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Transforming growth factor β mediates increase of mature transmembrane amyloid precursor protein in microglial cells

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Abstract

By using the immortalized microglial cell line BV-2, we show that the high expression of the βA4 amyloid precursor protein (APP), its biogenesis and metabolism is modulated by TGFβ, a cytokine with immunosuppressive activity, and by the microglia-stimulating agent LPS. TGFβ induces accumulation of cellular mature APP, the putative precursor of the amyloid subunit of Alzheimer's disease. LPS leads to an increase in cellular immature, non-amyloidogenic APP and secretion of also non-amyloidogenic APP fragments. We also demonstrate a functional involvement of ECM molecules in the regulation of microglial APP expression at mRNA and protein level by TGFβ and LPS.

Key words: Alzheimer's disease; βA4 amyloid; Amyloid precursor protein; TGFβ; LPS; Extracellular matrix

1. Introduction

Microglia are believed to be important effector cells in the immune response of the central nervous system [1–4]. They have attracted much attention because of their potential relevance for the pathogenesis of Alzheimer's disease. Activated microglia are able to synthesize large amounts of amyloid precursor protein (APP) [5–7] and are often associated with amyloid plaques in Alzheimer's disease [8–10]. The major component of the deposits is the βA4 peptide (for reviews, see [11,12]). This amyloidogenic peptide is derived by proteolytic cleavage from the larger transmembrane amyloid precursor protein [13]. Soluble βA4 protein can be detected in the extracellular mileu under normal, nonpathological conditions [33–35].

APP constitutes a family of different isoforms that are generated by alternative splicing [13–18]. The major primary translation products consist of 695, 751, and 770 amino acid residues as well as the recently described L-APP isoforms consisting of 677, 733, and 752 amino acids. All these APP/L-APP isoforms show the characteristic features of typical transmembrane glycoproteins. Major secreted forms of APP are generated by proteolytic cleavage within the amyloidogenic region [19,20].

Axotomy studies have shown that the βA4-amyloid precursor protein participates in immune responses of the central nervous system [6]. In injured brain, microglia possesses the immunomodulatory role of peripheral leukocytes [1–4]. Microglia share many antigenic markers and other properties with cells of the mononuclear phagocyte system [3]. Like peripheral macrophages, they produce and respond to a range of molecules that are involved in the modulation of immune response. TGFβ, a cytokine with potent immunosuppressive activity, has also been shown to be produced by microglia [21]. It has been suggested that TGFβ could be a negative regulator in cytokine network by inhibiting the function of microglia in inflammation or in immunoregulation [22,23]. However, cell responsiveness to growth and transforming factors is often controlled by components of the extracellular matrix (ECM) [24]. Especially, cells of the mononuclear phagocytic system undergo a rapid and extensive change in the pattern of gene expression upon adherence to substrata coated with ECM proteins [25].

In this study we have analyzed the effect of the immunosuppressive agent TGFβ and the effect of the microglia-stimulating agent lipopolysaccharide in regard to microglial APP expression, biogenesis and metabolism. For our analysis we used the immortalized microglial cell line BV-2 which shares several features characteristic of activated microglia in vivo [26]. We could demonstrate that microglial cell–cell interactions and cell–ECM interactions were essential for regulating microglial APP biosynthesis by the soluble mediators TGFβ and LPS. This

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could be an important mechanism for the regulation of microglial localization and function in health and disease.

2. Materials and methods

2.1. Cell culture

Cells of the mouse microglial cell line BV-2 [26] were studied in these experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine. For preparation of substratum-coated dishes, plates with a diameter of 60 mm were incubated for 12 h at 4°C with 20 μg/ml fibronectin, 20 μg/ml laminin, or 100 μg/ml collagen type I, respectively. To set up experimental cultures on ECM-coated dishes, BV-2 microglial cells were removed by scraping and seeded in suspension at 5×10^5 cells per ml in 3.0 ml medium consisting of optiMEM (Gibco/BRL, UK).

2.2. Mitogen treatment

Treatment of cells with mitogens was performed with 1 ng/ml transforming growth factor (TGFβ1) (Serva, Heidelberg, Germany), and 5 μg/ml lipopolysaccharide (LPS) (Sigma Chemical CO., St. Louis, MO) for 16 h.

