Curcumin inhibits dose-dependently and time-dependently neuroglial cell proliferation and growth

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Abstract

OBJECTIVES: Curcumin (CUR), the active chemical of the Asian spice turmeric, has strong anti-oxidant and anti-inflammatory properties. CUR inhibits proliferation and growth of several cell types, e.g. cancer cells. While CUR inhibitory effects on microglial cells are demonstrated, little is known of its effects on neuroglia, astrocytes (AST) and oligodendrocytes (OLG). Our work focuses on CUR's effects on neuroglial proliferation and growth in vitro, utilizing C-6 rat glioma 2B-clone cells, a mixed colony of both neuroglial cells, in 6 day trials.
METHODS: The doses studied included 4, 5, 10, 15, and 20 microM – concentrations slightly smaller than those shown to stimulate protein expression in ASTs. Automated particle counter was used to determine proliferation, and marker enzyme assays were used to determine AST and OLG activity.
RESULTS: CUR inhibited neuroglial proliferation, with the degree of inhibition correlated directly with the CUR concentration. Proliferative inhibition was observed after a concentration as low as 5 microM by day 6, while inhibition of 20 microM doses occurred by day 2 of culture. Proliferative inhibition is associated with morphological changes, e.g. cell elongation and neurite prolongation, and increased activity of a marker enzyme corresponding to differentiation of OLG and with a reduced activity of the marker enzyme for AST.
CONCLUSIONS: Our data suggests CUR acts continuously over a period of time, with low doses being as effective as higher doses given a longer period of treatment. It has been suggested that CUR's anti-inflammatory and anti-oxidant actions may be useful in the prevention-treatment of neurodegenerative diseases, e.g. Alzheimer's and Parkinson's Diseases. Given neuroglial involvement in these diseases, and CUR's observed actions on neuroglia, the data presented here may provide further explanations of CUR's preventative-therapeutic role in these diseases.

ORIGINAL ARTICLE

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Abbreviations and Symbols:

CUR	= curcumin
μM	= microMolar
mL	= milliliter
AST	= astrocytes
OLG	= oligodendrocytes
GS	= glutamine synthetase
CNP	= 2′ 3′-cyclic nucleotide 3′-phosphohydrolase
NF-κβ	= nuclear factor kappa beta
AP-1	= activator protein one
Aβ	= amyloid-beta
AD	= Alzheimer's Disease

Introduction

Several properties have been confirmed for curcumin (CUR), the active chemical in the curry spice turmeric. Extracellularly, CUR acts as a strong anti-oxidant [1, 2], an anti-inflammatory agent [3], and reduces free radical production [4]. CUR is a small, lipophilic molecule that can pass through the cell membranes and exert intracellular effects as well. CUR inhibits COX-I and COX-II [5] and several phospholipases [6, 7], enzymes involved in inflammation. CUR also inhibits transcription factors, including nuclear factor kappa beta (NF-κβ) involved in the expression of inflammatory cytokines [8], and activator protein one (AP-1) [9] associated with amyloid-beta (Aβ) peptide, a hallmark of Alzheimer's Disease (AD).

CUR's most observed property is its pronounced anti-proliferative action, described in several cell types, including colon [5] and microglial [10] cells as well as its ability to induce apoptosis in cancer cells [11]. Although CUR promotes expression of heme oxygenase-1 [12] and suppress the release of nitric oxide [13] in astrocytes cultures, its effects on neuroglial cell proliferation, maturation and function are still largely unknown. We investigated the effects of CUR *in vitro*, using C-6 rat glioma 2B-clone cells, a colony of mixed glial cells, which are precursors of both astrocytes and oligodendrocytes. We investigated (1) CUR anti-proliferative and maturation effects, (2) at which doses these effects are observable, utilizing a range of CUR concentrations – 4, 5, 10, 15, and 20 μM, and (3) the time interval for any CUR effects to occur.

