

Pro-inflammatory Cytokines Modulate Glial Apolipoprotein E Secretion

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Abstract: Alzheimer's disease (AD) is a neurological disorder characterized by plaques and an elevated immune response. Specifically, increased expression of interleukin (IL)-1 and tumour necrosis factor (TNF)- α , has been observed in AD cerebrospinal fluid and temporal brain tissue. Both of these immunomodulators were shown to carry genetic variants that increase the risk of developing AD. Studies have also established the apolipoprotein E (apoE) gene to be a risk factor for AD with $\epsilon 4$ carriers having been found to show lower levels of brain apoE. In the present study, treatment of primary rat mixed glial cell cultures with IL-1 β induced a significant increase in extracellular apoE protein. In contrast, treatment primary rat astrocyte and mixed glial cell cultures with TNF- α significantly reduced extracellular apoE protein levels. These results are consistent with the notion that elevated cytokine expression directly modulates immunosuppression and indirectly apoE-mediated neuronal remodeling.

Keywords: Apolipoprotein E, Alzheimer's disease, interleukin-1 β , tumor necrosis factor- α .

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder identified by the presence of neurofibrillary tangles, neuritic plaques composed of beta-amyloid (A β), dystrophic neurites, and cortical atrophy. Recently, the risk of developing sporadic AD has been associated with a well-known polymorphism in the apolipoprotein E (apoE) gene. Unlike rodents, three separate human apoE isoforms have been identified, namely apoE $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ [1]. Individuals bearing two copies of the apoE $\epsilon 4$ allele have been found to: (1) be at significantly greater risk for AD and (2) develop symptoms at an earlier age of onset [2,3]. In addition, apoE $\epsilon 4$ AD patients have been reported to have lower levels of apoE in the brain [4,5].

Functionally, apoE appears to facilitate internalization of lipid complexes, including very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL), via interactions with cell surface receptors [6]. Studies have established an increase in apoE protein [7] following brain lesions, as well as apoE modulation of neurite branching and extension *in vitro* [8,9]. Consequently, it has been suggested that increased levels of apoE may mediate membrane and synapse formation, as well as nerve regeneration and re-myelination, during development and following injury [7,10]. In AD, reduced levels of apoE may significantly inhibit apoE-mediated lipid transport and homeostasis, synaptic plasticity, and A β clearance [5,11].

In human AD brain, studies have illustrated up-regulation of complement proteins [12] and elevated expression of interleukin (IL)-1 and tumour necrosis factor (TNF)- α [13,14]. Functionally, increased cytokine expression in AD

has been suggested to facilitate chronic inflammatory processes via activation of astrocytes and microglia with subsequent glial production of pro-inflammatory mediators [15], all of which may contribute to neuronal damage. Several independent studies have now reported significant genetic associations between polymorphisms in the (IL)-1b and (TNF)- α genes and sporadic AD [16-18].

Prior work has established a relationship between apoE and the immune system. Specifically, TNF- α treatment of HepG2 [19], 3T3-L1 adipocytes [20], the tumoral astrocytoma CCF-STTG1 [21] has been found to decrease apoE expression and/or secretion to different degrees. Furthermore, IL-1 treatment of different cell lines including HepG2 [19], the astrocytoma CCF-STTG1 [21] and human astrocytes [22] has been shown to increase or have no effect on apoE expression and/or secretion. The variation in these results has led to the hypothesis that cytokine-mediated apoE modulation may be, in fact, a cell-type specific response [23].

Based upon the relationship between apoE and the immune system and the potential benefits of apoE in immune and lipid regulation, we hypothesize that elevated cytokine expression, as seen in AD, may modulate glial apoE production, thereby influencing apoE-mediated cholesterol homeostasis, as well as subsequent remodelling efforts. With various inconsistencies having been established in cell line experiments, the objective of the current study was to examine the effects of pro-inflammatory cytokines on apoE protein regulation in terminally differentiated primary rat glial cells.

