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Differential regulation of toll-like receptor mRNAs in amyloid plaque-associated brain tissue of aged APP23 transgenic mice

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ABSTRACT

Alzheimer's disease (AD) is characterized by the pathological deposition of amyloid-β protein in the aged brain. Inefficient clearance of amyloid-β from brain tissue is believed to play a major role in the pathogenesis of these deposits. Since amyloid-β clearance likely involves activation of microglial cells via toll-like receptors and since these receptors and their signaling pathways are regarded as potential therapeutic targets, we have studied the expression of toll-like receptor (tlr) mRNAs in an animal model of AD (APP23 transgenic mice). Laser microdissection was used to harvest plaques, tissue surrounding plaques and plaque-free tissue from cortex of aged APP23 transgenic mice and age-matched controls. Real-time RT-PCR was employed to quantify expression levels of different tlr mRNAs in these tissues. This revealed a strong upregulation of tlr2, tlr4, tlr5, tlr7 and tlr9 mRNAs in plaque material compared to plaque-free tissue. In contrast, tlr3 was not significantly upregulated. Plaque-free tissue did not show an increased expression of any tlr mRNAs compared to age-matched control mice. Double-immunofluorescence for TLR2 and the microglial marker Iba1 was used to demonstrate localization of TLR2 on plaque-associated microglia. Taken together, these data show a strong upregulation of mRNAs encoding surface TLRs in plaque-associated brain tissue of aged APP23 transgenic mice. Since TLR-upregulation is restricted to plaques, modifying TLR-signaling may be a promising therapeutic strategy for plaque removal.

A hallmark of Alzheimer's disease (AD) is the deposition of amyloid-β protein in brain [17,22]. In the vicinity of these amyloid-deposits, microglial cells are activated [2]. Although microglial activation is robust, it is insufficient to remove amyloid from the aged brain and may even cause damage to neighboring healthy tissue [1,2,5]. Since therapeutic strategies aimed at modifying the response of microglial cells to amyloid-β have been proposed, it is important to understand the interactions of microglial cells and amyloid-β at the molecular level.

Activation of microglia in AD likely involves toll-like-receptors (TLRs [9,12,14,21,23,24]). These receptors are a group of non-catalytic membrane molecules that belong to the interleukin-1 receptor superfamily [3]. They share the Toll-IL-1-receptor (TIR) domain and recognize molecules not normally found in healthy individuals. TLRs are expressed by immune cells and play a crucial role in the innate immune system. Several different TLRs have been described, which are expressed by different subgroups of immune cells and are activated by molecules sharing specific molecular patterns. In animal models of AD and under in vitro conditions, TLRs have been shown to be expressed by activated microglial cells [9,21,23,24]. In these studies, activation of TLR2, TLR4 and TLR9 increased amyloid removal by microglial cells, suggesting that enhancement of these receptors or enhancement of their signaling could be employed as a therapeutic strategy in AD.

In the present study, we investigated the topographical relationship between the expression of tlr mRNAs and amyloid plaques in APP23 tg mice, a well-established animal model of AD [20]. In these mice a strong microglial reaction can be observed in the vicinity of amyloid plaques [18]. Although the expression of some TLRs has already been qualitatively described in other transgenic mouse models of AD [9,21,23,24], neither the spatial pattern with regard to plaques nor the quantitative level of these changes has yet been analyzed. We, therefore, used the combination of laser microdissection (LMD) and quantitative RT-PCR (qPCR) to measure and compare gene expression of tlr mRNAs in the plaque vicinity and in plaque-free tissue.

20–30 months old male APP23 transgenic mice [20] bred on the C57BL/6 background and age-matched littermate controls were used (for LMD/qPCR: $n = 5–7$ transgenic; $n = 6$ controls; for immunofluorescence: $n = 3$ transgenic; $n = 3$ controls). For LMD/qPCR, mice were killed using an overdose of nembutal, the brain was

