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MIDKINE, A NOVEL NEUROTROPHIC FACTOR, IS PRESENT IN SENILE PLAQUES OF ALZHEIMER DISEASE

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Summary---An affinity purified antibody specific for midkine (MK) stained senile plaques in the brain of Alzheimer disease (AD) patients. After formic acid treatment, plaque staining was dramatically enhanced, and almost all ß-amyloid protein (BAP) deposits were also immunoreactive for MK. MK-immunoreactivity was not observed in normal cellular components nor in other pathological lesions including tangles in AD brain. Control brain sections were not immunoreactive for MK. The presence of MK in AD brain but not in control brain was confirmed by Western blotting. MK appears to be involved in the pathological process leading to senile plaque formation.

Alzheimer disease (AD) is a neurodegenerative disease in which neuronal loss is accompanied by two major pathological hallmarks; senile plaques and neurofibrillary tangles. Recent investigations have suggested that the neuronal degeneration in AD is also associated with attempted regeneration. Evidence in support of this view is that molecules with neurite outgrowth promoting activity are associated with the pathological lesions in AD. Basic fibroblast growth factor (bFGF), also known as a heparin-binding growth factor, is involved in AD pathology (1,2), as are heparan sulfate proteoglycans (HSPG) (3,4), which play a role in neurite extension (5).

A novel family of heparin-binding growth factors, which is distinct from the FGF family, has recently been identified (6-12). This family consists of two members, midkine (MK) (6-8) and heparin-binding growth-associated molecule (HB-GAM)/pleiotrophin (PTN) (9-12). MK and HB-GAM/PTN are cysteine-rich, basic proteins, which share 50% sequence identity with each other. They promote neurite outgrowth of embryonic brain cells (8,9,11). Furthermore, MK supports survival of dorsal root ganglion and spinal cord

Abbreviations: MK, midkine; BAP, β-amyloid protein; AD, Alzheimer disease; bFGF, basic fibroblast growth factor; HSPG, heparan sulfate proteoglycans; HB-GAM, heparin-binding growth-associated molecule; PTN, pleiotrophin; RA, retinoic acid.

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neurons from mouse embryos (Michikawa, M., et al., submitted for publication). Based on this evidence, we investigated whether MK is expressed in AD brain.

Materials and Methods

MK and anti-MK antibody---MK was purified from culture medium of cells transfected with MK cDNA (8). Anti-MK antiserum was raised in a rabbit by immunizing 4 times with 50 μg of MK together with 5 mg/ml heparin. Anti-MK antibody was obtained by absorbing the antibody to MK-glutathione S-transferase fusion protein (13) coupled to CNBr-activated Sepharose (3.65 mg/ml), and eluting it with 0.1M glycine-HCl buffer (pH 2.7). The affinity purified antibody reacted with MK but not with HB-GAM/PTN or with basic FGF (Muramatsu, H., et al., submitted for publication).
Immunohistochemistry---Brains from 8 cases with AD (aged 65-89 years) and 3 cases without neurological diseases (aged 63-75 years) were obtained within 2-21 h. after death. Small blocks of brain tissue were fixed for 2-3 days in 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) in the cold and then transferred to a maintenance solution of 15% sucrose in PB. Sections were cut on a freezing microtome at 30 μm thickness, collected in the maintenance solution, and stored until stained.

Sections were incubated for 72 h with primary antibody (0.7 μg/ml) in 0.01M phosphate buffered saline containing 0.3% Triton X-100 (PBS-T, pH 7.4) in the cold, and then treated with the Vectastain ABC second antibody system (Vector Lab. Burlingame, CA). A purple color was developed by incubating in a staining mixture containing 0.01% 3,3'-diaminobenzidine (Sigma), 0.6% nickel ammonium sulfate, 0.05M imidazole and 0.00015% H_2O_2 in 0.05M Tris-HCl buffer (pH 7.6). Washing of sections between incubation steps was done with PBS-T. Sections were mounted on glass slides, dried, dehydrated and coverslipped with Entellan (Merk). Some sections were pretreated for 5 min with 100% formic acid prior to immunohistochemical procedures. Adjacent sections were stained with anti-ß amyloid protein (BAP) antibody (R17, rabbit polyclonal, generously provided by Dr. T. Ishii (14), diluted 1:10,000).
Heparin-Sepharose affinity immunoabsorption---The antibody (3 ml, 0.7 μg/ml in PBS-T) preincubated with or without 0.5 μg/ml MK was applied to a column of heparin-Sepharose (0.5 ml, Sigma) equilibrated with PBS. The unabsorbed fractions were used for immunohistochemistry controls.
Western blotting--- Crude extracts from both control and AD brains were obtained by homogenizing tissue samples in 5 vol. of 50mM Tris-HCl (pH 7.4), 1mM EDTA (pH 8.0) and 2mM phenlmethylsulfonyl fluoride. In additional experiments, 1.5 g tissue samples were homogenized in 5 volumes of 6M guanidine-HCl, 0.1M Tris-HCl, 24mM dithiothreitol, 0.34mM EDTA, pH 8.0 and stirred at room temperature. After 24 h, each solution was centrifuged at 15,000 x g for 1 h at 4°C. The resultant supernatant was dialyzed against 1 L of PBS for 3 days. The dialyzed solution was centrifuged for 30 min at 15,000 x g at 4°C. The protein in the supernatant was precipitated with 10% trichloroacetic acid and then with 85% ethanol. The pellet was dissolved in SDS buffer and boiled for 5 min, prior to applying to a 13% polyacrylamide gel.