MATERIALS AND METHODS

Tissue Cell Culture

Cell culture solutions and supplies were purchased from Gibco (Grand Island, NY). All experimental protocols were granted ethics approval under the provisions of the McGill University Animal Care Committee and Canadian Council

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on Animal Care. Primary rodent cell cultures were prepared as previously described [24]. Briefly, primary astrocyte cell cultures were isolated from the cortices of one-day-old Sprague-Dawley rat pups (Charles River Laboratories Inc., Wilmington, MA). Following mincing in ice-cold dissection medium [Dulbecco's modified Eagle's medium (DMEM) with 20 mM HEPES, pH=7.6], incubation with dispase (10 mg/mL) and addition of Dnase I (1 mg/ml) (both from Boehringer Mannheim Corp., Indianapolis, IN), the cell suspension was then filtered through a 70 μ nylon mesh (Becton Dickinson, Franklin Lakes, NJ). Cells were suspended in growth medium [DMEM-F12 medium supplemented with 10% fetal bovine serum (Immunocorp, Montreal, QC), 1% penicillin/streptomycin, and 0.1% amphotericin B (fungizone), pH=7.6] and subsequently plated in 75 cm² flasks previously coated with poly-D-lysine (Sigma, St. Louis, MO). Upon reaching ~70% confluence, microglia and oligodendrocytes were removed via shaking. Shaking was repeated three to four days later to achieve ~95% astrocyte purity, as determined by glial fibrillary acidic protein (GFAP) immunoreactivity. All astrocyte cultures were utilized within three weeks of primary culture.

The mixed glial suspension derived from initial shaking was re-suspended in supplemented growth medium and plated in 75 cm² flasks. After 30 minutes, the cell culture medium was replaced so as to remove excess oligodendrocytes. Cell cultures were composed of ~70% astrocytes, 25% microglia, and 5% oligodendrocytes as assessed by immunolabeling with antibodies specific for the astrocyte antigen, GFAP, microglial antigen, ED-1, and the oligodendrocyte antigen, galactocerebroside. Subsequently, the cell culture medium was replaced every 3-4 days until ~85% confluence was achieved. All cell cultures were subsequently re-plated in 24-well cell culture plates at a density of ~ 50,000 cells/well. The supplemented cell culture growth medium was refreshed every 3-4 days until ~70% confluence was reached.

Cytokine Treatments

Cell cultures were treated with IL-1 β (Geneka Biotechnology Inc., Montreal, QC) or TNF- α (Geneka Biotechnology, Montreal, QC), for a period of 48 or 96 hours. Each cytokine was dissolved directly in fresh supplemented cell culture medium for treatment. Cell culture medium was collected upon completion of treatment and stored at -80°C.

Apolipoprotein E Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of extracellular apoE levels in all collected cell culture medium samples was achieved using a protein-specific ELISA assay [5]. Briefly, ELISA plates (Corning Costar E.I.A./R.I.A., Acton, MA) were coated with goat anti-human apoE capture antibody (International Immunology Corporation (IIC), Murrieta, CA, purified with a HiTrap Protein G Kit, Amersham Pharmacia Biotech, Baie d'Urfe, QC) in 10 mM sodium carbonate and stored overnight at 4°C. The capture antibody was subsequently blocked with 0.1% bovine serum albumin (BSA) in PBS and stored overnight at 4°C. Cell culture medium samples and recombinant apoE4 (Panvera Quality Reagents, Madison, WI) standards (50-

2000 ng/mL) were incubated in triplicate. Goat anti-human apoE antibody labelled with biotin (IIC, Murrieta, CA, purified with a HiTrap Protein G Kit, Amersham Pharmacia Biotech, Baie d'Urfe, QC, labelled with biotin, Boehringer Mannheim Corp., Indianapolis, IN) in 0.1% BSA in tris-base-salt-tween (TBS-T) was used. Subsequently, wells were incubated with alkaline phosphatase-streptavidin (Zymed Laboratories Inc., San Francisco, CA) and attophos reagent (Promega Corporation, Madison, WI). Measurements of emitted fluorescence were taken using a microplate fluorescence reader FL-600 (Bio-tek Instruments Inc., Winooski, VT), reading at a bandwidth of 450 nm/540 nm. Detectable levels of apoE protein were between 50-2000 ng/mL. As a positive control, astrocyte cell cultures were treated with 17- β -estradiol and ELISA analysis revealed a significant increase in extracellular apoE levels (not shown).

