Expression of Tumor Necrosis Factor α (TNFα) and Interleukin 6 (IL-6) mRNA in Adult Human Astrocytes: Comparison with Adult Microglia and Fetal Astrocytes

LOUISE LAFORTUNE, MSc, JOSEPHINE NALBANTOGLU, PhD, AND JACK P. ANTEL, MD

Abstract. Astrocytes and microglia are cell populations which are implicated as being capable of regulating and effecting immune responses within the central nervous system (CNS). These functions are postulated to be mediated at least in part by production of soluble protein molecules termed cytokines. In this study, we utilized dissociated cultures of glial cells prepared from adult and fetal CNS tissue and a combined in situ hybridization-immunocytochemical technique in order to compare expression of TNFα and IL-6 mRNA between adult and fetal astrocytes and between adult astrocytes and microglia. Our results, using digoxigenin-labeled riboprobes, indicate that in contrast to fetal astrocytes only adult astrocytes express TNFα and IL-6 transcripts under our serum-supplemented basal culture conditions. Activation with LPS and IFNγ increased the proportion of adult astrocytes expressing detectable TNFα and IL-6 mRNA signals; however, the proportion was significantly less than for microglia contained in the same cultures. These results suggest that microglia rather than astrocytes are more likely to be sources of these cytokines within the adult human CNS. Further studies of cytokine expression by glial cells will need to consider both the age and species of the glial cells used.

Key Words: TNFα; IL-6; Human astrocytes; Human microglia; In situ hybridization.

INTRODUCTION

Reactive glial cells, both astrocytes and microglia, are a pathologic hallmark of immune-mediated disorders of the central nervous system (CNS). Reactive glia are also a recognized feature of chronic neurodegenerative disorders such as Alzheimer’s disease and at sites of CNS injury (1, 2). The glial cells are postulated to have functional roles in regulating and effecting intracerebral immune responses, with cytokine molecules being implicated as the mediators of these activities (3, 4). Such molecules have been detected in the CNS under an array of inflammatory pathologic conditions, although the cell of origin of these molecules has been less clearly established (5–11).

Cytokine production by glial cells has most frequently been studied in vitro using purified cell populations or transformed cells (12–18). As regards astrocytes, studies of adult CNS-derived cells have been limited by lack of techniques to prepare purified cultures. However, purified populations of rodent and human astrocytes have been prepared from fetal or neonatal tissue specimens using techniques which take advantage of the relatively high in vitro proliferation rate of the immature astrocytes (19, 20). Initial studies documented that these rodent astrocytes could secrete an array of cytokines, including tumor necrosis factor (TNF) α and interleukin (IL) 6 (15–17). Similar findings were reported using human astrocytoma cells (12–14). In contrast, variable patterns of secretion and mRNA expression of these cytokines were described in studies of human fetal astrocytes maintained in dissociated cell culture, with differences perhaps related to age of donor tissue and detection techniques used (21–23).

Regarding cytokine production by microglia, our own previous studies have demonstrated production of TNFα and IL-6 by human adult CNS-derived microglia maintained in dissociated cell culture, findings which parallel those using rodent cells (24–26). As with systemic monocytes and macrophages, production is augmented by activation of microglia with agents such as lipopolysaccharide (LPS) and interferon (IFN) γ (27). Detection of TNFα, either at the mRNA or protein level, produced by microglia isolated from the fetal human CNS maintained in dissociated cell culture, has been inconsistent in the limited number of studies reported to date (21, 23, 28).

The purpose of the current study was to evaluate human adult CNS-derived astrocytes maintained in vitro as a source of the cytokines TNF and IL-6. Our aim was to compare these astrocytes with fetal human astrocytes maintained under the same culture conditions and with adult human microglia contained in the same cultures. The lack of culture techniques which yield purified populations of astrocytes from the adult human CNS precluded detection of cytokine protein secretion by biosay or mRNA expression by extraction of total RNA. To overcome the problem created by the need to use mixed glial cell preparations, we have resorted to a combined in situ hybridization-immunocytochemical technique to study cytokine expression in our glial cells under basal conditions and in response to LPS and IFNγ activation.

