In-vitro matured human macrophages express Alzheimer's βA4-amyloid precursor protein indicating synthesis in microglial cells

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Microglia which are consistently associated with Alzheimer's disease (AD) senile plaques are part of the mononuclear phagocyte system. In-vitro matured human monocyte-derived macrophages feature many immunological characteristics of microglia. We found strong constitutive expression of Alzheimer's βA4-amyloid precursor protein (APP) in human mononuclear phagocytes after terminal in-vitro maturation from monocytes to macrophages. Amyloid has previously been found to be associated with microglia in AD brains, however, it remained unclear whether the material was synthesized in or had been phagocytosed by the cells. The findings presented here support the assumption that brain microglia may contribute to APP synthesis in AD brain.

Macrophage; Microglia; Amyloid precursor protein; Alzheimer's disease

1. INTRODUCTION

β-Amyloid or A4 protein, now termed βA4 protein (βA4), is the major proteinaceous constituent of Alzheimer's disease (AD) cortical senile plaques [1] and cerebrovascular amyloid [2]. It is derived from a large precursor protein: βA4 amyloid precursor protein (APP) [3–5]. Three major APP protein forms of 695 [3], 751 [4], and 770 amino acids [5], respectively, are derived from an alternatively spliced hn mRNA. APP is synthesized in the brain as well as in several other organs and tissues of healthy individuals (for reviews, see [6,7]). Until now, only cortical and hippocampal neurons, astrocytes, and endothelial cells have been reported to be cortical sites of APP synthesis [5, 8–15].

Although numerous reactive microglia are consistently found in and around AD senile plaques [16–19] and although amyloid was found to be associated with them [17,18, 20] nothing is known so far as to whether this cell population of the brain may synthesize APP and thus may contribute to βA4 deposits. The question remained open whether the material was synthesized by the cells themselves or whether it had been phagocytosed. Our intention was to obtain evidence whether or not human microglia might be able to express APP and how it may be regulated. We applied a previously described special cultivation system to obtain terminally

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matured human macrophages from purified peripheral blood monocytes which feature many of the characteristics of human microglia [21,22]. The in-vitro matured monocyte-derived macrophages share morphological and immunological features with microglia: compared to monocytes, they show a typical macrophage-like ruffled cell membrane under the electron microscope [21], and express surface antigens such as MHC class II molecules, receptors for Fc, C3, transferrin, and the CD4 receptor [23,24]. In this report we show that monocyte-derived, in-vitro matured human macrophages constitutively express APP mRNA and synthesize APP protein.

2. MATERIALS AND METHODS

2.1. Human monocyte-macrophage cultures

Monocyte and monocyte-derived macrophage cultures were performed as previously described in detail [21,22]; monocytes were prepared from blood of healthy donors by centrifugation over Ficoll-Hypaque according to [25]. The mononuclear cell fraction was allowed to adhere to plastic dishes in supplemented RPMI 1640 medium (Seromed, Berlin, FRG) containing 10% human AB serum. Non-adherent cells (lymphocytes) were then removed resulting in highly monocyte-enriched cultures which were either used immediately for experiments or were further cultivated in order to obtain monocyte-derived macrophages. In the latter case, monocytes were incubated overnight and then removed from the dishes at 4°C by vigorous pipetting and placed into rectangular teflon bags (Biofolie 25; Heraeus, Hanau, FRG) in supplemented RPMI 1640 medium containing 10% human AB serum. After 8 days, the monocyte-derived macrophages exhibited features of typical tissue macrophages [23,24]. Cells were

removed from the bags, sedimented on plastic dishes, and could be used for further experiments.

2.2. Human neuronal cell cultures

The SH-SY5Y neuroblastoma clone [26] was a gift from Dr J. Biedler, Memorial Sloan-Kettering Cancer Center (New York, N.Y.). The cells were grown in RPMI 1640 medium containing 1% FCS (Gibco, Karlsruhe, Germany). Murine nerve growth factor β (NGF β) (a generous gift from U. Otten, Basel, Switzerland), retinoic acid (Sigma, Deisenhofen, Germany), and dibutyryl cAMP (db cAMP) (Sigma) were added to the medium for 5 days at a final concentration of 20 ng/ml, 10 μM, and 0.5 mM, respectively. Addition of these components has been previously reported to result in a differentiated phenotype of neuronal cells [27].