Samples, along with 100 ng of purified MK, were subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli (15). Proteins in the gels were transferred to nitrocellulose membranes according to the method of Towbin et al. (16). After incubation in 5% skim milk in PBS overnight at 4°C, the nitrocellulose sheets were incubated with the antibody (4 μg/ml) for 2 h at room temperature. After washing with PBS containing 0.1% Tween 20, the membranes were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (Jackson Immuno-research Laboratories) and stained with 4-chloro-1-naphthol.

Results and Discussion

Anti-MKantibody labeled senile plaques in all AD brains in the absence of formic acid pretreatment. MK immunoreactivity was observed in the cerebral cortex with some

preference for the superficial cortical layers, indicating some staining of diffuse amyloid deposits (Fig. 1A). In consolidated plaques, the staining was more intense in the core than in the periphery (Fig. 1D). Some diffuse amyloid deposits in the molecular layer of the cerebellar cortex also showed immunoreactivity (Fig. 1E). When sections were pretreated with formic acid, the staining of plaques was dramatically enhanced (Fig. 1B). With this pretreatment, almost all BAP deposits were also positive for MK (Fig. 1B, 1C). MK immunoreactivity was still not detectable either in normal cellular components or in other AD pathological lesions. Control brain sections failed to show specific MK immunostaining even after formic acid pretreatment.

Although the anti-MK antibody used in this study was affinity-purified, further control staining was done using anti-MK antibody preincubated with MK. Unexpectedly, the MK anti-MK complex stained not only plaques but also neurofibrillary tangles and neuropil threads (Fig. 2A). The same result was obtained when sections preincubated with 0.5 μg/ml of MK were subjected to MK immunohistochemistry. Thus, it is concluded that both plaques and tangles carry binding sites for MK.

To ascertain the specificity of the immunostaining, the MK anti-MK complex was applied to a heparin-Sepharose column to remove MK with the attached antibody. The pass through fraction from which the antibody had been removed failed to stain plaques. In contrast, when the MK antibody by itself was applied to the column, the pass through fraction stained tissues in the same fashion as when the antibody had not been exposed to the column. This indicates that the antibody was unaffected by the column. Therefore, the enhanced staining observed following incubation with the antigen-antibody complex must have been due to MK binding to specific sites in the tissue. In addition, plaque staining with the antibody was established to be specific for MK, because absorption of the antibody by antigen, which was trapped by the column, abolished the staining.

Figure 2B shows the result of immunoblotting with anti-MK antibody. When proteins were extracted without guanidine-HCl, the antibody did not detect any specific bands in extracts from either control or AD brain tissues. When a guanidine extract of AD brain was examined, a single band with the same mobility as MK was revealed by the anti-MK antibody. The molecular weight of MK was estimated to be 14 kDa after calibrating the effect of basicity using histones as standards, as has previously been described (8). In contrast, no band was detected in guanidine extracts of control brain. The result thus confirms that the immunoreactive substance detected in tissue sections of AD brain is MK.

The present observations have a number of important implications on the relationship between MK and amyloid deposits. First, enhancement of plaque staining of MK by formic acid pretreatment leads to the assumption that MK exists in a close relationship with amyloid deposits. Formic acid has been widely used to solubilize amyloid proteins and therefore may help to expose epitopes of proteins buried in the amyloid deposits (17). Second, the labeling of diffuse amyloid deposits with the anti-MK antibody suggests that association of MK is an early event in extracellular amyloid formation, since such deposits are believed to represent the earliest stage of plaque formation (18-20). Finally, the binding of MK, presumably to HSPG, may be closely related to plaque formation, as is the case with bFGF (21,22). HSPG has recently been shown to be a component related to early plaque biogenesis (3,4).