Statistics

Statistical analyses were conducted utilizing SPSS software (Chicago, IL, USA). All individual data points were expressed relative to non-treated mean protein values within trial, drug, and time. Two-way independent measures analysis of variance (ANOVA) tests were then used to assess the effects of drug concentration and duration of treatment across trials on extracellular apoE protein levels. Significant differences between non-treated and treated cells were then assessed using multi-sample 95% confidence intervals with the non-treated mean protein value arbitrarily set as a population mean of 1.0.

RESULTS

IL-1 β Treatment of Terminally Differentiated Rat Primary Glia Induced apoE Secretion

Results were derived from a minimum of three separate cell culture experiments. Rodent astrocyte and mixed glial cell cultures following 96h of cytokine treatment using the reported concentrations, displayed at least 95% cell viability, as assessed by acridine orange fluorescent staining. In a control experiment, IL-1 β protein quantification of non-treated mixed glial and astrocyte cell culture medium samples using an IL-1 β ELISA, revealed minimal levels of IL-1 β (typically less than 45 ng/ml).

Although no significant differences between treated and non-treated cells were detected following IL-1 β treatment of astrocytes (Fig. 1A), IL-1 β treatment of mixed glia resulted in significant main effects of time, $F(1,84) = 26.98$, $p < 0.0001$, and concentration, $F(5,84) = 3.00$, $p = 0.015$, respectively. Mixed glia treated with IL-1 β demonstrated significantly elevated levels of apoE protein when compared to non-treated cells (Fig. 1B) only 96h after the treatment and starting at a concentration of 0.01 picomolar of IL-1 β .

TNF- α Treatment Reduced apoE Secretion by Rat Primary Astrocytes and Mixed Glia

As opposed to the increase in apoE protein seen with IL-1 β , TNF- α treatment was associated with significant reductions in apoE protein. ANOVA analysis of astrocytes data showed a significant main effect of concentration, $F(5,42) = 2.92$, $p = 0.023$ but not of time, $F(1,42) = 2.42$, $p = 0.13$.

Comparisons of treated and non-treated cells across concentrations revealed significant decreases in apoE protein (Fig. 2A).

