

A β 1-42 induces production of quinolinic acid by human macrophages and microglia

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We hypothesized that the tryptophan catabolites produced through the kynurenine pathway (KP), and more particularly the excitotoxin quinolinic acid (QUIN), may play an important role in the pathogenesis of Alzheimer's disease (AD). In this study, we demonstrated that aggregated amyloid peptide A β 1-42 induced indoleamine 2,3-dioxygenase (IDO) expression and resulted in a significant increase in production of QUIN by human primary

macrophages and microglia. In contrast, A β 1-40 and prion peptide (PrP) 106-126 did not induce any significant increase in QUIN production. These data imply that local QUIN production may be one of the factors involved in the pathogenesis of neuronal damage in AD. *NeuroReport* 14:2311-2315 © 2003 Lippincott Williams & Wilkins.

Key words: A β 1-42; Alzheimer's disease; Human; Indoleamine 2,3-dioxygenase; Macrophages; Microglia; Quinolinic acid

INTRODUCTION

There is evidence that the kynurenine pathway (KP) might be involved in the neurotoxicity associated with Alzheimer's disease (AD) [1-3]. The KP is a major route of L-tryptophan catabolism, resulting in the production of various neuroactive intermediates [4,5]. Of these, the NMDA receptor agonist and neurotoxin quinolinic acid (QUIN) is the most important in terms of biological activity [4]. Macrophages and microglial cells are both able to produce QUIN in neurotoxic concentrations after immune activation [6-8].

AD is the most common cause of dementia. However, the etiopathogenesis of AD is unknown. Two significant abnormalities occur in the brains of AD patients: intraneuronal neurofibrillary tangles (NFT), and deposition of a protein called β -amyloid (A β), which forms extracellular amyloid plaques. Determining which of these is more important in the pathogenesis has been a subject of much controversy [9]. However, most investigators now consider that amyloid deposition in the form of neuritic plaques is the key neuropathological feature in AD. It should be remembered though that this always occurs in the context of inflammation in the form of activated microglia and astrocytes adjacent to the plaques in the vulnerable regions of the AD brain [10,11].

Microglial activation has been detected in the early stages of AD [12]. Indeed, this was almost simultaneous with the appearance of amyloid deposits. Furthermore, the density of

microglia and senile plaques were strongly interrelated. These local inflammatory phenomena may be amplified by the recent demonstration of recruitment of activated monocytes/macrophages from the blood in AD brains [13].

Given the importance of A β and microglia related inflammation in AD we hypothesized that A β could induce QUIN production by microglia and macrophages. We tested this by studying the expression of indoleamine 2,3-dioxygenase (IDO), the first and regulatory enzyme of the KP, using RT-PCR and we measured QUIN *de novo* synthesis using mass spectrometry. We confirmed these results using immunocytochemistry.

MATERIAL AND METHODS

Cell culture: Human peripheral blood mononuclear cells were isolated from blood of healthy volunteers using a standard Ficoll-paque density separation method as described previously [14]. Monocyte-derived macrophages (M ϕ M) were obtained using a classic adherence method. After 8 days *in vitro* the serum component of the medium was eliminated completely, and cells maintained in AIM-V (Life Technologies, Gaithersburg, MD, USA), a serum free medium containing no detectable QUIN. Human brains were obtained from 14- to 19-week-old fetuses collected after miscarriage or termination following informed consent and ethics approval. Microglia were prepared following a method described previously [15]. Both M ϕ M and microglia

were plated in 24-well plates at a density of 1×10^5 cells/ml/well.

Amyloid and prion peptides: A β 1-42, A β 1-42 scrambled and A β 1-40 were obtained from Bachem, Switzerland. The prion proteins PrP106-126, PrP106-126 scrambled were donated by Professor D. Dormont (France) and Prof. C. Masters (Australia) respectively. All the peptides were negative for endotoxin (< 10 pg/ml) as determined by *Limulus* lysate test (Sigma-Aldrich). The physical state of the amyloid peptides was determined using the thioflavin T fluorescence assay. A β 1-40 showed significant amounts of the fibrillar form as did A β 1-42.