Given the high levels of oxidation and inflammation that occur in AD, recent studies have investigated curcumin for prevention or treatment of this disease in Aβ-infused rats or with an Alzheimer transgenic AP-PSw mouse model (Tg2576), with promising results behaviorally [14], with reversed cognitive deficits, and biochemically [10], with a reduction in brain inflammation and senile plaques. Due to the significant role neuroglial cells play in neuronal metabolism and neurotransmission (AST), and myelin formation (OLG), and given curcumin's prevention-treatment potential of neurodegenerative diseases, we considered important to explore how CUR may affect neuroglia.

Material and Methods

Cell Culture. Curcumin-treated and non-treated (control) C-6 rat glioma 2B-clone cells were grown for a span of six (6) days in 7 mL of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin-fungizone, under conditions of 37°C, 5% CO_2, and 95% humidity. On days two, four, and six 7 mL of medium with CUR was replenished. The choice of the time in culture is based on data from our previous studies and those from other laboratories showing that by the sixth day, the cells have reached a stable proliferative optimum.

Cell proliferation was measured by using an automated particle counter, Coulter Particle Counter Z_1. Cell cultures were washed with phosphate buffer solution (PBS) and treated with trypsin and DMEM to form cell solutions. Cell counts were taken by diluting 200 μL of cell solution sample in 10 mL Isoton. Cell counts were taken on days 2, 4, and 6.

CUR stock solutions were made by dissolving various amounts of CUR into 10 mL of 100% ethanol. The amount of CUR measured into each stock solution was calculated on the basis that 20 μL of CUR stock solution would be added to 7 mL of DMEM to have concentrations of 4, 5, 10, 15, and 20 μM. Because of ethanol's own slight anti-proliferative properties, each control cell culture had 20 μL of 100% ethanol for 7 mL of medium.
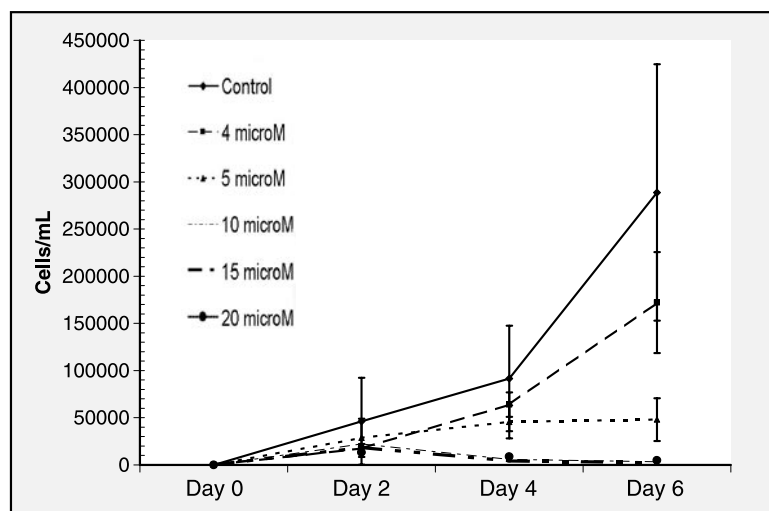