2.3. Radiolabeling and immunoprecipitation

Radiolabeling of the cell cultures and immunoprecipitation was done as described earlier in detail [29]: cells were plated into 40 mm plastic dishes (10⁶ cells/dish) in supplemented RPMI 1640 medium containing 1% FCS (neuronal cells) or 1% human AB serum (macrophages). Metabolic labeling was done by replacing media by methionine- and serum-free RPMI 1640 medium and by addition of [³⁵S]methionine (100 μCi/dish). Cells were allowed to incorporate the radioactive amino acid into newly synthesized proteins for 4 h except in the pulse-chase experiments, where cells were labeled with 200 μCi/dish for only 20 min followed by addition of an excess of unlabeled methionine (2 mM final concentration) in order to chase the radiolabeled methionine. For immunoprecipitation, media were separated from the cells. Cells were homogenized in 1 ml 25 mM Tris-HCl, pH 7.5; 20 mM NaCl; 1% sodium deoxycholate; 1% Triton X-100. Non-soluble material was removed by centrifugation at 12 000 rpm for 5 minutes. Cell homogenates and media were diluted with 2 volumes of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.6; 0.14 M NaCl; 5 mM EDTA; 1% Triton X-100). Total de-novo synthesized proteins were determined by TCA-precipitation of 20 μl aliquots of cell homogenates and media according to [30]. Specific proteins were extracted from equivalent amounts of total TCA-precipitable radioactivity (usually 0.5 × 10⁶ cpm from media, 2 × 10⁶ cpm from cell homogenates). In the pulse-chase experiments, specific proteins were extracted from total medium and from one half of the cell homogenate. Prior to extraction with specific antibodies, unspecifically binding material was absorbed by a sham extraction with non-immune control serum. After addition of an excess of monospecific polyclonal anti-APP₆₉₅ antibody [38] (usually 10 μl), the immune complexes were bound to Protein A Sepharose (Pharmacia, Freiburg, FRG), sedimented, and washed (2 times in immunoprecipitation buffer, 2 times in 50 mM sodium phosphate buffer, pH 7.5). The immune complexes were dissociated at 95°C for 5 min in 35 μl of dissociation buffer (0.1 M Tris-HCl, pH 6.8; 5% 2-mercaptoethanol; 5% sodium dodecyl-sulfate; 10% glycerol), subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to [31], and fluorography according to [32]. The specific radioactive bands were cut out from the gels, solubilized with protosol/water (9:1) and counted in a liquid scintillation counter.

2.4. DNA constructs

Standard cloning techniques were applied [33]. A partial βA4-amyloid precursor protein/APP₇₇₀ cDNA of 2.7 kb was isolated from a human fetal brain lambda gt10 cDNA library using radioactively endlabeled oligonucleotide probes specific for the APP₇₇₀ cDNA as well as a BamHI/XhoI restriction fragment (−47 to +1135, [3]), which was labeled by random priming [34,35]. A 1.7 kb EcoRI fragment of this cDNA, where the 5'-EcoRI restriction site derived from the adaptor (EcoRI-XhoI-SalI) used during construction of the cDNA library, and the 3'-EcoRI site at position +2020 from the APP₇₇₀ cDNA, was subcloned into the EcoRI restriction site of the pUC19 polylinker, resulting in the recombinant plasmid p2.21. Digestion of plasmid p2.21 with XhoI resulted in a fragment with 815 bp of APP₇₇₀ cDNA. This fragment was cloned into the SalI restriction

site of the M13mp18 polylinker, resulting in recombinant phage M8.11.