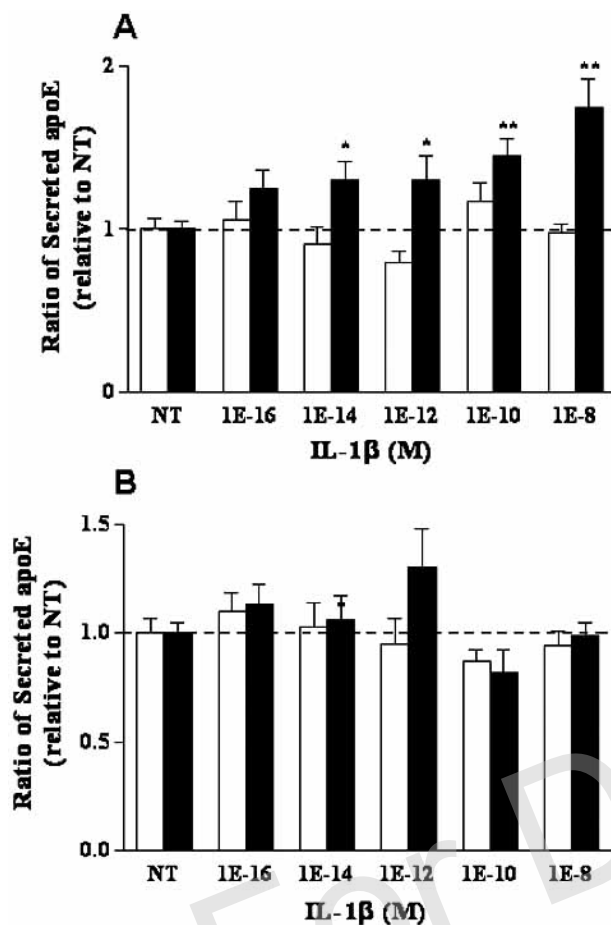


Fig. (1). Mean (\pm SEM) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of interleukin-1 β treatment concentration at 48 (open bars) or 96 (solid bars) hours. All data points were expressed relative to the mean of non-treated (NT) cells. Mean protein values were derived from an average of $n \geq 6$ cell culture wells. The mean apoE protein values of treated cells were then compared after normalization to mean levels of NT cultures. Significant differences in extracellular apoE protein levels relative to those of NT cells are indicated by * $p < 0.05$ and ** $p < 0.01$.

Analysis of mixed glial data also revealed a main effect of TNF- α concentration $F(5,57) = 3.21$, $p = 0.013$ with no significant effect of time, $F(1,57) = 3.22$, $p = 0.078$. As with astrocytes, mixed glia exhibited decreases in apoE protein following treatment with TNF- α (Fig. 2B).

DISCUSSION

Pharmacological or genetic manipulation of apoE concentrations has been shown *in vitro* to facilitate A β clearance [5,25] and, *in vivo* to modulate neurite extension and branching [26-29]. However, the interaction between apoE and immune modulators in the context of AD remains largely unexplored. In the present study, treatment of primary rat mixed glial cell cultures with IL-1 β induced significant in-

creases in apoE protein and no change in purified astrocyte cultures. On the other hand, TNF- α mediated significant apoE decreases in both astrocyte and mixed glial cell cultures which were of about the same magnitude. The results of the present study are consistent with prior *in vitro* studies showing IL-1 β can induce apoE secretion from neonatal rat mixed glia [30] but has no effect on astrocyte cultures [22,23]. Altogether these results support the idea of a cell-type specific response. It is indeed interesting to note that while TNF- α induced a similar effect on both cell culture types, IL-1 β only induced apoE secretion in mixed glial cultures.

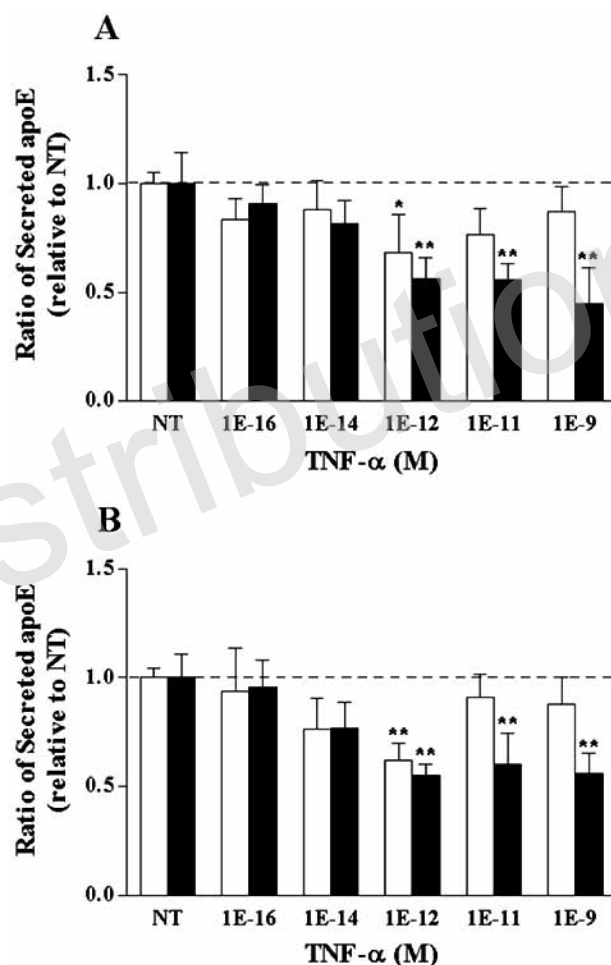


Fig. (2). Mean (\pm SEM) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of tumor necrosis factor- α treatment concentration at 48 (open bars) or 96 (solid bars) hours. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte and mixed glial protein values were derived from an average of $n \geq 9$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared after normalization to mean levels of NT cultures. Significant differences in apoE protein levels relative to those of NT cells are indicated by * $p < 0.05$ and ** $p < 0.01$.