2.3. Immunoprecipitation and immunoblotting

Conditioned medium was removed from the cell cultures, cleared by centrifugation and stored at −20°C. 2×10^6 cells were collected by scraping in 1.5 ml phosphate-buffered saline (PBS) and were washed once with PBS. For lysis, cells were resuspended in 0.2 ml lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 1% Triton X-100, 2 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1,6 mg/ml iodoacetamide) and incubated for 30 min on ice. Cell lysates were centrifuged at $10,000 \times g$ for 5 min and the resulting supernatants were stored at −20°C until further use. The extraction pellet was discarded.

For analysis of APP, cell lysates and conditioned media were subjected to immunoprecipitation. The cell lysate was diluted with an equal volume of ice-cooled PBS; conditioned medium was supplemented with 75 μl medium buffer (1 M Tris (pH 8.0), 100 mM EDTA, 10% Nonidet P40, 100 mM PMSF). Lysate and medium were preincubated for 2 h at room temperature (RT) with 10 μl pre-immune serum and 3 mg protein A-Sepharose (Pharmacia, Uppsala, Sweden). The insoluble complexes were discarded after centrifugation. Lysates and supernatants were then incubated for 1 h at RT with 5 μl of undiluted anti-CT and anti-FdAPP antiserum, respectively. Anti-FdAPP is a polyclonal rabbit antiserum raised against purified *E. coli* FdAPP fusion protein [27]. The polyclonal anti-CT antiserum was raised against a synthetic peptide corresponding to the C-terminal 43 residues of APP.

After addition of 2 mg of protein A-Sepharose the mixture was incubated for 30 min at RT. Non-bound proteins were removed from sepharose beads by washing with TSA solution (20 mM Tris (pH 8.0), 150 mM NaCl). The immunoprecipitates were fractionated by 7% SDS-PAGE [28] and transferred onto nitrocellulose [29]. The nitrocellulose sheet was soaked in PBS containing 1% bovine serum albumine (BSA) for 1 h at RT and then incubated at RT for 2 h with monoclonal antibody 22C11 (1:10000) [27,38]. The nitrocellulose sheet was then washed three times with Tris-buffered saline (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20). Specific binding was visualized with the Protoblot alkaline phosphatase system (Promega, Heidelberg, Germany). Quantitation of individual bands was performed by densitometric scanning.

2.4. Isolation of RNA, Northern hybridization

Total RNA was prepared as described [30]. For each sample, 20 μg of total RNA was separated in a denaturing formaldehyde-containing 1% agarose gel, and vacuum blotted to Gene-Screen-Plus (DuPont) nylon membrane following the manufacturer's protocol. Filters were hybridized with an *Eco*RI-APPcDNA and a GAPDH cDNA restriction fragment labelled by nick translation using [α-^{32}P]dCTP (Amersham). Prehybridization and hybridization buffer contained 0.5 M Na_2HPO_4 (pH 7.4), 1% sodium dodecylsulfate, and 1% BSA. Hybridization was performed overnight at 65°C, and filters were washed three times in 40 mM Na_2HPO_4, pH 7.4 and 1% SDS at 65°C, and exposed to Kodak X-AR film for 4 d at −80°C. For quantitation of individual bands, gels were analyzed on a phosphorImager (Molecular Dynamics).

3. Results

3.1. Effect of extracellular matrix, TGFβ and LPS on morphology of BV-2 cells

The microglial cell line BV-2 showed a different morphology when cultivated on different ECM-coated plastic dishes (Fig. 1). Cultivation of microglia cells on plastic induced both spindle- and round-shaped morphologies (Fig. 1a). Microglia cells cultivated on fibronectin showed a marked increase in extending processes (Fig. 1b). Microglial cells lost their characteristic morphology after being plated on laminin and collagen (Fig. 1c,d). Round and small microglial cells were visibly lacking filopodial projections. Attachment to laminin was weak and not followed by spreading. On collagen cells were grown in suspension by forming partly big cell aggregates.

Microglial cells cultivated on plastic and fibronectin revealed a significant morphologic transition upon treatment with the soluble mediators TGFβ and LPS. The change in phenotypic bevahior was strongest by BV-2 cells cultivated on fibronectin (Fig. 1e,f). As shown in Fig. 1e, addition of the TGFβ to the culture medium induced the 'ramified' phenotype (Fig. 1e) whereas bacterial lipopolysaccharide (LPS) induced the 'ameboid' phenotype (Fig. 1f). Microglial cells cultivated on laminin displayed only slight morphologic alteration after treatment with TGFβ and LPS. No change in morphology was observed by microglia grown on collagen substratum (not shown).