Cell stimulation: At 10–11 days old primary human macrophage cultures were treated for 24, 48 and 72 h with 100 IU/ml IFN- γ (R&D Systems, Minneapolis, MN, USA), PrP106-126 (50 μ M), PrP106-126 scrambled (50 μ M), A β 1-42 (25 and 50 μ M), A β 1-42 scrambled (50 μ M), and A β 1-40 (50 μ M) in AIM-V. mRNA and culture supernatants were collected after 6, 16, 24, 48, and 72 h. Each experiment was performed in triplicate using cultures derived from three different donors. Each treatment was performed in triplicate.

Microglial 7–9-day old cultures were stimulated with 100 IU/ml IFN- γ , A β 1-42 (25 μ M), A β 1-42 scrambled (25 μ M), and A β 1-40 (25 μ M) in AIM-V. RNA and culture supernatants were collected after 24, 48, and 72 h. Each experiment was performed in triplicate using cultures derived from two different fetal brains. Each treatment was performed in duplicate. Cell viability was estimated using a standard trypan blue staining method.

Immunocytochemistry: The method for brain cell characterization has been described previously [15]. Immunocytochemical studies have shown that more than 99% of cultured microglia strongly express the monocytic markers CD14, CD11c and CD68 at day 5 after seeding. No cells stained for GFAP, galactocerebroside, MAP2 or factor VIII (data not shown). In the macrophage cultures, after 11 days, 98% of the adherent cells strongly expressed the macrophage markers including CD68, CD11c and CD16. No CD3 staining was detected (data not shown).

Mdm were grown in permanox chamber-slides for 2 to 3 days. After 24 h control (untreated) and treated (A β 1-42 or IFN- γ) cells were fixed with acetone/methanol (vol/vol) for 20 min at -20°C . Cells were then rinsed three times with PBS and a gentle membranous permeabilization was performed by incubation with 0.025% Triton X-100 in PBS for 10 min at room temperature. After washing, cells were incubated with 5% normal goat serum (NGS) in PBS for 45 min at room temperature, rinsed twice with PBS and incubated for 1 h at 37°C with selected primary antibody mAb IDO (donated by Professor O. Takikawa, Japan) or pAb QUIN (Chemicon, Australia, VIC) diluted in 5% NGS. Cells were then washed with 5% NGS solution and incubated for 1 h at 37°C in the dark with the appropriate labelled secondary antibodies (Goat anti-mouse IgG1 or goat anti-rabbit coupled with FITC or Texas Red; from Southern Biotechnology Associated, Birmingham, AL, USA). In order to quantify the percentage of labeled cells,

nuclear staining was performed using DAPI at 1 μ g/ml for 10 min at room temperature. After several washings with PBS at 37°C , the coverslips were quickly mounted on glass slides with Fluoromount-G, and were examined with an Olympus BX60 fluorescence microscope associated with a digital SensiCam. The following three controls were performed for each labelling experiment: (1) isotypic antibody controls, (2) incubation with only the secondary labeled antibodies, and (3) estimation of auto-fluorescence of unlabelled cells.

GC-MS quantification of QUIN production: After 72 h culture supernatants were assayed for QUIN as described previously [14]. QUIN samples were analyzed by GC-MS with the spectrometer operating in electron capture negative ionisation mode. Selected ions (m/z 273 for PIC and m/z 277 for d4-PIC) were then monitored. All results are expressed as the mean \pm s.e.m.

RT-PCR of IDO mRNA expression: This method has been described previously [16]. Experiments were performed in triplicate.

Statistical analysis: Mean values and standard errors were calculated for each treatment at all time points, and the results were plotted on a histogram (Fig. 3). Unpaired *t*-tests were performed on the results. *p*-values were generated comparing QUIN production from the various treatments to the negative controls. *p* < 0.05 was regarded as statistically significant.

