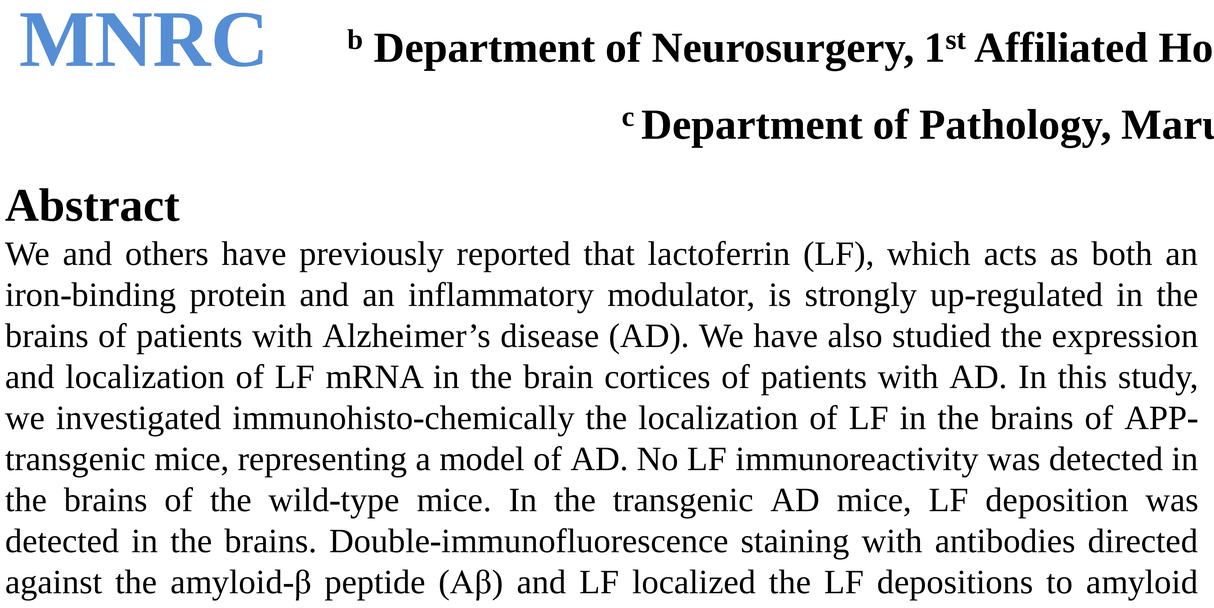
Abstract

We and others have previously reported that lactoferrin (LF), which acts as both an iron-binding protein and an inflammatory modulator, is strongly up-regulated in the brains of patients with Alzheimer's disease (AD). We have also studied the expression and localization of LF mRNA in the brain cortices of patients with AD. In this study, we investigated immunohisto-chemically the localization of LF in the brains of APP-transgenic mice, representing a model of AD. No LF immunoreactivity was detected in the brains of the wild-type mice. In the transgenic AD mice, LF deposition was detected in the brains. Double-immunofluorescence staining with antibodies directed against the amyloid-β peptide (Aβ) and LF localized the LF depositions to amyloid deposits (senile plaques) and regions of amyloid angiopathy. Senile plaque formation precedes LF deposition in AD. In the transgenic mice aged <18 months, most of senile plaques were negative for LF. LF deposits appeared weakly at about 18 months of age in these mice. Both the intensity and number of LF-positive depositions in the transgenic mice increased with age. Double-staining for LF and thioflavin-S revealed that LF accumulated in thioflavin-S-positive, fibrillar-type senile plaques. The up-regulation of LF in the brains of both AD patients and the transgenic mouse model of AD provides evidence of an important role for LF in AD-affected brain tissues.



Materials and Methods

Transgenic mice

APP-transgenic mice (mouse strain: C57B6/SJL; ID no. Tg2576, Taconic Inc., Germantown, NY) expressing human APP695 containing a double mutation (K670N, M671L) and wild-type mice. These mice develop both amyloid deposits (starting at 12 months of age) and memory deficits. Twelve transgenic mice of different ages (<18 months, n=3; 18-20 months, n=3; >20 months, n=6) were used in this study. Six wild-type mice were also used (13-22 months).