Figure 1. Cell proliferation was measured using cell counts by automated particle counter. By day 6 all concentrations of 5 μM and higher showed significant cell proliferative inhibition. The higher the dose, the greater and quicker the inhibition, with 20 μM showing inhibition by day 2, while 5 μM not showing significant inhibition until day 6. The concentration of 4 μM did not statistically significantly inhibit proliferation by day 6. (All cultures started with 10^6 cells. For graphical representation the starting cell number is 0.)
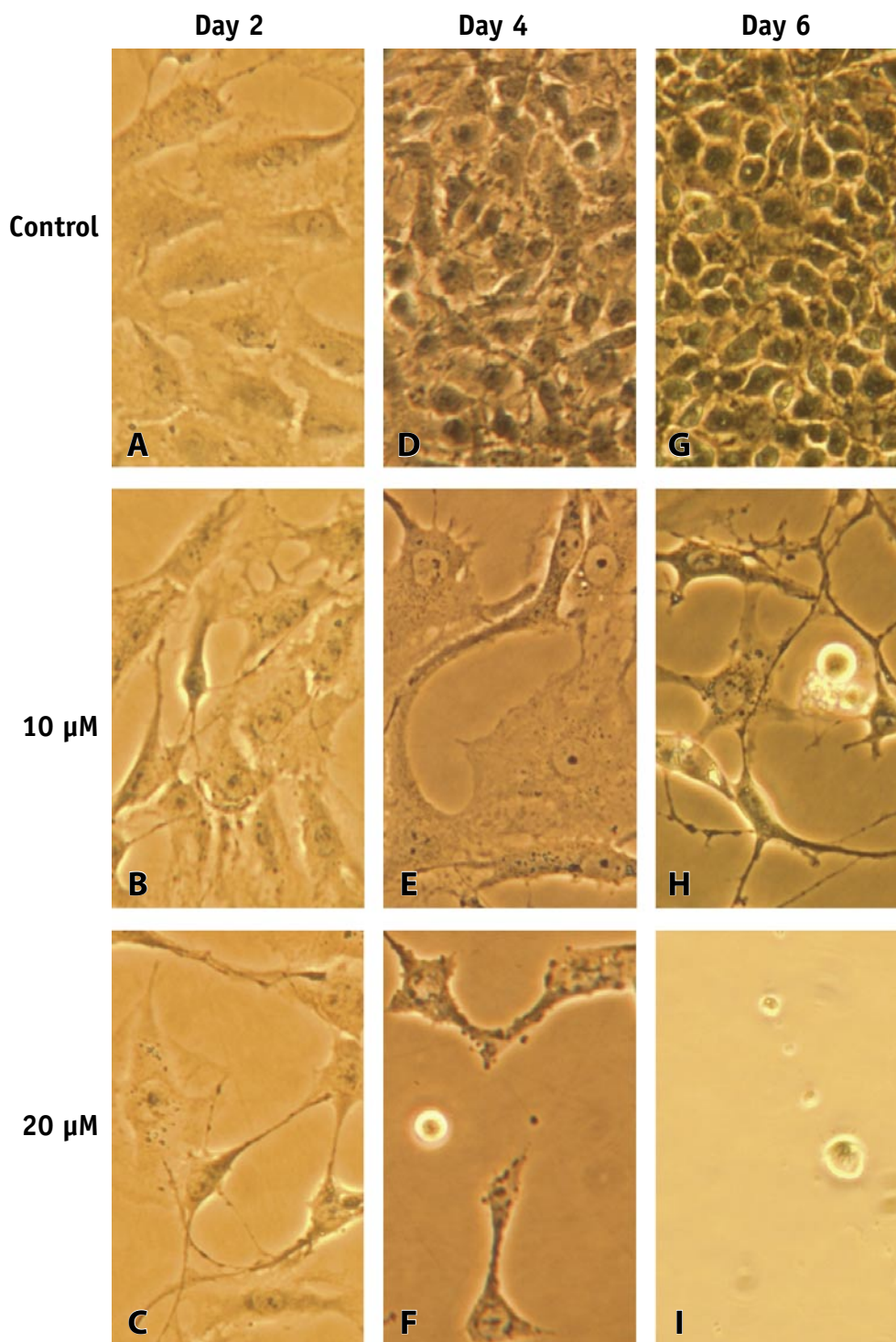
Figure 2. Morphology. Change in morphology is observed in curcumin treated cells (200X). The control cells (2A, 2D, 2G) remain, circular, and tightly packed throughout the 6 days. In contrast, both the 10 μM and 20 μM cultures show cells that are much larger, more loosely packed, with significantly more cell elongations and neurites. Toxic effects are also noticeable in the 20 μM culture by day 6 (2I).

Commercial Sources of Curcumin. Two commercial sources of curcumin were tested to determine if there was any variance in efficacy between companies. Curcumin was supplied by Acros Organics (Pittsburg, PA; Catalog No. 458-67-7) and Sigma-Aldrich (St. Louis, MO; Catalog No. C-7727). Both sources showed nearly identical effects (concentrations tested at 5 μM and 10 μM). We preferred to use the Acros curcumin because it was less expensive, had a higher purity level (minimum purity of 98%, versus Sigma-Aldrich with

a minimum purity of 94%), and Acros curcumin dissolved better in ethanol.