2.5. RNA extraction and S1 nuclease protection assay

Total RNA was extracted from cells according to [36]. The S1 nuclease protection assay was performed as described [37]. A uniformly labeled single-stranded DNA probe was synthesized by annealing 1 pmol of M13 phage specific oligonucleotide primer to 250 ng of single-stranded template DNA of M8.11. This primer was extended with 1 unit of Klenow polymerase (Boehringer, Mannheim, Germany) in the presence of 25 mM dATP, dGTP, dTTP, 2 mM dCTP, and 40 μCi of [α-³²P]dCTP (300 Ci/mmol) for 15 min at 37°C. Extended products were digested with SacI for 45 min. The labeled single-stranded DNA-probes were purified on a 5% denaturing polyacrylamide gel. Probes were visualized by autoradiography. Gel slices corresponding to the bands on the autoradiograph were cut out and electroeluted (Elutrap, Schleicher and Schüll). Excess probe M8.11 (50 000 cpm) was hybridized overnight to 15 μg of total RNA at 55°C (75% formamide, 0.4 M NaCl, 20 mM Tris-HCl, pH 7.4, and 1 mM EDTA). S1 nuclease digestion (1200 units/sample; Boehringer, Mannheim, Germany) was performed for 2 h at 37°C (0.3 M NaCl, 3 mM ZnSO₄, 60 mM NaAc, pH 4.5, 0.5 μg denatured calf thymus DNA). The resulting products were once phenol- and once phenol/CHCl₃-extracted, ethanol-precipitated and resolved on a 5% denaturing sequencing gel. Autoradiography was done with Kodak X-OMAT AR films.

2.6. Immunocytochemical staining

Monocyte-derived macrophages were plated in 1640 medium on thermanox plastic coverslips (Nunc, Ill, USA) and allowed to adhere. Cells were then washed with phosphate-buffered saline (PBS) and fixed with 4% formalin PBS. After incubation with a 1:10 dilution of the monospecific primary antibodies: monoclonal anti-βA4-amyloid precursor protein (Boehringer, Mannheim, Germany) or polyclonal anti alpha 2-macroglobulin [28], cells were treated with the Vectastain ABC-peroxidase detection system (Vector Lab., Burlingame, CA). Cells were mounted in glyceringelatine (Merck, Darmstadt, Germany) and viewed with a Zeiss III RS microscope.

3. RESULTS

In-vitro matured macrophages were obtained by subjecting purified human peripheral blood monocytes to cultivation in hydrophobic teflon foils for 8 days. During this time, the cells increase in size (Fig. 1A) and develop features of tissue macrophages [23,24]. In freshly isolated blood monocytes synthesis of βA4-amyloid precursor protein (APP) was nearly undetectable (Fig. 1B, lane 1). However, after terminal maturation macrophages express high levels of APP both on the protein (Fig. 1B, lane 2) as well as at the mRNA level (Fig. 2). If RNA extracted from macrophages was analyzed by S1 nuclease protection analysis, APP mRNA coding for the 751 and for the 770 amino acid forms of the APP protein were found to be predominant, while only a faint signal was detected corresponding to the mRNA coding for the 695 amino acid form of the protein (Fig. 2).