RESULTS

A β 1-42 peptide induces mRNA expression of IDO in human fetal microglia and adult macrophages: Using RT-PCR (Fig. 1), unstimulated macrophages (Fig. 1a) and microglia (Fig. 1b) did not express IDO mRNA. After stimulation with IFN- γ 100 IU/ml, macrophages strongly expressed IDO mRNA at 6, 16, 24, 48, and 72 h. However, IDO mRNA was only transiently expressed and detected at 16 h after stimulation with A β 1-42 (25 μ M). IDO expression induced by A β 1-42 was 11 fold lower than IDO expression induced by IFN- γ (Fig. 1c). Similarly, microglia stimulated with A β 1-42 (25 μ M) and IFN- γ 100 IU/ml expressed IDO transcripts but in the case of A β 1-42 this only occurred after 16 h. In contrast with macrophages, IDO expression induced by A β 1-42 in microglia was only 1.7-fold lower than that induced by IFN- γ (Fig. 1c).

A β 1-42 peptide induces production of IDO protein in human fetal microglia and adult macrophages: We confirmed that unstimulated macrophages did not express IDO and did not produce any detectable amount of QUIN using immunocytochemistry (Fig. 2). After treatment with A β 1-42 (25 μ M) for 24 h, IDO expression (red staining) was induced and the protein was detectable with a perinuclear localization. QUIN production (green staining) was also detected within the cytoplasm and appeared mostly concentrated around the nucleus. After treatment with IFN- γ (100 IU/ml) for 24 h, the IDO expression was very high and the protein was detectable within almost all of the cytoplasm. QUIN

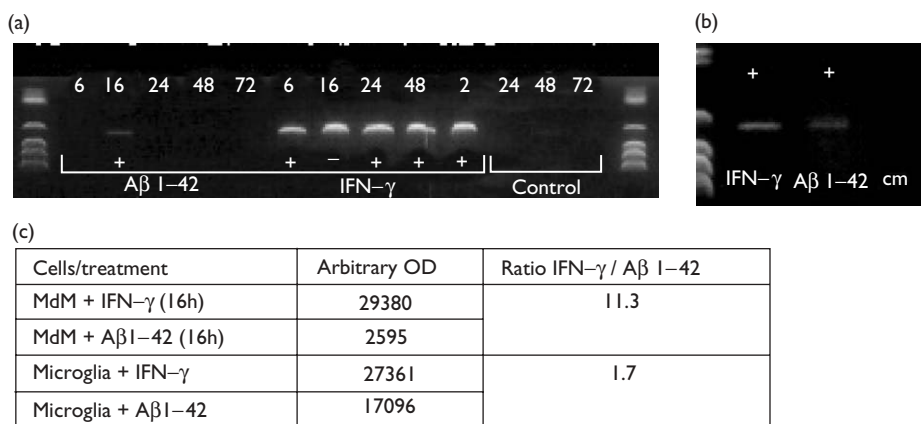


Fig. 1. Detection of IDO mRNA expression using RT-PCR. (a) Time scale (h) of IDO expression in Monocyte-derived macrophages (MdM) after stimulation with Aβ1-42 (50 μM), IFN-γ (100 IU/ml), and unstimulated (control). (b) IDO expression in microglia after stimulation with IFN-γ (100 IU/ml), Aβ1-42 (50 μM) and unstimulated (control). (c) Semi-quantification of mRNA expression. The intensity of RT-PCR signals has been quantified using the application ImageJ 1.3, NIH, USA. Intensity of the IDO mRNA expression 16 h after stimulation with Aβ1-42 (25 μM) was compared with the IDO mRNA expression 16 h after stimulation with IFN-γ (100 IU/ml) for both macrophages and microglia.