MATERIALS AND METHODS

Source of Glial Cells

CNS tissue derived from 6- to 12-week-old fetuses following Medical Research Council (MRC) of Canada-approved guidelines served as the source of human fetal astrocytes. Temporal...
lobe tissue, containing both white and gray matter, obtained from surgical resections carried out to ameliorate non-tumor-related intractable epilepsy in young adult patients (age range 20–45 years) served as the source of adult human glial cells. Tissues used were derived from regions requiring resection to reach the precise epileptic focus and were distant from the main electrically active site.

Glia1 Cell Preparations

Fetal glia: These cultures were prepared by carefully stripping CNS material of meninges and blood vessels, mechanically dissociating the tissue with scalpel blades, and then treating with trypsin (0.25%) and DNase (50 μg/ml) (both from Boehringer Mannheim, Laval, Quebec) at 37°C for 45 minutes (min) (27). Dissociated tissue was then passed through a 130 μm nylon mesh and washed twice in phosphate-buffered saline (PBS). Cells were suspended in culture medium consisting of Eagle's MEM supplemented with 5% fetal bovine serum (FBS), 0.1% glucose, and 20 μg/ml gentamicin (all from Gibco, Burlington, Ontario), and plated onto poly-L-lysine-coated plastic Petri dishes. Cultures were split when confluent (approximately every 7 days). Experiments were conducted after the second or third cell passage in culture. At this time, 50,000 cells in 100 μl were seeded onto adjacent chambers of Nuac chamber slides (Naperville, Illinois) containing the adult glial cell preparations described below. Cultures were used within a few days, a time at which they were becoming confluent.

Adult glia: Our method for preparing adult human glial cell cultures has been previously described (29). Briefly, tissues were treated with trypsin (0.25%) and DNase (50 μg/ml) followed by Percoll gradient centrifugation (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 15,000 RPM for 30 min. The dissociated cells at the interface were washed several times with PBS and were then suspended in the same culture medium as used for the fetal astrocytes and seeded (50,000 cells in 100 μl) onto individual chambers of Nuac chamber slides. The following day, oligodendrocytes remained floating and were removed. Remaining adherent cells, consisting of astrocytes and microglia, were allowed to develop morphologically for 7 to 14 days. Culture medium was changed every 2 to 3 days, probably resulting in some further selective loss of the less adherent astrocytes.

Activation of Glial Cells

For some adult and fetal glial cell cultures, 100 U/ml of human recombinant IFNγ (Genzyme, Intermedico, Markham, Ontario) and 5 μg/ml of LPS (Sigma, St Louis, MO) were added either 4 or 24 hours (h) prior to analyzing the cultures for TNFα or IL-6 mRNA expression. These concentrations of IFNγ and LPS were previously found to be optimal for stimulating cytokine production in adult microglia.

Immunocytochemistry to Identify Glial Cells

These studies involved single- or double-immunostaining to identify astrocytes or microglia. In single-immunolabeling studies to detect glial fibrillary acidic protein (GFAP) in astrocytes, cells were fixed for 20 min in 4% paraformaldehyde (5% glacial acetic acid), rinsed in PBS and incubated for 45 min in polyclonal rabbit anti-GFAP IgG (1:100) (Dakopatts, Dimension Laboratories, Mississauga, Ontario) diluted 1:100 in Hank’s balanced salt solution (HBSS) supplemented with 2% horse serum and 10% goat serum in 1 mM Hepes (HHC). The cells were then rinsed in PBS and incubated 1 h in Texas Red-labeled anti-rabbit IgG (1:100) (Jackson Immunologicals, BioCan, Mississauga, Ontario) in HHC. In double-immunolabeling studies to detect microglia as well as astrocytes, the initial single-label procedure was continued by incubating the cells for 1 h with EBM11 (anti-CDS8) antibody (Dakopatts), diluted 1:40 in HHC, and followed by 1 h incubation in FTC-labeled goat anti-mouse IgG (1:80 in HBSS) (TAGO, Immunocorpb, Montreal, Quebec). Finally, the cells were washed in PBS, mounted, and analyzed using a Leica fluorescent microscope.

In situ Hybridization Combined with Immunocytochemistry

These studies were carried out simultaneously on cultures containing fetal astrocytes and adult glial cells which had been maintained under basal or activating conditions and then fixed as described above.