Cell Differentiation. Glutamine synthetase (GS) is a marker enzyme for astrocytes. GS converts toxic glutamate to non-toxic glutamine. The GS marker enzyme assay [15] detects a color change which is proportional to enzyme activity. The 2'3'-Cyclic Nucleotide 3'-Phosphohydrolase (CNP) assay [16] was used to detect oligodendrocytes. CNP is involved in the myelination of neuronal axons. This assay also involves a

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color change which is proportional to enzyme activity. All results were normalized to total protein content. Assays were performed on day 6, and only curcumin concentrations of 10 μM and 15 μM were tested.

Results

There are two options for a cell that stops proliferating: i) differentiate into a more mature cell, or ii) undergo apoptosis. Both effects are observed with CUR treatment, depending on the concentration and the duration of treatment. Over a 6 day trial, CUR inhibits neuroglial cell proliferation dose-dependently at concentrations as low as 5 μM, with the higher the concentration, the greater the inhibition (Figure 1). This inhibitory action also follows a timetable depending on concentration, with the higher the dose, the shorter the onset time of inhibition of proliferation. All concentrations tested showed significant inhibition by day 6, except for the 4 μM concentration (lowest concentration tested). Although the proliferative inhibition is not statistically significant in the 4 μM group, cell numbers start to decline by day 6 relative to day 0 (Figure 1).

This time-dependent action is also observed in changes of morphology. The control cells remain small and circular, and continue to proliferate throughout the 6 days (Figures 2A, 2D, 2G). In contrast, both the 10 μM and 20 μM cultures show cells that are much larger with significantly more cell elongations and neurites, and are less densely packed than the control cells. However, morphological changes are observable in the 20 μM culture by day 2 (Figure 2C), while it is not until day 4 that morphological changes are seen in the 10 μM culture (Figure 2E). It is only on day 6 that the 10 μM culture (Figure 2H) truly resembles the morphology exhibited in the 20 μM CUR treated cells seen on day 2 (Figure 2C).

CUR treated cells showed an increased expression of the marker enzyme CNP at both concentrations tested (Figure 3). This is indicative of a more differentiated and mature neuroglia, and coincides with the morphologic changes (Figure 2). At the same time, there was a reduction of the marker enzyme GS in the higher CUR concentration (15 μM) treated cells, indicative of an inhibitory effect on AST (Figure 4).
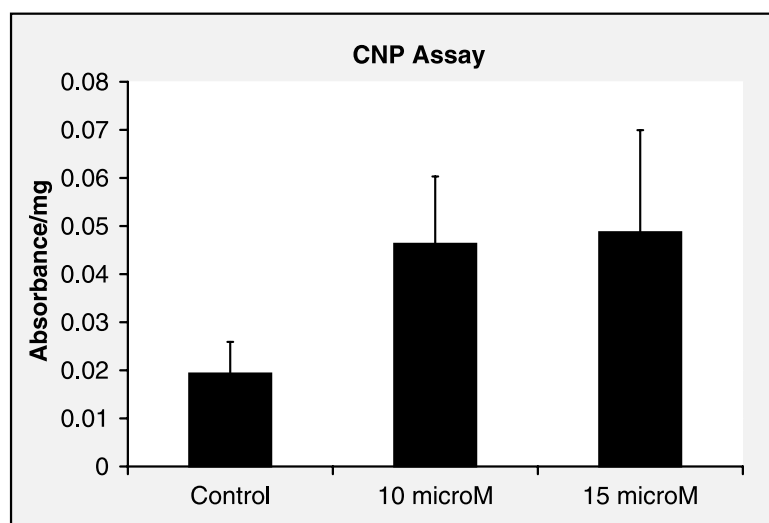