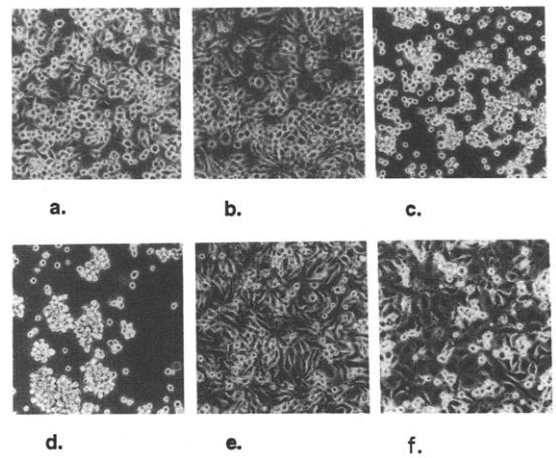


Fig. 1. Morphology of the microglial cell line BV-2 grown on different substrates. Phase contrast micrographs of these cells were photographed after 16 h of cultivation in FCS-reduced medium (magnification 1:50): a. cell culture plastic, b. fibronectin (20 μg/ml), c. laminin (20 μg/ml), d. collagen (100 μg/ml). Morphologic alterations of cells cultivated on fibronectin in presence of TGFβ and LPS were shown in e and f, respectively.

3.2. Effect of TGFβ and LPS on APP gene expression

The effects of TGFβ and LPS on total APP gene expression in microglial cells cultivated on different matrices were analyzed by Northern blotting using an APP cDNA restriction fragment and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA restriction fragment as a control. Visual inspection of autoradiogramms indicated (Fig. 2A) that TGFβ and LPS increased the absolute amount of APP mRNA (detected as a 3,4 kb band) transcripts under all investigated culture conditions, but the strength of APP expression differed in dependence on the chosen coating protein. However, changes in level of GAPDH mRNA transcripts (detected as 1,6 kb band) reflecting the overall rate of protein synthesis, were also observed after treatment with TGFβ and LPS but in other extent than observed for APP transcripts. The selective effects on APP expression of microglial cells were uncovered by comparison of the steady-state levels of APP transcripts and levels of GAPDH transcripts. For this RNA bands were quantitated by use of a PhosphorImager and the ratio of APP to GAPDH mRNA band intensity calculated (Fig. 2b). Changes in this ratio reflect specific changes in APP mRNA expression. Microglia cultivated on non-coated plastic culture plates exhibited the highest ratio of APP/GAPDH expression. The ratio of APP/GAPDH was generally 50% to 70% lower in microglia cultivated on ECM-coated plates. The selective effects of TGFβ and LPS on APP expression of microglial cells cultivated on fibronectin, laminin, collagen and non-coated plates are graphically shown in Fig. 2c. Treatment of microglia grown on plastic and collagen with TGFβ led to a decrease of APP expression of about 17%. An increase in APPmRNA expression was observed by microglia grown on laminin (+ 30%) and in highest extent by microglia grown on fibronectin (+ 80%). LPS induced under all cultivation conditions an increase in APP expression but to different extents. The LPS-induced increase in APP expression was lowest by microglia grown on uncoated plates (+ 7%), and reached maximum values by microglia cultivated on laminin and fibronectin (+ 103 and + 116%, respectively). The results demonstrate a functional involvement of ECM molecules in mediating cellular responses to TGFβ and LPS with regard to microglial APP expression. Regarding the distribution of alternatively spliced APP mRNA isoforms, however, quantitative RT-PCR analysis [18] revealed no significant differences within these differently cultivated microglial cells (results not shown).

3.3. Effect of TGFβ and LPS on APP biogenesis and metabolism

To investigate whether TGFβ and LPS actions are associated with changes in APP biogenesis and metabolism, we examined cells and conditioned media with regard to APP content by immunoprecipitation and subse-

