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ALZHEIMER AMYLOID PROTEIN PRECURSOR ENHANCES PROLIFERATION OF NEURAL STEM CELLS FROM FETAL RAT BRAIN

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SUMMARY: We have investigated the effect of the Alzheimer amyloid protein precursor (APP) on the proliferation of neural stem cells. Two secretory forms of APP (sAPP770 and sAPP695, with and without the Kunitz-type serine protease inhibitor domain, respectively) were purified from conditioned media of COS-7 cells transfected with genetically modified APP cDNAs. Both secretory APPs promoted the growth of neural stem cells, and the effect of sAPP770 was greater than that of sAPP695. sAPP770 and known growth factors in combination exerted a cooperative stimulation of the stem cell proliferation. These results suggest that APP, especially APP possessing the protease inhibitor domain, regulates the growth of neuronal precursor cells during development of the nervous system.
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A number of studies have suggested that APP is closely associated with the pathogenesis of Alzheimer's disease. We have demonstrated that overexpression of APP in full-length APP cDNA transfectants causes cellular degeneration associated with the abnormal processing of APP (1 , 2). Little is known, however, about physiological functions of APP in the brain.

We have previously reported that neuronal differentiation markedly increases expression of APP mRNA, especially that of APP695 mRNA (i.e., the major transcript expressed predominantly in neurons) (3). The APP gene is expressed during early periods of embryogenesis in mouse (4) and Xenopus (5).

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In addition, expression of the Drosophila ß-amyloid protein precursor-like gene is restricted to the nervous system (6). These findings suggest physiological functions of APP during neural development. We report here that the extracellular domain of APP, which is secreted into the extracellular space after proteolytic processing within the ß/A4 domain (7), promotes the proliferation of neural stem cells in cooperation with various growth factors.

MATERIALS AND METHODS

Neural stem cell culture: Neural stem cells were prepared from the striata of Wistar strain rats at the age of embryonic day 14 (8) . The cells were cultured at 5 x 10^4 cells per well (48 multiwell plate, Falcon) in a serum-free medium containing Dulbecco's Modified Eagle Media (DMEM) and Ham F12 (Gibco) (1:1), N2 supplements, vitamin E (100 nM), T3 (1 nM), vitamin B_{12} (0.67 µg/ml), 0.1% bovine serum albumin, penicillin (50 U/ml), and streptomycin (50 µg/ml). The culture medium was changed every three days. These stem cells were positively stained with an antibody against nestin, an intermediate-sized filament specific for neural stem cells (8). To estimate the number of neural stem cells in each well, 1/10 vol. of alamarBlue solution (BioSource International) was added to the medium. The absorbance of the conditioned medium incubated for 8 hrs was measured at 570 nm and 595 nm, and the cell number was determined using a standard curve of differential absorbance (OD_{570} - OD_{595}) vs. actual cell count.

Purification of secretory APP from cDNA transfected cells: Human full length APP cDNAs (APP695 and 770) (1) were subcloned into the Hind III site of Bluescript II. A universal terminator d(pCTAGTCTAGACTACT) (New England Biolabs Inc.) was inserted into the internal EcoR I site of APP cDNA. The resultant cDNAs encoding the peptide sequences 1-600 of APP695 (sAPP695) and 1-675 of APP770 (sAPP770) were digested with Hind III, and cloned into the Hind III site of the expression vector pKCRH (9). COS-7 cells were cultured in DMEM medium supplemented with a 10% Nuserum (Collaborative Biochemical) in 5% CO_2/95% air at 37 °C, and transfected with the expression vectors by the DEAE dextran method (10). After transfection, the cells were cultured for 72 hrs in the serum-free defined medium. The conditioned media were pooled from 300 culture dishes, and secreted sAPP695 and sAPP770 were purified by column chromatographies on dextran sulfate-Sepharose, DEAE Sepharose, and Sephacryl 400 according to the procedure of Van Nostrand and Cunningham (11). The purified sAPP695 or sAPP770 was added to the medium 3 hrs after starting the cultures. Growth factors and protease inhibitors used were obtained from the following companies: basic fibroblast growth factor (bFGF) from Bachem California; platelet-derived growth factor (PDGF) from Chemicon International Inc ; epidermal growth factor (EGF) and acidic fibroblast growth factor (aFGF) from Promega Co. ; aprotinin, α-1-antitrypsin, and soybean trypsin inhibitor from Sigma; urinastatin from Mochida Pharmaceutical Co., Tokyo.