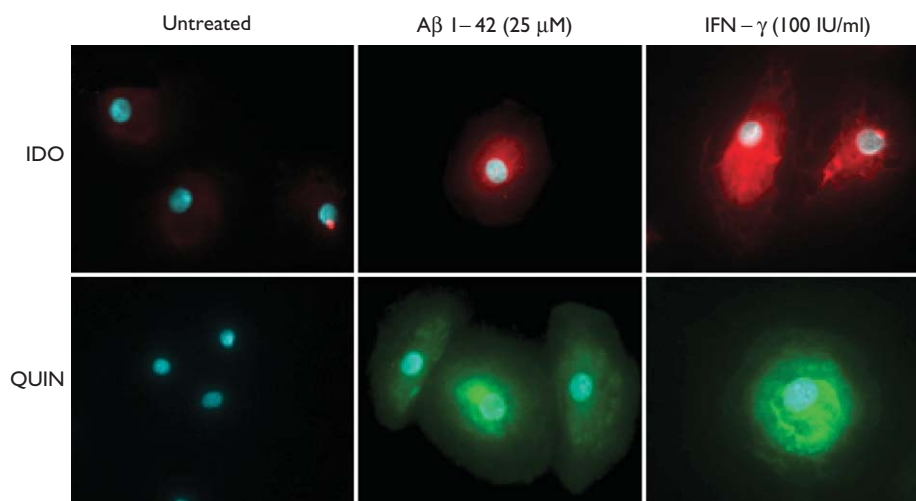


Fig. 2. Immunocytochemical detection of IDO expression (Upper line, red) and QUIN production (Bottom line, green) in untreated Monocyte-derived macrophages (MdM) (left), stimulated with Aβ1-42 (25 μM; center), and IFN-γ (100 IU/ml; right). Magnification × 400.

production was also higher and present within the cytoplasm mostly concentrated around the nucleus.

IFN-γ (100 IU/ml) were used as positive controls for QUIN production.

Aβ1-42 peptide induces human fetal microglia and adult macrophages to produce QUIN (Fig. 3): After 72 h only stimulation with Aβ1-42, but not Aβ1-42 scrambled, PrP106-126, PrP106-126 scrambled or Aβ1-40 induced human adult macrophages to produce significant amounts of QUIN (Fig. 3a). Moreover, this production was dose dependent: Aβ1-42 (50 μM) led to synthesis of 345 nM QUIN and Aβ1-42 (25 μM) to 178 nM QUIN (a 51% difference). Similarly, human fetal microglia produced significant amounts of QUIN after stimulation by Aβ1-42 (25 μM) but not Aβ1-40 (25 μM; Fig. 3b). After 72 h unstimulated macrophages and microglial cells produced only a very low amount (basal) of QUIN (24 nM). Macrophages and microglia stimulated with

DISCUSSION

The present study demonstrated that amyloid peptide Aβ1-42 can induce IDO expression, the main regulatory enzyme of the KP, and lead to QUIN production in potentially neurotoxic concentrations by human microglia and macrophages.

In inflammatory conditions affecting the brain, there is evidence suggesting a major role for resident microglia and infiltrating macrophages in the overproduction of neuroactive metabolites, particularly QUIN, generated through the KP [17,18]. Elevated serum concentrations of kynurenine (KYN) have been found in AD with the degree of elevation correlating with the level of cognitive impairment [1,2]. The