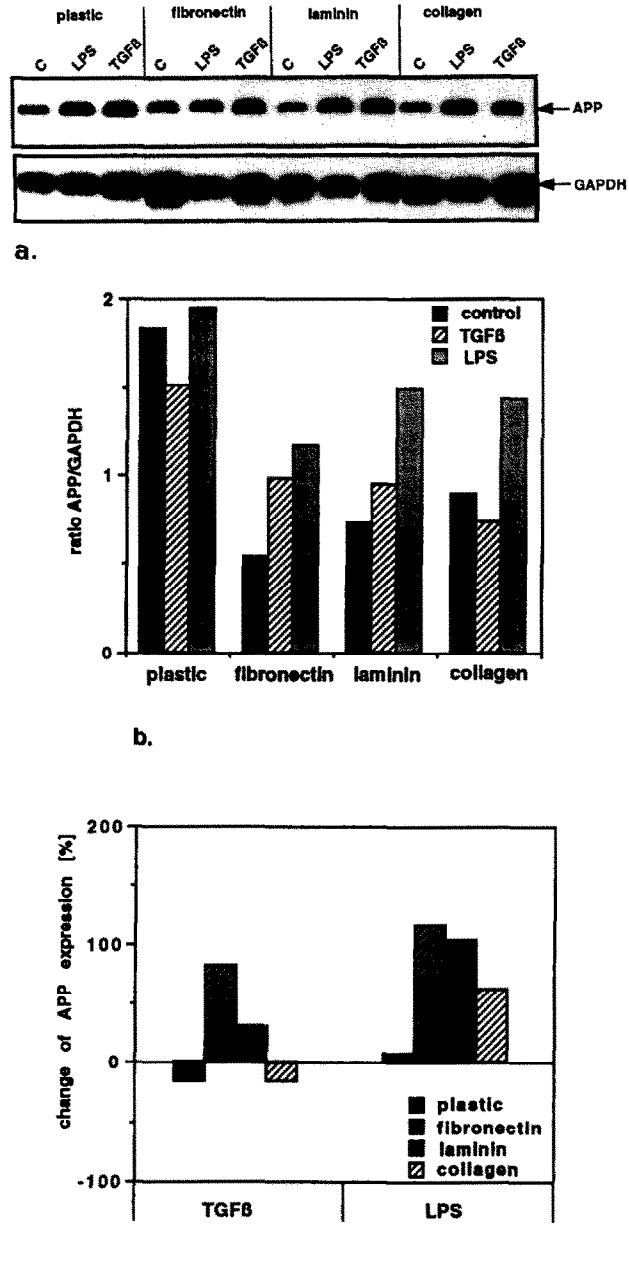
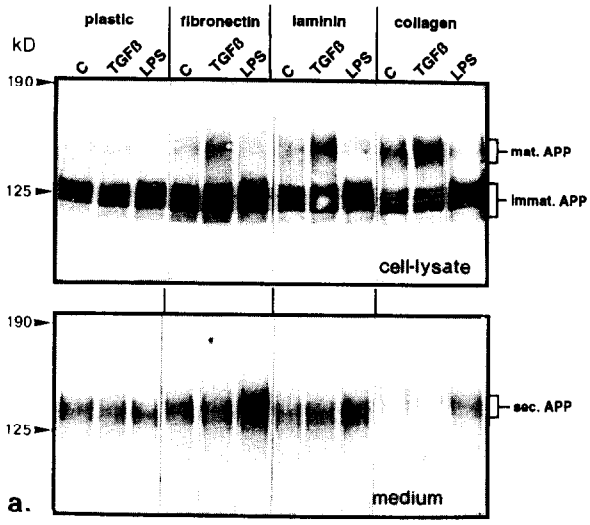
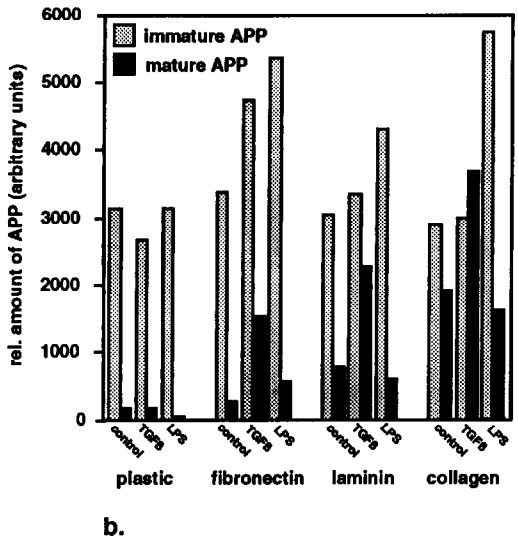
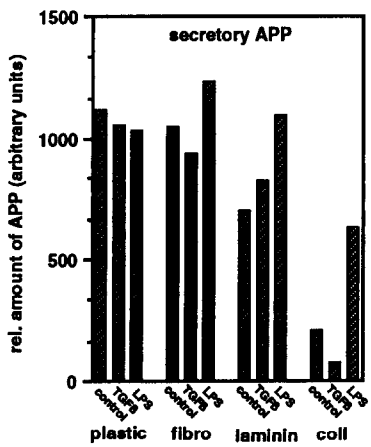