MK is the product of a retinoic acid (RA) responsive gene, and is intensely expressed during the midgestation period of mouse embryogenesis (23). From the mode of its

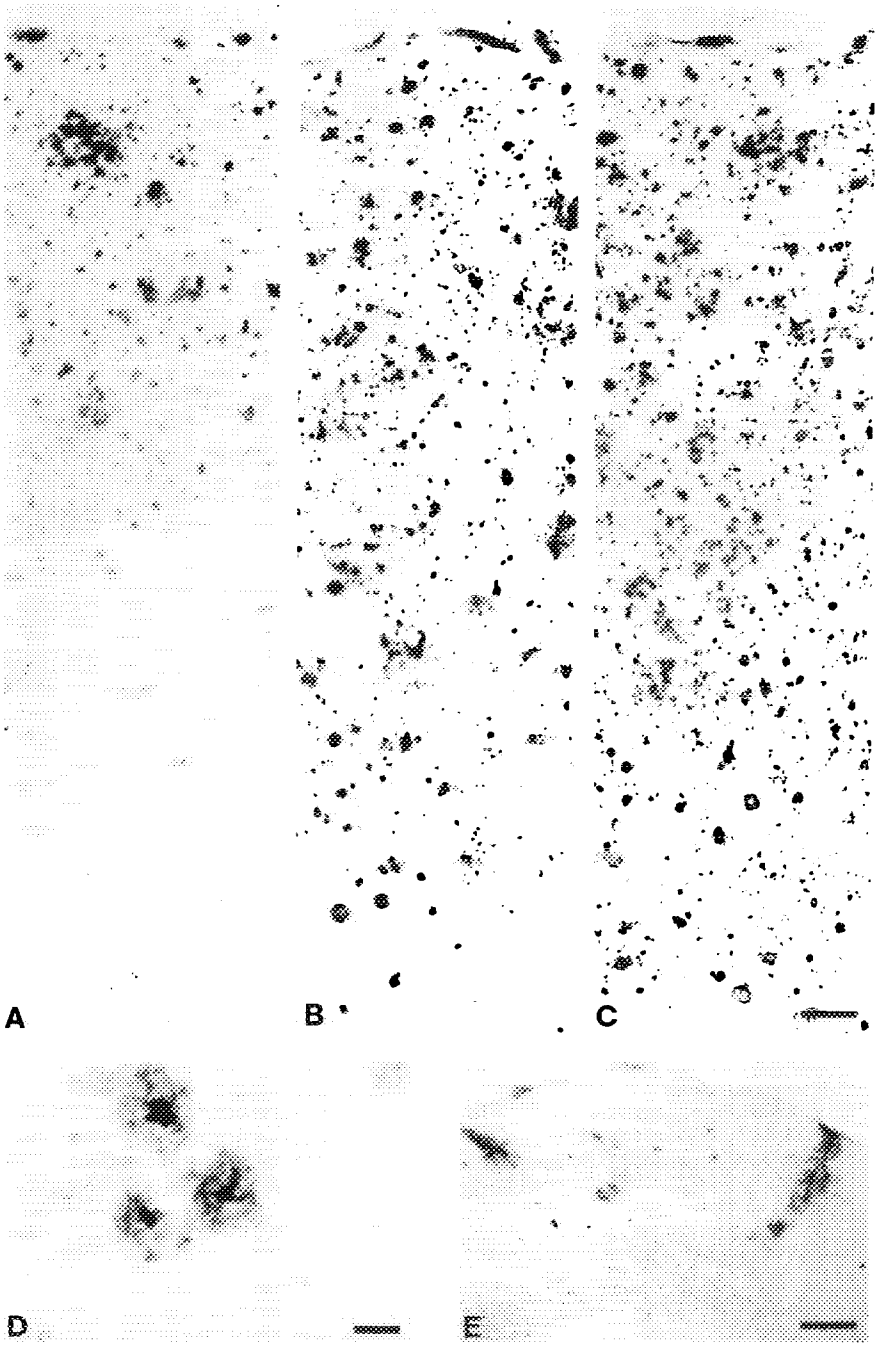

Fig. 1. Immunostaining of MK (A, B, D, E) and BAP (C) in AD brain. Sections were stained without (A, C, D, E) or with formic acid pretreatment (B). **A, B, C :** Adjacent sections of the middle frontal cortex stained for MK (A, B) and for BAP (C). Without formic acid pretreatment, MK-positive plaques are located preferentially in the superficial cortical layers (A). After formic acid pretreatment, MK-immunoreactivity is dramatically enhanced (B). Note that MK-immunoreactivity is detectable in all forms of BAP deposits (Compare B with C). **D :** Higher magnification of MK-positive plaques, showing intense reaction in the core of plaques. **E :** Diffuse amyloid deposits in the cerebellar cortex positive for MK.
Bars = 100 μm.