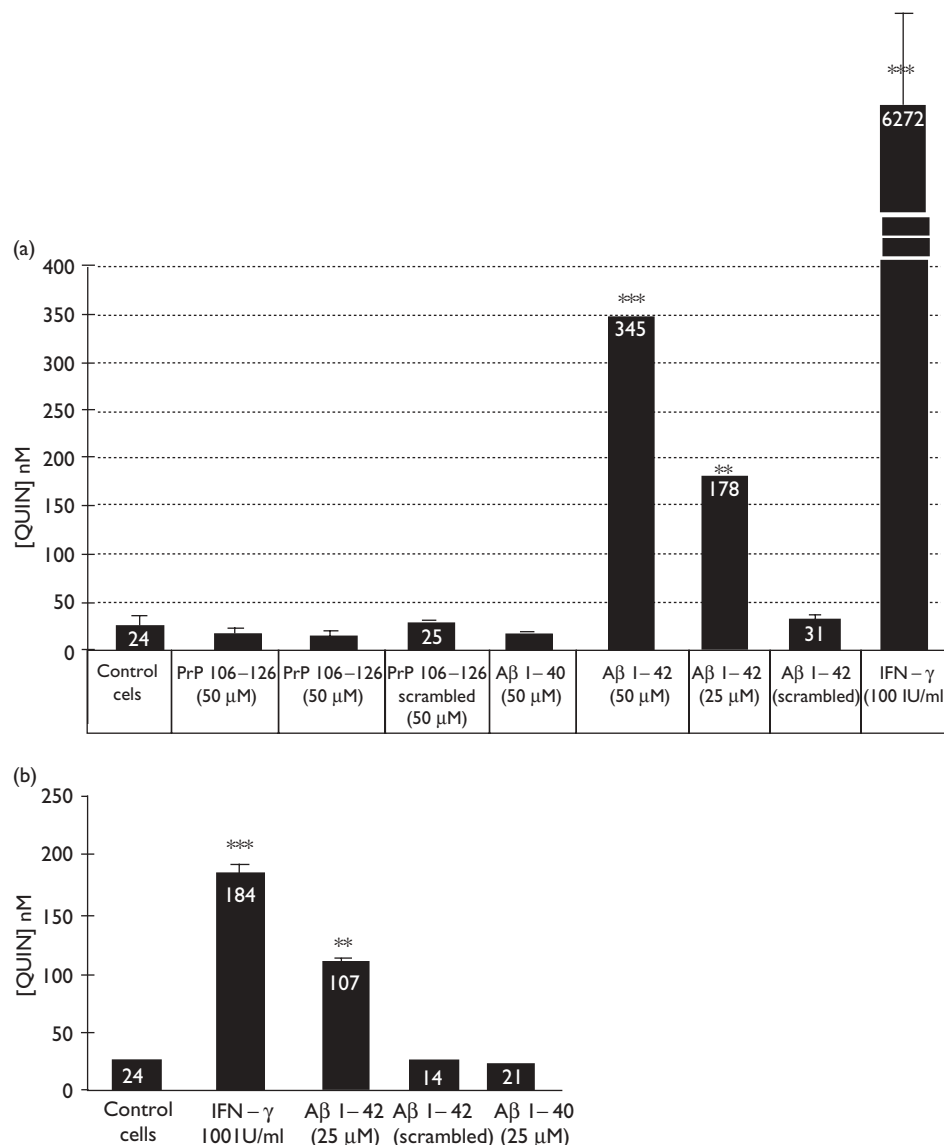


Fig. 3. Quantification of QUIN production using gas chromatography-mass spectrometry. (a) Monocyte-derived macrophages (Mdm): *significant vs respective control cells (no treatment); ** $p < 0.01$; *** $p < 0.005$ (Mann-Whitney test). (b) Microglia: *significant compared with respective control cells (no treatment); ** $p < 0.05$; *** $p < 0.01$ (Mann-Whitney test).

KP is switched on when microglia, macrophages and astrocytes are activated. Given that there are increased numbers of activated astrocytes, microglia and infiltrating macrophages around senile plaques, it is biologically plausible that the KP is important in AD. There are several recent lines of evidence that over-production of QUIN can amplify local inflammation around senile plaques and augment the associated neurotoxicity. We recently found that QUIN induces astrocytes to produce large quantities of chemokines and upregulates chemokine receptor expression in astrocytes [19]. Chemokines and chemokine receptors are increasingly being recognized as important in this aspect of AD pathogenesis [20]. We have previously shown that QUIN can induce IL1- β mRNA expression in astrocytes and macrophages [21]. A β can induce the production of certain cytokines that have been found in association with plaques:

IL1- β , IL-6 and TNF- α [22]. Each of these cytokines is directly or indirectly neurotoxic and associated with astrocytes and microglia activation and proliferation. Additionally, QUIN-induced experimental injury of rat brain leads to an immediate increase in extracellular glutamate [23]. The importance of this is highlighted by the fact that QUIN and A β are both able to inhibit glutamate uptake in astrocytes [24] thereby augmenting glutamate associated neurotoxicity.

CONCLUSION

These results imply that QUIN may be, locally and probably indirectly, one of the factors involved in the pathogenesis of neuronal damage in AD. This will open a new and important therapeutic door using different KP inhibitors

already available [25] to limit the local QUIN related inflammation and associated neuronal death thereby leading to neurological improvement in AD patients.

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