Probes

The human IL-6 cDNA and the human TNFα cDNA were kindly provided by Dr J. Gauldie, McMaster University. Both probes were subcloned in the bluescript II (Stratagene) vector containing bacteriophages T3 and T7 RNA polymerase promoters. The template DNA for TNFα was linearized by digestion to completion with the restriction enzymes ApaI and MboII (Promega) for the sense and antisense probes, respectively. The RsaI and Avall restriction enzymes (Gibco BRL, Burlington, Ontario) were used to linearize the IL-6 sense and antisense probes. Initial TNFα studies were conducted with radiolabeled sense and antisense probes prepared with [32P]-UTP using the in vitro transcription system of Ambion (Austin, Texas). Subsequent studies were conducted using digoxigenin (DIG)-labeled probes.

The DIG-labeled RNA transcripts were synthesized from the linearized DNA segments using the T3 or T7 polymerases (Promega, Mississauga, Ontario) for the sense and antisense probes, respectively. DNA (1 μg) was added to the DIG-RNA labeling mixture (Boehringer Mannheim, Laval, Quebec) (10X transcription buffer: 400 mM Tris-HCl, pH 8 (20°C); 60 mM MgCl2, 100 mM NaCl, 1 U/ml RNAse Inhibitor), 40 U of T3 or T7 RNA polymerase in a final volume of 20 μl. The mixture was incubated for 2 h at 37°C, followed by a 15 min incubation at 37°C with 20 U of DNAse I. The reaction was stopped with 0.2 M EDTA containing glyoxylate. The labeled RNA was precipitated with the addition of 4 M LiCl and ethanol at −70°C for 30 min, dissolved in TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA) containing 20 U of RNase inhibitor, and stored at −20°C until use.

Prehybridization and Hybridization

The cells were rehydrated in a series of graded ethanol concentrations, incubated for 20 min in 0.2 M HCl, rinsed briefly in PBS (pH 7.4), and incubated for 30 min in 2× SSC (1× SSC is 0.15 M NaCl, 0.075 M sodium citrate) (70°C). The cells
were then digested for 15 min in 0.0625 mg/ml of pronase in P buffer (1 M Tris, pH 7.5; 0.1 M EDTA), incubated in 0.2% glycine to stop the reaction, rinsed in PBS, and fixed for 20 min in 4% paraformaldehyde (pH 7.6). After several rinses in PBS, the cells were acetylated for 10 min in a fresh mix of acetic anhydride in 0.1 M triethanolamine, pH 8.0, and incubated 2 to 3 hours in 1× salts, 0.3 M NaCl; 0.01 M Tris-HCl; 0.01 M NaH₂PO₄ (pH 6.8); 5 mM EDTA; 0.02% w/vol Ficoll 400; 0.02% w/vol polyvinylpyrrolidone; 0.02% w/vol BSA fraction V) containing 100 μg/ml of rRNA.

The hybridization with DIG-labeled probe was carried out overnight at 55°C in a mixture of 30% formamide, 1× salts, 8.5% dextran sulfate, 1.25 mg/ml rRNA and 5 ng/ml probe. In some experiments, samples were hybridized overnight in presence of 1× 10⁶ cpm of radiolabeled probe.

Following the overnight hybridization, the cells were washed with 20% formamide, 1× salts, 10 mM DTT at 55°C, incubated in 0.5 M NaCl in TE buffer for 15 minutes at 37°C, followed by incubation in 20 μg/ml RNAse A in 0.5 M NaCl in TE and washed in the same buffer. Finally, several washes were carried out in 2× SSC before the immunological detection of the probe and the immunocytochemical staining of the cells.

Immunocytochemistry

Following the post-hybridization washes, the cells were incubated for 20 min in 0.5% Blocking Solution (Boehringer Mannheim), followed by 1 h incubation in mouse anti-DIG-IgG (Boehringer Mannheim) diluted (1:100) in 0.1 M Tris-HCl; 0.15 M NaCl. The cells were then washed several times in 0.1 M Tris-HCl; 0.15 M NaCl and incubated for 30 min at 37°C in biotinylated antimouse IgG (1:100) (Beckton Dickenson, Mississauga, Ont.); followed by 1 h incubation in Streptavidin-FITC (1:100) (Boehringer Mannheim) diluted to 0.1 M Tris/HCl; 0.15 M NaCl.