Figure 3. 2'3'-Cyclic Nucleotide 3'-Phosphohydrolase (CNP) Assay. This assay detects a marker enzyme for oligodendrocytes. Both curcumin concentrations of 10 μM and 15 μM statistically significantly increased CNP enzyme activity over the control, suggesting that the CUR cultures have a larger presence of oligodendrocytes. All values normalized to total protein content (n = 6).
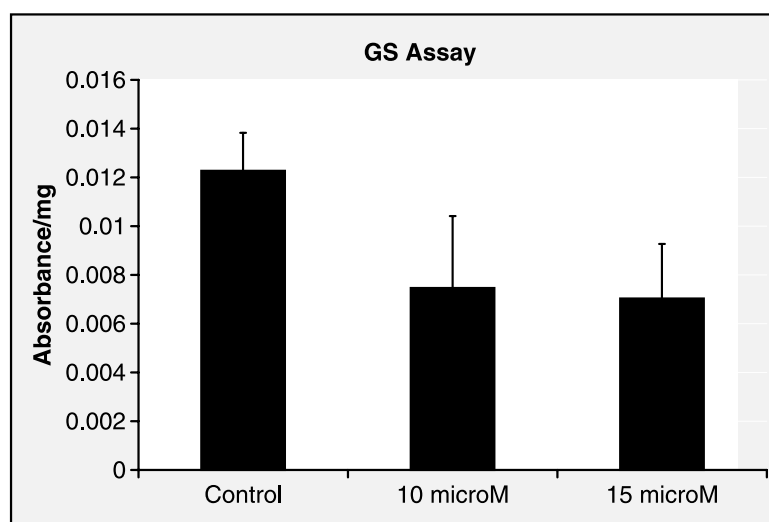

Figure 4. Glutamine Synthetase (GS) Assay. This assay detects a marker enzyme for astrocytes. The 15 μM CUR concentration showed a statistically significantly decrease of GS activity over the control. All values normalized to total protein content (n = 6).

Discussion

It is generally recognized that the inflammation with increased microglia and astrocytic gliosis that surrounds the amyloid plaques, the neurofibrillary debris and other pathologic lesions characteristic of neurodegenerative diseases may contribute to their etiology and progressive worsening [17]. The beneficial effects of curcumin in prevention/treatment of these diseases may be due to its antinflammatory actions through inhibition of microglia and astrocytic proliferation. The reduction of the astrocytic marker enzyme GS is consistent with previous studies of CUR *in vivo* that showed reduction of another astrocytic marker, GFAP [10]. The association of a decline in cell proliferation with the acquisition of adult morphology and the rise in specific enzyme activity of CNP activity between CUR-treated and control cells (Fig. 3), suggests a preferred stimulatory action of curcumin on OLG, and an inhibitory effect on AST. In our present data, curcumin appears to act on neuroglia cells by promoting oligodendritic differention, improving myelinogenesis, and reducing astrocytic proliferation.

The range of concentrations used was based on a report by Scapagnini et al. (see citation 12) that cited concentrations of $15 \mu M$ to $30 \mu M$ as optimal concentrations, while concentrations above 50uM promoted toxicity in astrocyte cultures. The data presented here suggest concentrations as low as $5 \mu M$ are effective on neuroglial cultures, with necrotic or apoptotic effects observable in the $20 \mu M$ concentration. The $20 \mu M$ concentration showed a decrease in cell count numbers by day 6 relative to its original cell concentration, accompanied by a dramatic change in morphology from the large cells with many projections as seen on day 2 (Figure 2C) to very few and small round cells as seen on days 4 and 6 (Figures 2F and 2I). This difference between our findings and the reported literature may simply be due to the difference in duration of treatment, as the Scapagnini study tested CUR for only 6 to 24 hours, while our study was for 6 days (6 to 24 times longer period), further indicative of CUR's time-dependent action.