The biosynthesis and secretion of APP was analyzed by a pulse-chase study. A comparison of synthesis and secretion in macrophages (Fig. 3, lanes 1-4) and human neuronal (SH-SY5Y neuroblastoma) cells (Fig. 3, lanes 5-8) revealed, that macrophages synthesize intracellular
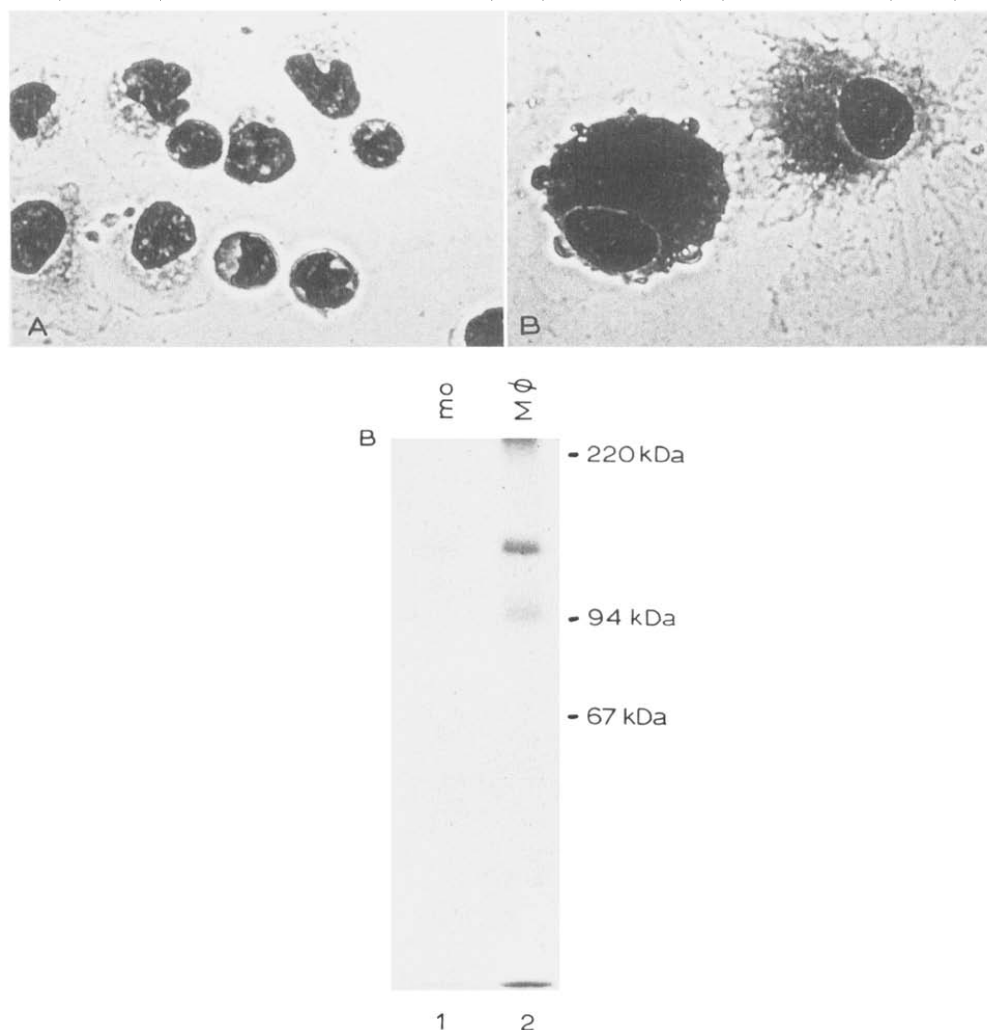
Fig. 1. (A) Morphological characterization of in-vitro matured human macrophages compared to freshly isolated human blood monocytes. Pappenheim-stained freshly prepared human blood monocytes (left) and macrophages after in-vitro maturation (right) were viewed by oil immersion microscopy at the same magnification (×2400) for both pictures. (B) Secretion of βA4-amyloid precursor protein (APP) by freshly isolated human blood monocytes and by in-vitro matured macrophages. Adherence-purified human blood monocytes (lane 1) and in-vitro matured macrophages (lane 2) were metabolically radiolabeled. APP was immunoprecipitated from standardized amounts of secreted TCA-precipitable radioactivity (i.e. from standardized amounts of de-novo synthesized proteins) as described in Section 2, subjected to SDS-PAGE and autoradiography.

APP forms with an apparent molecular weight between 110 kDa and 130 kDa (Fig. 3, lane 1) corresponding to the N-glycosylated forms of APP_{751} and APP_{770} (about 110 kDa) and to the N- and O-glycosylated thyrosine sulfated mature membrane protein APP_{751} and APP_{770} (about 130 kDa) [38]. The same pattern was found in neuronal cells (Fig. 3, lane 5), however, these cells contained an additional APP form of approximately 98 kDa (lane 5, arrowhead). Most likely, this neuron-specific form represents N-glycosylated APP_{695} ([38] see below). Secretory APP forms in the macrophage-conditioned medium had a molecular weight between 110 kDa and 125 kDa (Fig. 3, lane 4). Again, a neuron-specific additional APP_{695} form gives rise to an additional band with a molecular weight of approximately 92 kDa (lane 8, arrowhead).