The cells were then incubated for 45 min in a 1:100 dilution of polyclonal anti-GFAP IgG (Dakopatts) in 0.1 M Tris-HCl; 0.15 M NaCl, rinsed several times in the same buffer, incubated for 45 min in goat anti-rabbit Texas Red (Jackson Immunologicals), washed in PBS, mounted in Poly-Aquamount (Polysciences) and analyzed with a Leica fluorescent microscope and Leica Confocal Laser Scanning Microscope (CLSM) Imaging Software.

Confocal microscopic images were acquired, processed, and analyzed using an inverted Leica Diaplans microscope coupled to a 2–50 mV argon-ion laser and linked to a MC 68020/68881 computer system equipped with a VME bus and a Leica CLSM imaging software. The laser was set at 488 nm for excitation of FITC and Texas Red. The emission filter was a highpass from 550 nm. For the analysis of colocalization, scanning was made at 1.0 electronic zooming so that at a magnification of 40X (using a fluor oil immersion objective) the final (x, y) resolution was of 1 pixel per 0.245 μm. Final images were derived from three consecutive 1 μm-thick optical sections for a total thickness of 3 μm and were scanned for each fluorophore (FITC/Texas Red) simultaneously. From these data, single-merged images were generated using the 3D image reconstruction function.

The double-labeled images were generated by the combination of the image processed for the Texas Red fluorochrome with its corresponding image processed for the Texas Red fluorophore. All images were stored on an optical disk and printed using a Focus camera system.

Statistics

Data regarding proportion of fetal astrocytes and adult microglia (GFAP⁺ cells in the adult gial cultures) expressing a detectable mRNA signal were determined by counting a total of 50 cells on each of at least 3 coverslips derived from separate cultures, unless otherwise indicated. Due to the limited number of cells present, fewer GFAP⁺ cells were counted in the adult gial cultures.

RESULTS

Cell Cultures

The purity of cultures was assessed by double-staining for the astrocyte marker GFAP and the macrophage/microglia marker CD68 with EMB11. Previous immunocytochemical studies indicated that the microglia in culture expressed an array of molecules characteristic of this cell type, including Fc receptors and complement receptors (30, 31). As shown in Figure 1A, virtually all the fetal glial cells were GFAP⁺. No EMB11⁺ cells were detected. In the adult gial cells cultures, only a minority of cells (5 to 15%) were GFAP⁺; the majority of cells were microglia (GFAP⁺, EMB11⁺) (Fig. 1B, C). These two cell types accounted for almost all of the approximately 50 to 100 cells present in each of the chambers. Thus, in the in situ hybridization studies described below, microglia were defined as GFAP⁺ cells, although we cannot exclude that a small number of GFAP⁺, EMB11⁺ cells were present in our cultures.

In situ Hybridization Studies

TNFa mRNA detection: Fetal glial cells: In initial studies, we utilized a radiolabeled riboprobe to detect TNFa mRNA expression in the fetal astrocytes (Fig. 2A). However, combined in situ hybridization/immunocytochemistry proved difficult to perform, and for subsequent studies of both fetal and adult CNS cultures we utilized DIG-labeled riboprobes. As indicated in Table 1, we detected TNFa mRNA expression, using the DIG-labeled probe, in virtually all the fetal astrocytes under basal as well as activation conditions. Figure 2B and C illustrate such expression, determined by confocal microscopy, using in situ hybridization, either alone or in combination with GFAP immunocytochemistry, respectively. mRNA signal intensity did not differ significantly between cells which were or were not also immunostained for GFAP reactivity.

Adult gial cells: All these studies were performed using combined in situ hybridization with DIG-labeled probes and GFAP immunocytochemistry. As summarized in Table 1, under basal culture conditions we could rarely detect a specific hybridization signal for TNFa mRNA in
any of the astrocytes (GFAP+ cells). In contrast, almost all the microglial cells (GFAP− cells) in the same cultures gave a detectable signal for TNFα mRNA. In the cultures treated with IFNγ and LPS, there was an apparent increase in the proportion of GFAP+ cells with a positive TNFα mRNA hybridization signal, although the majority remained negative (Table 1). In cultures treated for 24 h with IFNγ and LPS, the proportion of GFAP+ cells with a positive signal was increased to 33%. Figure 2D and E present examples of negative and positive GFAP+ cells derived from cultures activated for 24 h. Microglia in the activated cultures all had detectable expression of TNFα mRNA. No detectable signal was found for the sense probe (Fig. 2F).