Immunohistochemistry

For simultaneous visualization of LF and Aß, a double-immunofluorescence staining method was used. The sections were incubated with a goat polyclonal anti-LF antibody (1:200 dilution; N-20, sc-14431; Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) and a rabbit polyclonal anti-Aß antibody (1:1000 dilution; Immuno-Biological Laboratories) overnight at 4°C. The sections were then incubated for 2 h at room temperature with a mixture of Alexa 488-conjugated anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR) and Alexa 594-conjugated anti-goat IgG (1:500; Molecular Probes).

Quantification of LF-positive SPs

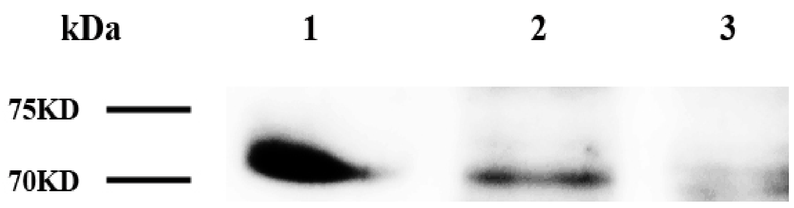
Three cortical sections (20-µm in thickness) from each mouse were visualized separately under the fluorescence microscope. The sections were divided into a square of area 3,384,039 µm^2, and the photographic data were acquired using a CCD camera and saved on a computer. LF-positive SPs in the whole cortical region of a section were counted, and the data were converted into the number of labeled plaques per square. All data shown are mean ± SEM. Statistical significance was assessed using the one-way ANOVA test. Significance was set at $P<0.05$.

Western Blot

The brains of transgenic and wild-type mice of the same age were homogenized in five volumes of lysis buffer, which consisted of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 µg/ml of pepstatin and proteinase complex (Complete Min; Roche Diagnostics, Mannheim, Germany). The homogenates were centrifuged at 20,000 g for 20 min at 4ºC, and the supernatants were collected as a crude cytosolic protein fraction. Protein samples (100 µg) and human LF (5 ng) were subjected to SDS-PAGE (10%-20% gradient gels; Wako Pure Chemicals Co. Ltd., Osaka, Japan) under non-reducing conditions, and then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Nippon Millipore Ltd., Tokyo, Japan). Molecular weight markers were used in the gels (Precision Plus; Nippon BioRad Laboratories, Tokyo, Japan). Non-specific protein binding to the membrane was blocked by a 30-min incubation at room temperature in 5% bovine serum albumin in 25 mM Tris-buffered saline (TBS; pH 7.4). This was followed by overnight incubation at 4ºC with a goat polyclonal antibody directed against LF, which was diluted 1:200 in 25 mM TBS that contained 0.1% Tween-20 (TBST). The blots were washed three times for 10 min each with 25 mM TBST, and then incubated for 1 h at room temperature with horseradish peroxidase-labeled anti-goat IgG (ImmunoPure, 1:20000; Pierce, Rockford, IL, USA). After extensive washing with 25 mM TBST, the blots were visualized by the chemiluminescence method with the ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