Molecular weight heterogeneity of APP has previous-

ly been shown to be due to the facts: first, that 3 differentially spliced major APP mRNA forms (coding for APP proteins with 695, 751, and 770 amino acids) are derived from hn mRNA [3–5]; second, that APP is subjected to posttranslational modifications including N- and O-glycosylation, sulfation, and proteolysis [38–40]. The secretory APP forms found in the conditioned medium are produced by a proteolytic cleavage C-terminal to residue 16 of the βA4 part of the mature APP transmembrane forms [39, 40].

The presence of APP in macrophages was finally demonstrated by immunocytochemical staining. Figure 4A shows strong intracellular (mainly perinuclear) βAPP immunoreactivity in macrophages. In addition, cells from the same preparation stain also for alpha-2-macroglobulin (Fig. 4B), a previously described marker of terminally matured macrophages [28].
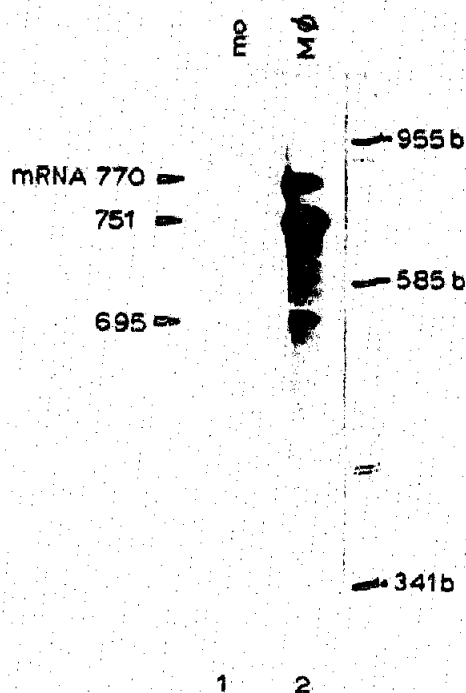
Fig. 2. Detection of APP mRNA in freshly isolated human blood monocytes and in-vitro maturated macrophages by S1 nuclease protection assay. 15 µg of total RNA from freshly isolated human blood monocytes (lane 1) and from in-vitro maturated macrophages (lane 2) were hybridized to an excess of APP probe M8.11 and digested with S1 nuclease. The resulting protected fragments were subjected to a 5% denaturing polyacrylamide gel electrophoresis. The protected fragments of 815 bases for the βAPP_{770} mRNA, of 714 bases for the βAPP_{751} mRNA and of 546 bases for the βAPP_{695} mRNA are indicated.

Fig. 3. Comparison of the APP forms synthesized and secreted by in-vitro maturated human macrophages and by human neuronal (neuroblastoma) cells. In-vitro maturated human macrophages (lanes 1–4) and human SH-SY5Y neuroblastoma cells were pulse-labeled with 200 µCi of [³⁵S]methionine for 20 min. Then the cultures were harvested either immediately (lanes 1,3,5,7) or cultivated for 4 more hours in the presence of an excess of unlabeled methionine (2 mM final concentration) in order to chase the incorporated radioactive methionine (lanes 2,4,6,8). Cultures were harvested by separating culture media from cells. APP was immunoprecipitated from half volume of cell homogenates (lanes 1,2,5,6) and from total media (lanes 3,4,7,8), subjected to SDS-PAGE and autoradiographed. Unspecifically binding material was preabsorbed from cell homogenates (P_c) and media (P_m) using non-immune control serum. Arrowheads point to an APP form produced in neuronal cells only.