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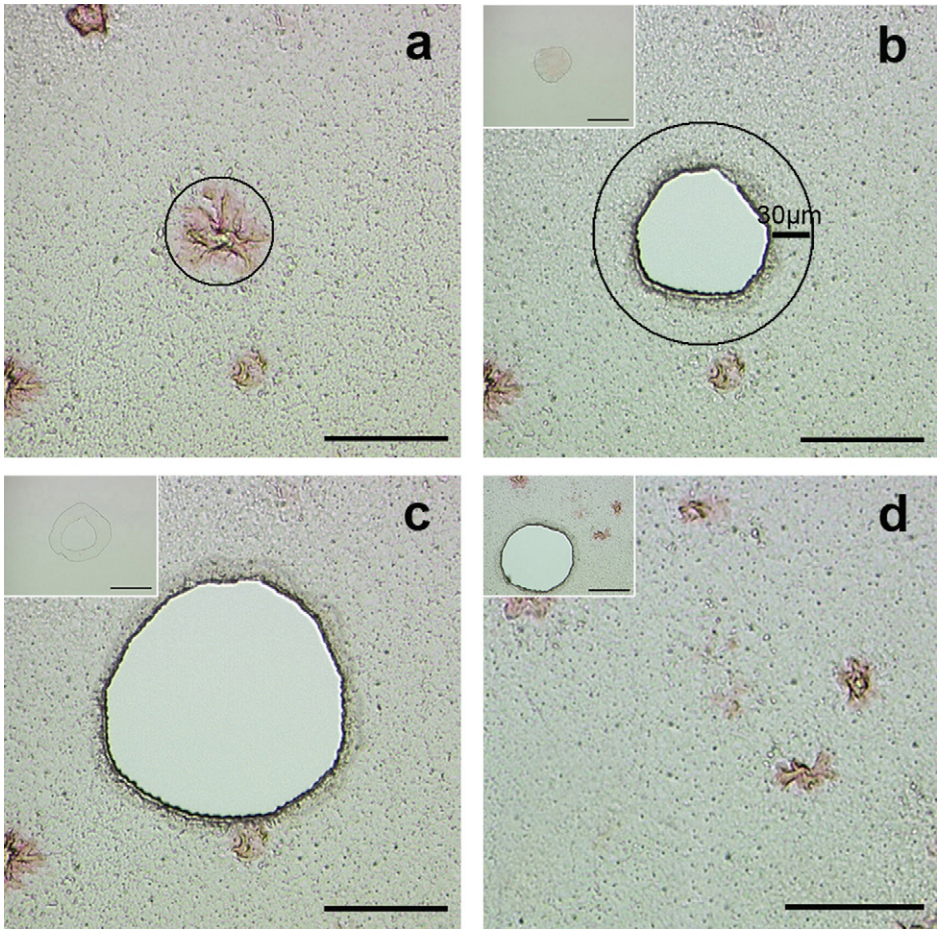

Fig. 1. Microdissection of tissue samples from cortex of aged APP23 transgenic mice. Congo Red-stained plaques were identified in aged APP23 transgenic mice. Plaques, 30 μm wide tissue rings surrounding plaques and plaque-free tissue areas were microdissected. (a) Congo Red-stained plaque in the cortex prior to microdissection. The cutting line is indicated. (b) Same tissue area after the plaque (inset) has been harvested. The cutting line for the tissue ring is shown. (c) Same tissue area after the tissue ring (inset) has been collected. (d) Plaque-free area before and after (inset) microdissection. Scale bars: 100 μm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

removed, embedded in tissue freezing medium and immediately frozen at −40 °C. Frozen tissue was stored at −80 °C until sectioning. 14–20 μm thick sections were obtained by cryostat sectioning at −20 °C, brush-mounted onto autoclaved PET (Polyethylene terephthalat) foil stretched on a metal frame (Leica Microsystems, Wetzlar, Germany), dried on a heater at 40 °C for 8 min and, finally, subjected to histochemical staining. To visualize amyloid plaques, Congo-Red solution (0,2%; Merck, Darmstadt, Germany) was prepared in 0.9% NaCl in 80% ethanol using RNase-free water [8,11]. The solution was applied directly onto the mounted section, incubated for 10 min at RT, and rinsed briefly in DEPC water. After another brief rinse in 80% ethanol, sections were dried on a heater at 40 °C for 10 min and immediately subjected to LMD. LMD was used to harvest plaques, plaque-associated tissue and plaque-free tissue, as previously described (Fig. 1) [8]. Similar amounts of tissue were isolated from plaque-free cortex of non-transgenic age-matched controls of the same genetic background. For a single analysis, a total of approximately 500–1000 samples (e.g., plaques) were picked on serial sections and pooled. Samples were transferred by gravity alone into a microcentrifuge tube cap filled with a guanidine isothiocyanate (GITC)-containing buffer (Buffer RLT, RNeasy Mini Kit, Qiagen, Hilden, Germany) to ensure isolation of intact RNA. Tissue collection was verified by inspecting the tube cap (Fig. 1). Total RNA was isolated using commercially available RNA-extraction kits (Qiagen). RNA integrity of selected samples was determined using the Agilent RNA 6000 PicoLab Chip (Agilent Technologies, Waldbronn, Germany) prior to reverse transcription using TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) to control RNA quality. cDNAs were subjected to qPCR (Abi Prism 7000 sequence detection system or StepOnePlus, Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). Primers were selected spanning two 'exons' in each gene to prevent detection of genomic DNA (Assays on demand, Applied Biosystems; tlr2: Mm00442346_m1; tlr3: Mm00446577_g1; tlr4: Mm00445274_m1; tlr5: Mm00546288_s1; tlr7: Mm00446590_m1; tlr9: Mm00446193_m1). For normalization eukaryotic 18S ribosomal RNA primers and probe were used (Assay on demand, Applied Biosystems: Hs99999901_s1). For all amplifications, a standard amplification program was used (1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min). After normalization with 18S rRNA expression levels for each cDNA, a relative quantitation of target cDNA was performed using $2^{-\Delta c_t}$ values (data are means ± SEM). Statistical analysis of qPCR data was performed using GraphPad Prism and the Mann–Whitney test was used to test for statistical significance ($p < 0.05$).

For double-immunofluorescence labeling against TLR2 and the microglial marker Iba1 standard immunolabeling protocols were employed. In brief, mice were transcardially perfused (0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) and brains were removed. After post-fixation overnight, brains were embedded in 5% agar and cut on a Vibratome (VT 1000S; Leica).