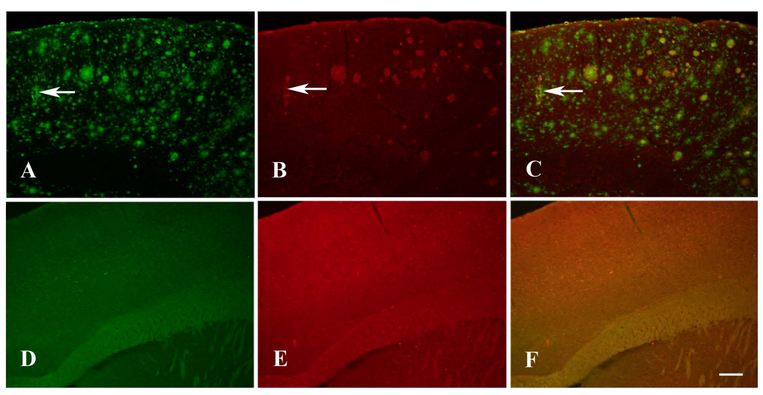
Results

1. Western blot analysis of the brain homogenates



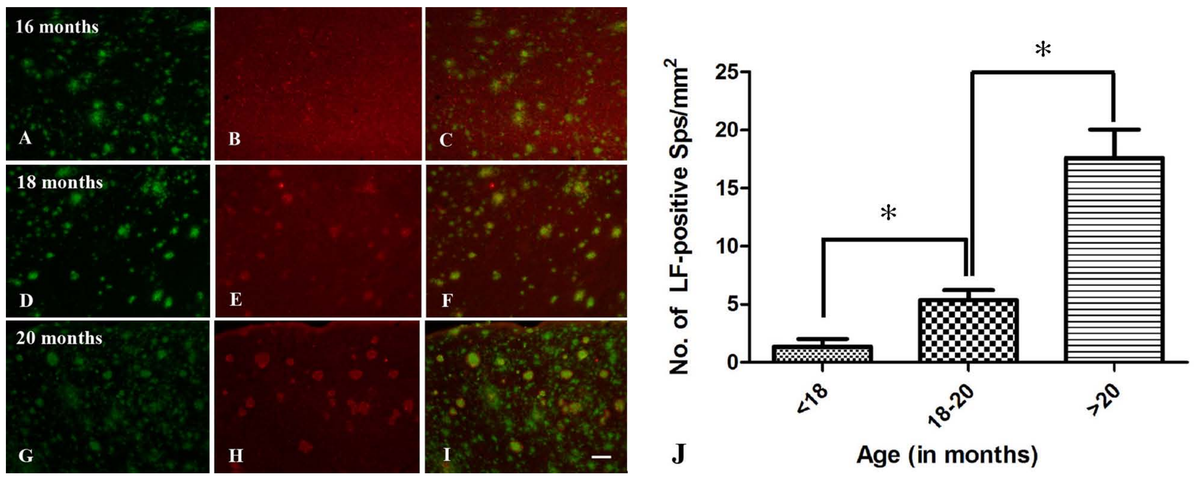
Western blot analysis of LF (lane 1), a lysate of the brain of a transgenic mouse (lane 2), and a lysate of the brain of a wild-type mouse brain (lane 3). An immunoreactive band of molecular mass of approximately 70 kD is present in all the lanes.

2. Double-immunofluorescence staining for Aß and LF



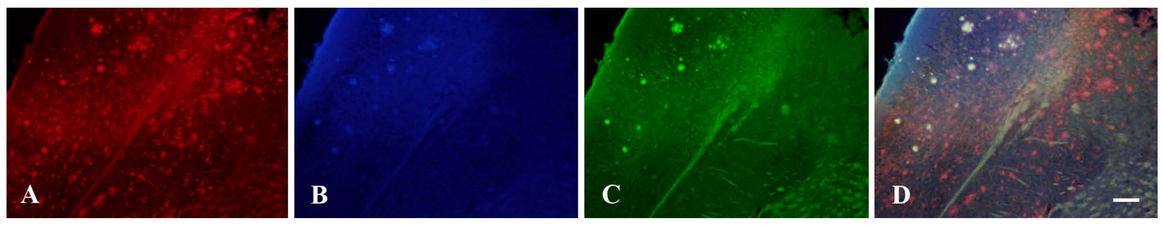
Double-immunofluorescence staining for Aß (A, D) and LF (B, E), and merged images (C, F) in mouse brain cortex. A-C, LF deposits are apparent in areas of the Aß-positive senile plaques that also show amyloid angiopathy (arrows in A-C). D-F, No positive staining is seen in the controls. Scale bar, 200 µm.

3. Differential expression of LF according to mouse age



The number and intensity of LF-positive senile plaques increase gradually with age (A-I). A, D and G for Aß; B, E and H for LF; as well as C, F and I for merged images. Quantitative analysis of LF-positive senile plaques detected in the cortex. Data shown represent the mean ± SEM. *, P<0.05 (J). Scale bar, 100 µm.

4. Staining with the anti-Aß, anti-LF antibodies and thioflavin-S



LF deposited in thioflavin-S-positive plaques (a fibrillar plaque type). Scale bar, 200 µm.

Conclusion

The fibrillar-type SPs in transgenic mouse models of AD are associated with massive inflammatory responses, including the influx into the plaques of inflammatory proteins, such as cyclo-oxygenase-2, complement components, and α2-macroglobulin. In addition, activated microglia and astrocytes accumulate in the fibrillar-type SPs. As stated above, LF may play a functional role in the inflammatory process, since it markedly inhibits the classical C3 convertase, thereby suppressing the complement activation pathway active in the brains of AD patients. LF also suppresses the production of pro-inflammatory cytokines, such as interleukin-1 and tumor necrosis factor. Taken together, these results suggest that LF prevents cell injury and tissue damage, and protects the integrity of the brain through a combination of anti-inflammatory and antioxidant functions. In the present study, we show that both the number and intensity of LF-positive SPs increased gradually with age.