RESULTS

The stem cells prepared from the fetal striata survived well, but proliferated poorly in a serum-free culture medium (Fig 1A). Addition of bFGF (Fig 1B), a typical growth factor promoting the proliferation of neural stem cells (12), or sAPP770 (Fig 1C) increased the cell number, and resulted in the formation of colonies. Most of the cells in colonies displayed a neuron-like appearance 5 days after withdrawal of sAPP770 (Fig 1D). These neuron-like cells were positively stained with antibodies against MAP-2 and neurofilaments, both typical markers for differentiated neurons (data not shown). These results suggest that the stem cells proliferated by the sAPP treatment are indeed neural precursors.

To confirm the growth-promoting effect of sAPP in a quantitative manner, we examined the effects of sAPP695 and sAPP770 on the number of the stem cells

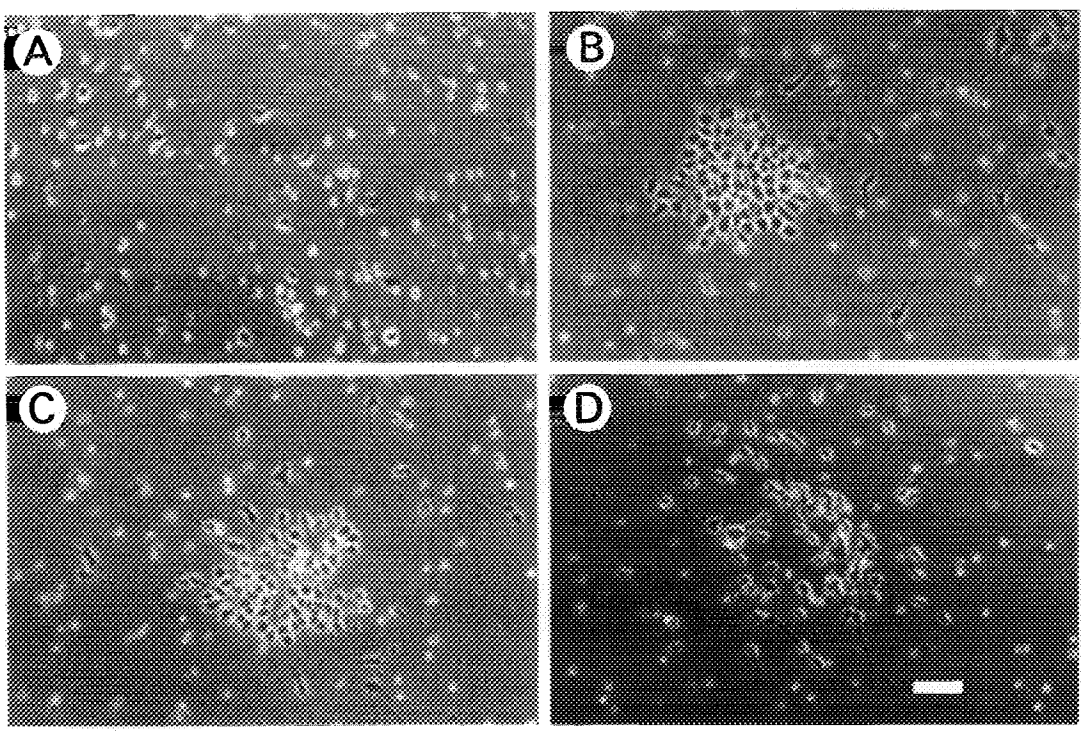

Fig.1. Phase contrast micrographs of primary cultured neural stem cells from the fetal rat striatum. Cells were prepared from the striata of embryonic day 14, and cultured for 5 days in the presence of APP or bFGF. A: control (no treatment), B: treated with bFGF (5 ng/ml), C: treated with sAPP770 (30 μg/ml), D: 5 days after withdrawal of sAPP770, with which the cells were pretreated for 5 days at 30 μg/ml.

(Fig 2). Purities of sAPP preparations were examined by SDS polyacrylamide gel electrophoresis; major single bands at 95 and 105 kDa for sAPP695 and sAPP770, respectively, were detected (Fig.2A). Although minor bands at 90-100 kDa were found in the sAPP770 preparation, these bands reacted with an antibody raised against the amino-terminus of APP (data not shown), suggesting that these bands are of processed or degraded forms of sAPP770. As shown in Fig.2B, sAPP695 increased the number of stem cells about twice the control at the concentrations of 13 and 26 μg/ml, whereas sAPP770 showed a marked stimulation (6.5 times the control at 53 μg/ml) in a dose-dependent manner.