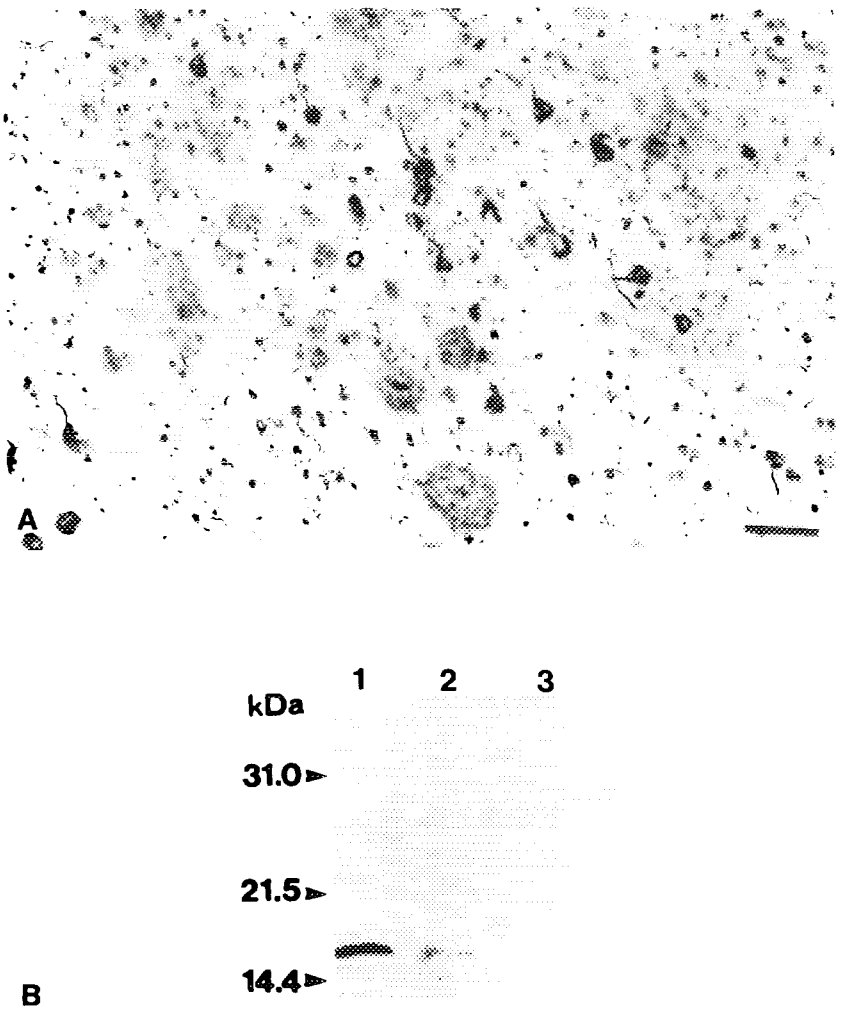

Fig. 2. **A :** Immunostaining with the MK anti-MK complex in the angular cortex of an AD patient, showing that plaques as well as neurofibrillary tangles are intensely labeled with the complex. Neuropil threads are also stained. Bar = 50 μm.
B : Identification of MK in AD brain by Western blotting. Samples of MK (lane 1 : 0.15 μg), guanidine extracts (40 μl, each) from the middle frontal cortex of AD (lane 2) and control brain (lane 3) were run on a 13% polyacrylamide gel under reducing conditions. After transfer to a nitrocellulose sheet, the protein was stained by anti-MK antibody. The molecular weight marker proteins indicated in the left side are : carbonic anhydrase (31 kDa) ; soybean trypsin inhibitor (21.5 kDa) ; and lysozyme (14.4 kDa). A single band with the same mobility as MK is detected in guanidine extract of AD brain, but not in that of control brain. It should be noted that MK migrates slowly due to its basic charge.

developmentally regulated expression, MK is thought to play a role in brain development. In control brain, we were unable to detect any MK-immunopositive structures. However, a recent report (24) has suggested that MK immunoreactivity occurs in selected neurons and astrocytes, as well as strongly in senile plaques and tangles in AD brain. Although the cause of this discrepancy is unclear, the present study indicates that MK is up-regulated in AD brain and may participate in the neurodegenerative process of AD.

RA is involved in fundamental aspects of the development of the central nervous system and has neurotrophic activity (25-27). It is tempting to speculate that RA is

involved in early plaque formation by activating the MK gene. A possible role of RA in AD has already been proposed (28).

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