4. DISCUSSION

As the resident macrophages of the brain, microglia are linked to the mononuclear phagocyte system (for recent reviews see [41–43]). Here we describe that cells of the mononuclear phagocyte system, linking bone marrow precursors, blood monocytes, and the different tissue macrophages, strongly express APP upon terminal maturation from monocytes to macrophages. Treatment of the macrophages with endotoxin (a classical stimulans) or glucocorticoids did not affect APP synthesis (not shown). Like in other non-neuronal cells [44–46], APP_{751} and APP_{770} are the major APP forms in macrophages. Expression of APP_{695} is nearly exclusively restricted to neuronal cells where it is expressed in a [751 + 770] : [695] ratio of about 1 : 1 [44–46]. Several reports describe an increased ratio of [751 + 770] : [695] APP mRNA in AD brain [47–49]. This finding may be explained by an age-dependent alteration [50] and/or by gliosis which is commonly observed in AD [48]. It remains to be elucidated whether the changed ratio in APP expression is an early or late event of Alzheimer's disease pathophysiology.
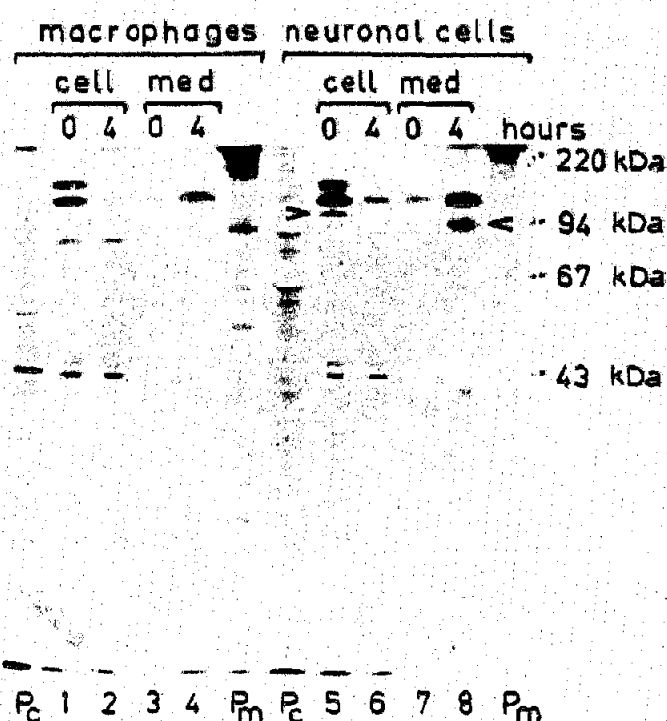
In AD brain, senile plaques are consistently found to contain and to be surrounded by reactive microglia [18–20]. Resident microglia are derived from immigrating monocytes which then undergo terminal maturation into resident microglia [18–20]. This immigration occurs mainly in the perinatal period, but a low degree of immigration has been assumed to occur throughout life [18–20]. Injury or infection in the central nervous system give rise to the appearance of both reactive microglia (i.e. activated resident microglia) and activated macrophages (i.e. mononuclear phagocytes recruited from the circulation) [41–43]. The local environment may influence the behaviour of resident macrophages in the different tissues. Therefore, data obtained with in-vitro matured macrophages must be interpreted with caution. It is impossible to determine to which of the microglial cell types (resident microglia, activated microglia, or activated macrophages) our in-vitro matured macrophages are best correlated. However,
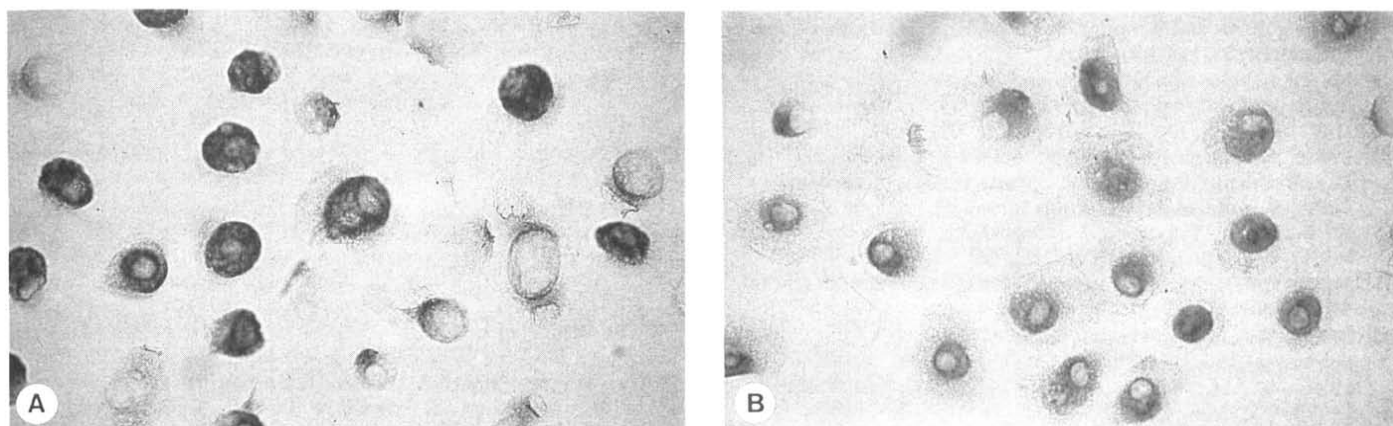
Fig. 4. Immunocytochemical staining for APP of in-vitro-maturated macrophages with a monoclonal antibody directed against βA4-amyloid precursor protein (A) and a polyclonal antibody directed against alpha 2-macroglobulin (B). Control stains with rabbit non-immune serum were completely without stain (not shown). Magnification ×373.