We then examined the effects of sAPP695 and APP770 in combination with bFGF (Fig. 3). These factors in combination showed additive effects on the stem cell proliferation. Even in the presence of a maximally effective dose of bFGF (5 ng/ml), both sAPP695 and sAPP770 showed additive effects (data not shown). Therefore, it is unlikely that the stimulating effects of sAPPs are attributable to a trace of bFGF contaminated in the sAPP preparations. Cooperative effects of

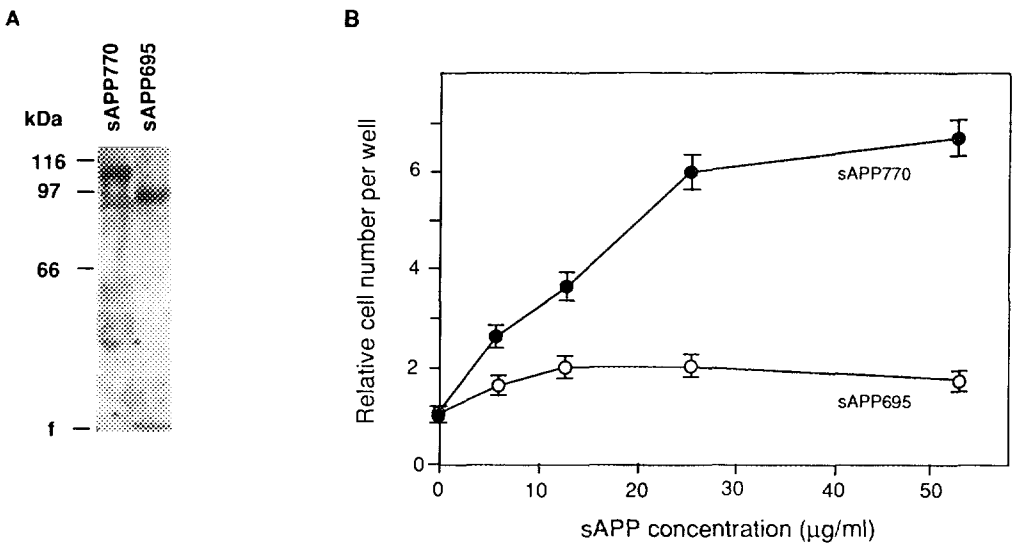

Fig.2. Effects of sAPP695 and sAPP770 on proliferation of neural stem cells. Panel A: Silver staining of purified sAPP695 and sAPP770. sAPPs purified from conditioned media of cDNA transfected cells were analyzed by SDS polyacrylamide gel electrophoresis. The numbers on the left are molecular masses in kDa. f, dye front. Panel B: Dose-response curves of stimulating effects of sAPP695 and sAPP770 on neural stem cells. The cells were cultured for 8 days in the presence of various amounts of sAPPs, and the cell number was determined by the alamarBlue assay. Each point represents the mean (±SEM) of 5 experiments.

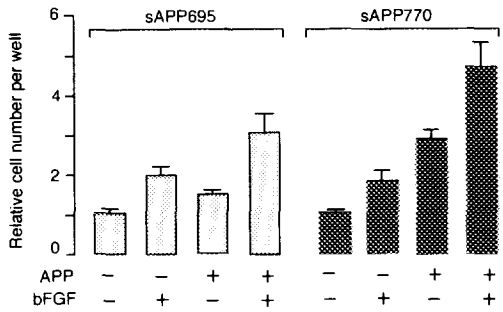

Fig.3. Effects of sAPPs and bFGF in combination on proliferation of neural stem cells. The cells were cultured in the presence or absence of sAPPs (30 μg/ml) and bFGF (1 ng/ml) for 4 days. The cell number was determined by the alamarBlue assay. Each point represents the mean (+SEM) of 5 experiments.

sAPP770 and various growth factors in combination are shown in Fig.4. All the factors tested markedly increased the colony number, and the cooperative effect of sAPP770 was evident. These results suggest that APPs activate the cell proliferation through a mechanism distinct from those of known growth factors.