Main effects analysis generally revealed increases in apoE protein with longer incubation time in both rodent astrocytes and mixed glial cells. Recent studies have suggested that tissue culture conditions, in particular the presence of serum, may play a key role in the effect of cytokines on

apoE expression [21]. However, the inclusion of FBS in the present study was an attempt to mimic physiological conditions in which glia receive substantial growth support from the environment. In addition, given that cell cultures were maintained for as long as 96 hours, FBS was utilized in order to minimize apoptotic processes upon reaching maximal confluence. Increased levels of apoE protein in response to IL-1 β exposure may have been the result of cell proliferation. Indeed, IL-1 β has been demonstrated to stimulate cell proliferation *in vitro* [11,17] as well as gliosis *in vivo* [12], thereby increasing cell number and, consequently, apoE production. However, considering the results we report for TNF- α , this doesn't seem very likely because TNF- α was also reported to induce glial proliferation [17,27]. Thus modulation of apoE secretion may be indicative of a yet unidentified cytokine signal for apoE-mediated remodelling following injury, as well as a means of immunoregulation as originally proposed by Finch *et al.*, 1993 [31].

Numerous studies have demonstrated increased cytokine and cytokine receptor expression following brain injury [32,33] as well as in AD brain tissue [34] with concentrations in the picomolar (10^{-12} M) range (~0.6-130 pg/ml for IL-1 and ~300 pg/ml for TNF). Similarly, apoE protein and mRNA levels have been shown to significantly increase following hippocampal deafferentation *in vivo* [7,33]. Temporally, IL-1 β expression has been identified up to two days following injury [35] with apoE induction having been shown to peak 6 days following hippocampal deafferentation in rats [7]. As a pro-inflammatory cytokine, IL-1 β most probably triggers microglia activation and subsequent degradation of damaged terminals and synapses. In turn, such catabolic process could potentially release a wide assortment of lipids like cholesterol, thereby requiring later apoE-mediated removal and transport. This is consistent with the temporal pattern of apoE induction with apoE mRNA levels increasing a few days after heightened cytokine expression. Subsequent apoE protein expression could also facilitate cholesterol transport and internalization via the apoE - apoE receptor pathway in neurons for membrane and synapse formation, and ultimately, neuronal synaptic remodelling. Thus, from an AD perspective, heightened levels of cytokine expression in the brain may trigger apoE-mediated recovery mechanisms, thereby potentially countering some of the detrimental effects of prolonged cytokine exposure.

In contrast, a reduction in apoE secretion was observed following TNF- α treatment. Interestingly, TNF- α was also shown to modestly inhibit apoE mRNA expression in differentiated monocyte cultures as well as reduce mRNA expression and apoE secretion by adipocytes [36]. The effects of TNF in adipocytes were mediated by elements in the proximal promoter of the *APOE* gene and seem to require NF κ B [37]. Though the mechanism underlying this decrease in glial cells remains speculative, in light of the apoE induction observed with IL-1 β treatment, TNF- α -mediated decreases could act as a counter-balancing force with which to prevent unfettered increases in apoE production. Further studies aimed at exploring the dichotomous effects of IL-1 β versus TNF- α are clearly warranted as well as analyses of the mRNA prevalence changes in response to immunomodulation.

The present study demonstrated that IL-1 β induced a significant increase in apoE secretion by mixed glial cell cultures while TNF- α treatment significantly reduced extracellular apoE in both mixed glial and astrocyte cell cultures. From an AD perspective, elevated apoE expression may be indicative of an endogenous recovery mechanism that is triggered following cytokine up-regulation in order to promote neuronal remodelling and A β clearance, thereby limiting further cytokine synthesis and inflammation. Thus, the current results may be indicative of a relationship between inflammation and apoE induction whereby initial inflammation following injury may be followed by apoE induction so as to promote plasticity and, ultimately, recovery.

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