since APP expression was not altered upon stimulation of our cells with the classical activator endotoxin, the state of activation seems to be of minor significance for APP expression. Instead, APP expression seems to critically depend upon the terminal step of mononuclear phagocyte maturation. We assume that APP expression of in-vitro matured macrophages may serve as evidence for synthesis in microglial cells. Very recently, this assumption was supported by immunocytochemical detection of APP in primary cultures of human microglia (P. Gebicke-Haerter, J. Bauer, B. Volk, in preparation).

The physiological functions of APP are still unclear. The secretory (N-terminal) part of APP_{751} (which, like APP_{770}, bears a Kunitz-type protease inhibitor domain) has been found to be identical to protease nexin II [51,52]. Functions of this molecule include inhibition of coagulation factor XI a, plasmin, trypsin, chymotrypsin, and nerve growth factor gamma (which is involved in NGF processing) [5,51-55]. In addition, binding to epidermal growth factor binding protein and to transforming growth factor beta has been observed [51,52,54,56]. Since neurite outgrowth depends on a precarious balance between proteases and their inhibitors [57], it appears conceivable that the protease inhibitors APP_{751} and APP_{770} may interfere with neuronal plasticity. With respect to the βA4 peptide, its effects on undifferentiated neurons seem to be beneficial and to support neuronal differentiation [58,59]. In contrast, βA4 was found to exert neurotoxic effects on differentiated neurons [59-61].

The mechanisms by which APP contributes to the pathology of Alzheimer's disease are not yet fully understood. A detailed analysis of APP biosynthesis in cultured cells revealed that APP, after its insertion into the cell membrane as a transmembrane protein [3, 38, 62], is cleaved *inside* the βA4-sequence of the mature APP

protein by a still unknown secretase resulting in the secretory release of the large N-terminal portion of APP [39,40]. Therefore, it can be concluded that the formation of the βA4 peptide is prevented under normal physiological conditions. Vice versa, it appears conceivable that a protease inhibitor may inhibit the APP secretase in Alzheimer's disease (AD) and may thus preserve the βA4 peptide from being cleaved. Pathologic APP breakdown could then give rise to βA4 which due to its tendency to aggregate may contribute to plaque formation. In fact, we recently found that human neuronal cells synthesize and secrete the most potent of the known endogenous protease inhibitors, alpha2-macroglobulin (a2M), upon stimulation with the inflammatory cytokine interleukin-6 (IL-6) [63]. a2M was found to inhibit APP secretion [63] and both a2M and IL-6 could be detected in AD senile plaques (B. Volk and J. Bauer, submitted).

Once normal APP processing is disturbed, APP from any source inside the brain might contribute to βA4 and subsequent plaque formation. Our data show that tissue macrophages contribute to APP synthesis and thus provide evidence that APP may be synthesized also by brain microglia. However, further studies have to be carried out to elucidate how exactly microglial cells may contribute to the pathophysiology of Alzheimer's disease.

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