Fig. 2. Effect of TGFβ and LPS on APP mRNA expression of microglial cells cultivated on different ECM. (a) Analysis of total APP mRNA expression of BV-2 cells cultivated under different cultivation conditions. BV-2 cells were cultivated in the absence (control) or in the presence of TGFβ and LPS, respectively, on different ECM-coated substrata for 16 h. Cells were harvested and total cellular RNA was isolated. RNA samples (20 μg) were separated on agarose gels, transferred onto nitrocellulose membranes, and subjected to Northern blot hybridization using ^{32}P-labeled cDNA probes specific for human APP, or GAPDH as a control. (b) Normalization of APP mRNA expression. For normalization of APP mRNA, individual signals corresponding to APP transcripts and GAPDH transcripts were quantitated on a phosphorimager by overnight exposure. The relative change in APP expression was expressed as the ratio of APP transcripts to GAPDH transcripts. (c) Specific effect of TGFβ and LPS on APPmRNA expression. For the detection of specific effects of TGFβ and LPS on APPmRNA expression, changes in the ratio of APP to GAPDH transcripts in comparison to the respective control culture in the absence of TGFβ and LPS were estimated for different ECM substrata (in %).







quent Western blotting. The results are shown in Fig. 3a. BV-2 expressed transmembrane APP isoforms in the predominant molecular mass range of 95–130 kDa and 140–145 kDa, corresponding to all described major translation products of the APP gene. APPs in the molecular weight range of 95–130 kDa represent mainly immature (partially glycosylated) APP whereas the higher APP isoforms represent mature (fully glycosylated) transmembrane products [5,27]. Secretory APP isoforms, released in conditioned medium, were detected in the molecular weight range of 125–135 kDa. Relative levels of immature and mature APP isoforms and the level of respective secretory APP observed under different cultivation conditions were determined by densitometric scanning (Fig. 3b and c).

Cells cultivated on tissue culture plastic did not show significant differences in intracellular APP biogenesis in the presence of TGFβ and LPS. No significant differences in the ratio of immature APP to mature APP were observed. APP secretion was only slightly affected. In contrast, APP biogenesis of cells cultivated on fibronectin, laminin and also collagen highly sensitive to the mitogens used. Treatment of cells with the cytokine TGFβ increased the appearance of mature transmembrane APP isoforms. Cells cultivated on laminin and collagen showed the greatest accumulation of mature transmembrane APP in the presence as well as in the absence of TGFβ. The ratio of mature transmembrane APP to immature transmembrane APP was in general markedly higher in TGFβ-treated cells than in control cells. This might indicate that transmembrane APP accumulated on the cell surface of TGFβ-treated cells. Secretion was slightly suppressed by cells cultivated on fibronectin and collagen. LPS, known to induce phagocytic activity in microglial cells in vitro, caused an increase in the amount of cellular immature transmembrane APP. In the presence of LPS, the amount of mature transmembrane APP was comparable to control cells (in the case of laminin or collagen) or slightly increased (in the case of fibronectin). High amounts of immature and, concomitantly, low amounts of mature APP may reflected a higher turn over rate of transmembrane APP. This is also evidenced by the significant in-

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Fig. 3. Effect of TGFβ and LPS on APP biogenesis of microglial cells cultivated on different ECM. (a) Western blot analysis of APP from cell lysates and supernatants. Microglial cells were cultivated for 16 h on different substrates without or in the presence of TGFβ (1 ng/ml) and LPS (5 μg/ml). APP of detergent extracts from cells (top panel) and conditioned media (bottom panel) was immunoprecipitated, electrophoresed on a 7% SDS-gel and analyzed by Western blotting as described in section 2. (b) Quantitation of cellular immature and mature APP isoforms. Quantitation was done by densitometric scanning analysis of the immunoblot presented in the top panel of a. The relative amounts of APP are represented in arbitrary units. (c) Quantitation of secretory APP. Quantitation of the bottom panel of a was done as described in b.

crease of APP secretion after treatment with LPS. The fact that cells cultivated on laminin and collagen generally revealed a higher level of mature transmembrane APP and a lower level in secretory APP can be explained by an impairment in APP metabolic and catabolic processes based on a disorganization of the cytoskeleton (unpublished observations).