The present data suggest that dose and time factors should be considered in further CUR research. For neuroglial cell culture and other *in vitro* experiments, concentrations between $15 \mu M$ and $30 \mu M$ are more effective for short trials (< 24 hours), while concentrations between $5 \mu M$ and $15 \mu M$ are better suited for longer studies (4 to 6 days). It is foreseeable that the $4 \mu M$ concentration would show inhibition of proliferation if treated for a longer period (8 to 10 days). Our data may have implications for clinical research and other *in vivo* research involving CUR. As CUR may be a preventative-treatment for neurodegenerative diseases, including AD, due to its anti-inflammatory and anti-oxidant properties, and for cancer treatments due to its anti-proliferative properties, CUR's activity and bioavailability concentration outside of the gastrointestinal tract is a concern. It is possible that very small doses ($\leq 1 \mu M$) may be as effective as higher doses if used for a longer period.

REFERENCES

1 Subramanian M, Sreejayan, Rao MN, Devasagayam TP, Singh BB. Diminution of singlet oxygen-induced DNA damage by curcumin and related antioxidants. Mutat Res 1994; **311**:249–255.

2 Mukundan MA, Chacko MC, Annapurna VV, Krishnaswamy K. Effect of turmeric and curcumin on BP-DNA adducts. Carcinogenesis (Oxford) 1993; **14**:493–496.

3 Huang MT, Lysz T, Ferraro T, Abidi TF, Laskin JD, Conney AH. Inhibitory effects of curcumin on in vitro lipoxygenase and cyclo-oxygenase activities in mouse epidermis. Cancer Res 1991; **51**: 813–819.

4 Zhao BL, Li XJ, He RG, Cheng SJ, Xin WJ. Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. Cell Biophys 1989; **14**:175–185.

5 Ramsewak RS, DeWitt DL, Nair MG. Cytotoxicity, antioxidant and anti-inflammatory activities of Curcumins I-III from Curcuma longa. Phytomedicine (Jena) 2000; **7**:303–308.

6 Yamamoto H, Hanada K, Kawasaki K, Nishijima M. Inhibitory effect of curcumin on mammalian phospholipase D activity. FEBS Letters 1997; **417**:196–198.

7 Rao CV, Rivenson A, Simi B, Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res 1995; **55**:259–266.

8 Xu YX, Pindolia KR, Janakiraman N, Chapman RA, Gautam SC. Curcumin inhibits IL1 alpha and TNF-alpha induction of AP-1 and NF-kB DNA-binding activity in bone marrow stromal cells. Hematopathol Mol Hematol 1998; **11**:49–62.

9 Huang TS, Lee SC, Lin JK. Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. Proc Natl Acad Sci USA 1991; **88**:5292–5296.

10 Lim GP, Chu T, Yang F, Beech W, Frautschy SA, Cole GM. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J Neurosci 2001; **21**: 8370–8377.

11 Ruby AJ, Kuttan G, Babu KD, Rajasekharan KN, Kuttan R. Anti-tumour and antioxidant activity of natural curcuminoids. Cancer Letters 1995; **94**:79–83.

12 Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: A novel class of heme oxygenase-1 inducers. Mol Pharmacol 2002; **61**:554–561.

13 Soliman KFA, Mazzio EA. In vitro attenuation of nitric oxide production in C6 astrocyte cell culture by various dietary compounds. Proc Soc Exp Biol Med 1998; **218**:390–397.

14 Frautschy SA, Hu W, Kim P, Miller SA, Chu T, Harris-White ME, Cole GM. Phenolic anti-inflammatory antioxidant reversal of Abeta-induced cognitive deficits and neuropathology. Neurobiol Aging 2001; **22**:993–1005.

15 Rowe B, Ronzio RA, Wellner VP, Meister A. Glutamine Synthetase (Sheep Brain). Meth Enzymol 1970; **17**:900–902.

16 Prohaska JR, Clark DA, Wells WW. Improved rapidity and precision in the determination of brain 2′,3′-cyclic nucleotide 3′-phosphohydrolase. Anal Biochem 1973; **56**:275–82.

17 Griffin WST, Sheng JG, Royston MC, Gentleman SM, McKenzie JE, Graham DI, et al. Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. Brain Pathol 1998; **8**:65–72.