**IL-6 mRNA Detection**

Fetal glial cells: Under basal culture conditions, IL-6 mRNA was detected in 43% of the cells (Table). Following activation for either 4 or 24 h, a large proportion (95%) of cells had detectable expression (Fig. 2G).

Adult glial cultures: Under basal conditions, none of the GFAP+ cells and only rare GFAP− cells demonstrated a positive hybridization signal for IL-6 (Table 1 and Fig. 2H). In adult glial cell cultures treated with IFNγ and LPS for 4 h, a rare GFAP+ cell positive for IL-6 could be detected; in these cultures, 95% of the GFAP− cells had a detectable hybridization signal (Fig. 2I). This latter proportion decreased to 28% in cultures activated for 24 h.

**DISCUSSION**

The present study was conducted using glial cells derived from both human adult and fetal CNS tissue. The availability of these tissues provided the opportunity to directly compare cytokine RNA expression between adult and fetal astrocytes maintained under the same culture conditions and between adult astrocytes and microglia present in the same cultures. The age of the adult donors from whom glial cells were prepared corresponds rather well to that of the peak age of occurrence of the human autoimmune CNS diseases, multiple sclerosis, and AIDS encephalopathy. Under our basal culture conditions, which contained 5% serum supplementation, we could rarely detect either TNFα or IL-6 mRNA in adult astrocytes. In contrast, the large majority of fetal astrocytes expressed TNFα mRNA signal and almost half expressed IL-6 mRNA. Activation of the adult glial cultures with LPS and IFNγ did induce expression of these cytokine transcripts in a minority of the astrocytes, indicating that these cells are at least a potential source of TNFα and IL-6. Without purified cultures, we could not evaluate protein production by the adult astrocytes.
Our results regarding TNFα and IL-6 cytokine expression in fetal astrocytes can be compared with those contained in previous reports, although there are differences in ages of the fetal tissues and in techniques used for cytokine detection. Aloisi et al, using human fetal astrocytes prepared from 8- to 9-week old embryos, stated that these cells constitutively express elevated levels of TNFα transcripts, as determined by Northern blot analysis, but do not secrete TNFα protein even after stimulation, suggesting a post-translational block in TNFα synthesis (22). We also could not detect significant levels of TNFα in supernatants of our fetal astrocytes (27). Lee et al, using tissue of 16- to 24-week old gestation, found that IL-1b induced TNFα transcripts...
In previous studies using enriched microglial cell cultures, we have shown that these cells can secrete TNFα and IL-6, with marked augmentation in response to IFNγ (24, 27). Our finding that astrocytes in the stimulated adult mixed glial cell cultures, which contain TNFα-secreting microglia, fail to upregulate IL-6 mRNA suggests that adult astrocytes are less responsive to TNF than are the fetal astrocytes (33).

Our data on human adult microglia can be compared with that derived by Sebire et al (23) and Lee et al (21) using purified fetal microglia cultures. Sebire et al were unable to detect TNFα protein in either basal or LPS-stimulated cultures, in contrast to the detectable levels found in blood monocytes (23). IL-6 protein was detected, even under basal conditions; IL-6 transcripts were detected in 50% of cells under basal conditions and 70% after stimulation. Lee et al found that LPS was a potent inducer of both TNFα and IL-6 in fetal microglia (21). Maximal TNFα induced in microglia was greater than in astrocytes. In agreement with Lee et al (21), we found that IL-6 mRNA expression following stimulation peaked later than did TNF mRNA expression.

Our study provides insight into the properties of the adult astrocytes as related to cytokine production relative to other astrocyte preparations often used as models for defining the biologic properties of this cell type and to microglia, a cell type with which they share the CNS environment. Our data, taken together with previous reports cited above, indicate that studies of cytokine regulation in astrocytes will need to consider variables related to age and species used. Within the adult human CNS, the microglia, rather than the astrocytes, are likely to be the most potent glial cell source of the pro-inflammatory cytokines IL-6 and TNFα.

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