sAPP770 has the Kunitz-type protease inhibitor (KPI) domain, which may be important for the promotion of stem cell proliferation. We therefore tested the effects of KPI members on the stem cell proliferation (Fig.5). Each KPI member

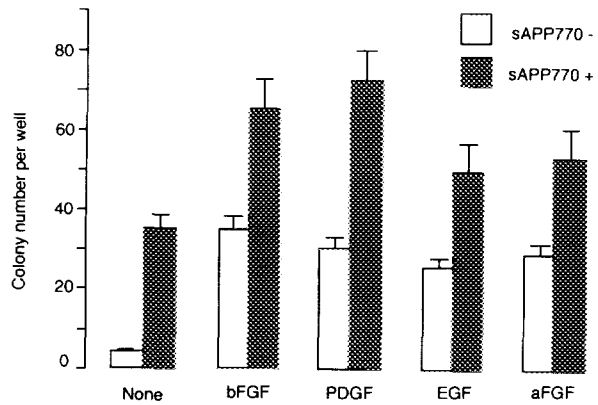

Fig.4. Effects of sAPP770 on growth factor-induced proliferation of neural stem cells. Each growth factor was added to the stem cell culture in the absence (sAPP770-) and presence (sAPP770+) of sAPP770 (30 μg/ml). The number of colonies was counted 5 days after addition of the compounds. Each growth factor was added to the medium at the final concentration of 1 ng/ml. Each point represents the mean (+SEM) of 5 experiments.

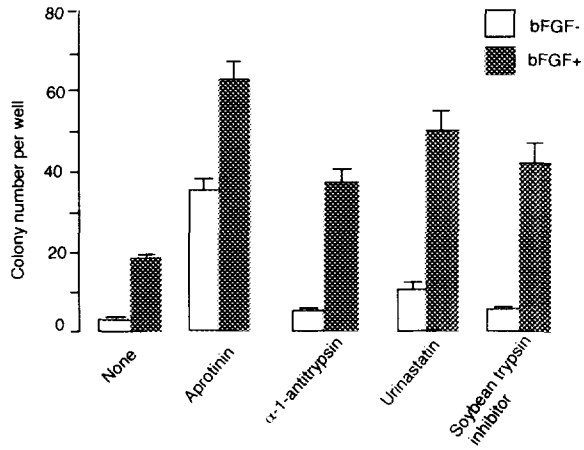

Fig.5. Effects of various protease-inhibitors in the KPI family on proliferation of neural stem cells. Each inhibitor was added to the culture at the final concentration of 30 μg/ml in the presence and absence of bFGF (1 ng/ml). The colony numbers were counted 5 days after addition of the compounds. Each point represents the mean (+SEM) of 5 experiments.

tested stimulated the colony formation to a different extent (aprotinin > urinastatin > soybean trypsin inhibitor > α-1-antitrypsin). These inhibitors and bFGF in combination showed additive or synergistic effects on the stimulation of colony formation. Non-KPI protease inhibitors such as leupeptin, E-64, and antipain showed neither stimulating effects nor enhancement of the growth effect of bFGF (data not shown). Thus, the effect of sAPP770 on the stem cell proliferation may be, at least in part, derived from its KPI activity.

DISCUSSION

The present study has demonstrated that APP exerts positive regulatory effects on the proliferation of neuroectodermal stem cells. APP770 and APP751, which are both major APP species containing the KPI domain, are present during early embryonic periods preceding the neural differentiation (4). Therefore, it is possible that the secretory forms of APP are involved in controlling the number of undifferentiated stem cells. On the other hand, APP has been reported to have neurotrophic effects on primary cultured neurons (13). These findings suggest that APPs have dual actions: the promotion of proliferation of undifferentiated stem cells and the trophic effect on differentiated neurons.

The sAPP concentrations that promote the proliferation of the stem cells were much higher than those of bFGF or other growth factors. The expression of the APP gene during development of the nervous system is extremely high (1). In addition, APP binds to the extracellular matrix (14), which may serve as a reservoir of APP at the extracellular space. Therefore, it is likely that the concentrations of extracellular APPs are high enough to regulate the neuronal growth under physiological conditions.

All the KPI members examined in the present study promoted the growth of neural stem cells. Noggin, whose molecular structure resembles those of KPI members, has been recently reported to show neural tissue-inducing activity (15). Thus, these KPI members might function as endogenous modulators to regulate the development of the nervous systems in vertebrates.

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