In addition to a transcriptional regulation of APP expression by TGFβ and LPS, the results also point to a possible posttranslational control of APP biogenesis and metabolism by soluble mediators.

4. Discussion

Based on the observations made in this study on the retroviral immortalized microglial cell line BV-2 we conclude that APP is an important component in the functional behavior of microligal cells within the immune response of the central nervous system. We revealed a critical role of cell–ECM interaction in regulating microglial APP biogenesis and metabolism upon addition of growth and differentiation factors. The responsiveness of microglial cells to soluble factors is controlled by components of the extracellular matrix. Microglial cells cultivated on plastic culture dishes do not show significant changes in APP mRNA expression as well as APP biogenesis and metabolism after treatment with TGFβ and LPS. In contrast, microglial cells grown on ECM substrata like fibronectin, laminin and collagen were highly sensitive to TGFβ and LPS treatment. Transforming growth factor β which has been proposed to have immunosuppressive effects on microglia cells led to an increase in cellular mature transmembrane APP. In contrast, the microglia activating mitogen lipopolysaccharide enhanced secretion of APP under all ECM cultivation conditions, but not on untreated plastic culture dishes. RNA analysis of the appropriate microglial cells has shown that both TGFβ and LPS have an enhancing effect on microglial APP mRNA expression, but generally LPS to a higher extent than TGFβ. Since microglial cells grew in suspension by forming cell aggregates when culture dishes were precoated with collagen, we suggest that cell–cell interactions, possibly based on interactions of cells with cell-associated extracellular matrix, are responsible for increased responsiveness to TGFβ and LPS, rather than interactions of cells with collagen. This is also supported by the fact that microglia cells grown on plastic culture dishes in high density cultures (increased cell–cell interactions) show a significantly higher responsiveness to TGFβ and LPS than microglia grown in low density cultures (impaired cell–cell interactions) (unpublished observations).

Our findings support the hypothesis that growth and differentiation factors like TGFβ and LPS may act synergistically or complementary through signals initiated by ECM–cell or cell–cell interactions. Microglia requires at least one additional signal for modulating APP biosynthesis in response to soluble mediators. The parallel effects of TGFβ and LPS on APP gene expression and APP biogenesis suggest the involvement of transcriptional as well as posttranscriptional events in this response.

The results presented here confirm that APP plays a fundamental role in the migratory behavior of microglial cells. Nevertheless, transmembrane APP and secreted APP possess different functional acitivity within microglial behavior. It is likely that regulative factors like TGFβ and LPS change the balance of the two functional states of APP by modulation of APP expression and processing. As shown in this study, mature transmembrane APP forms were predominantly observed upon addition of TGFβ. Treatment of BV-2 cells with TGFβ in the presence of fibronectin induced the 'ramified' phenotype which often reflects an inactive state of microglia [31]. Indeed, it has been shown that TGFβ suppresses activation and proliferation of microglia [22,23]. Therefore, we suggest that mature transmembrane APP might be needed directly as cell–surface receptor transducing extracellular signals, e.g. chemotactic signals or activation signals, to the interior of the cells. In contrast, activation of microglia with LPS (activated 'ameboid' phenotype) induced increased secretion of APP pointing to a key role of secretory APP in initial pathfinding activities, target recognition and binding. This is in excellent agreement with results obtained from the analysis of APP biosynthetic behavior of peripheral blood T-lymphocytes [5,32]: APP as an inducible molecule is secreted by T-lymphocytes directly upon stimulation with mitogenic lectins, such as phytohemagglutinin (PHA) suggesting an important role of secretory APP in initial processes of T-cell related immune responses.

Thus, the results presented here confirm and extend our earlier conclusion that APP plays a fundamental role in the initial processes of immune responses of the periphery and the central nervous system. Since microglia are often closely associated with amyloid plaques in Alzheimer's disease [8–10], it is suggested that microglial APP and APP fragments are involved in the generation of amyloid plaques. Accumulation of cellular transmembraneous (amyloidogenic) APP caused by environmental changes might be a prerequisite for starting amyloidogenesis. Since it is known that expression of TGFβ is associated with immune responses of the central nervous system [36,37], it is an intriguing hypothesis that TGFβ might play a modulatory key role in these processes. High expression of TGFβ in association with changes in the extracellular matrix, such as increased expression of collagen upon brain injury, could cause an increase in amyloidogenic transmembrane APPs and of its break-down product, the soluble βA4. This could start the process of preclinical amyloid generation if the

concentration of βA4 surpasses the threshold for its aggregation.

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