SECRETION OF IL-6 BY ASTROGLIOMA CELLS IN RESPONSE TO TNF-α

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DEDICATION

This thesis is dedicated to the following:

Michelle Worthington : mother and full time inspiration.

The other members of the MCR : and yes I still won.

To all the bunny wabbits and cute widdle cweatures : tasty.

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CELLS IN RESPONSE TO TNF-α

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ABSTRACT

Reactive astrocytosis or astrogliosis is associated with traumatic and pathologic injury to the central nervous system and is characterized by quiescent astrocytes becoming hypertrophic and hyperplasic. Studies of the signaling pathways involved have implicated microglial production of tumor necrosis factor-alpha (TNF-α), which, among other effects, stimulates astrocytes to secrete interleukin-6 (IL-6). The time course and intracellular secretory pathway of IL-6 in response to TNF-α has not previously been determined. The objective of the study reported in this thesis was to determine the time course and pathway of TNF-α stimulated secretion of IL-6 and to test the hypothesis that cultured astroglioma cells could be used as rapid detectors of TNF-α in plasma or cerebrospinal fluid samples *in vitro*. Immuno-techniques (ELISA, immunogold) were employed to track IL-6 in 8 day-old cultures of U87 MG cells (human astroglioma) exposed to 100ng/ml TNF-α for times ranging from 5 minutes to 4 hours. IL-6 synthesis was detected by immunogold localization associated with rough endoplasmic reticulum within 90 minutes after TNF-α stimulation. Because the U87 MG cells became highly vesiculated when exposed to TNF-α for times longer than 90 minutes, it was not possible to track nascent IL-6 through its secretory path to the cell exterior other than to note association with the vesicles. ELISA of culture supernatant showed statistically significant increases in IL-6 levels within 2 hours of TNF-α exposure. Based upon these results, use of cultured astrocytes in a rapid detection system developed for a clinical setting may be feasible.

INTRODUCTION

Although many of the functions of astrocytes in a normal, uninjured central nervous system (CNS) have been characterized (for review see Ransom *et al.*, 2003), the function of astrocytes during and following injury is not as well understood. Astrocytes undergo several morphological and physiological changes upon either chemical or physical injuries, including hyperplasia and hypertrophy. Physiological changes representing aspects of the activation of astrocytes include secretion of interleukin-6 (IL–6) and the up-regulation of glial fibrillary acidic protein (GFAP). Increased GFAP production is considered a marker for reactive astrocytes, which in turn indicates a condition known as astrocytosis or astrogliosis (for review see Eng and Ghirnikar, 1994). Certain pathologies have this condition in common, e.g., multiple sclerosis (Benveniste and Benos, 1995) and Alzheimer's disease (Forloni *et al.*, 1997; Griffin *et al.*, 1998), among others.

Part of the response to injury is the activation of microglia, which in the CNS have many of the same functions of macrophages (Aloisi, 2001). Microglia respond to CNS injury by retracting their many processes and assuming a rounded morphology, migrating to the site of injury and secreting cytokines. A major cytokine secreted is TNF-α, which has long been known to stimulate IL-6 production in astrocytes (Benveniste *et al.*, 1990). However, astrogliosis as defined by an increase in GFAP apparently is not caused directly by TNF-α stimulation, since stimulation with TNF-α has been reported to cause a decrease in GFAP mRNA both *in vitro* and *in situ* (Murphy *et al.*, 1995). Nevertheless, TNF-α secretion from microglia is definitely part of the injury response. Others

(Benveniste *et al.*, 1990; Eskes *et al.*, 2003; Jean Harry *et al.*, 2002; Norris *et al.*, 1994; Sallmann *et al.*, 2000; Van Wagoner and Benveniste, 1999; Vanden Berghe *et al.*, 2000) have shown expression of IL-6 may be induced by chemicals such as TNF-α, lipopolysaccharide, interleukin-1β, trimethyltin, or depolarization of neurons.

Stimulation of astrocytes by TNF-α elicits a signaling cascade leading to activation of a complex array of proteins, including those involved in activation and apoptosis. Astrocytes preferentially express tumor necrosis factor receptor (TNFR)-1 (or 55), the receptor most associated with activation of astrocytes. Once stimulation occurs SODD (Silencer of Death Domains), an inhibitor of TNFR-1, dissociates. Following this, many proteins become active such as FADD (FAS associated death domain), TRADD (TNF receptor-associated death domain), and RIP (receptor-interacting protein). These proteins recruit other proteins like caspase-8 (part of the apoptotic pathway) and cIAP (an anti-apoptotic protein). One of the end results of this cascade is activation of the nuclear transcription-activating factor (NF)-κB, a DNA binding protein that activates numerous genes, including those of IL-6 and proteins that prevent apoptosis (for review see Chen and Goeddel, 2002).

Interleukin (IL)-6 is a pleiotropic cytokine produced by many different kinds of cells with many different effects. Many isotypes of IL-6 have been demonstrated, all with the same amino acid sequence but differential patterns of glycosylation of O- and N- groups and the degree and location of phosphorylation. It has also been demonstrated that each IL-6 form is specific to a type of cell or tissue (Bauer *et al.*, 1988; Gross *et al.*, 1989; May *et al.*, 1988). IL-6, formerly known as B cell stimulatory factor (BSF)-2, is important in differentiation and proliferation of B cells (Muraguchi *et al.*, 1988) as well as being part of the process of activation of cytotoxic T lymphocytes (Takai *et al.*, 1988).

There is increasing evidence of the importance of IL-6 in neuronal regrowth and CNS development (Marz *et al.*, 1998; Parish *et al.*, 2002). IL-6 is important in the inflammatory response as well as to further astrocyte activation as marked by increased levels of GFAP (Maeda *et al.*, 1994; Marz *et al.*, 1999a; Marz *et al.*, 1999b; Penkowa *et al.*, 2001). IL-6 is not constitutively expressed (at least, not in cultured astroglioma cells as shown in this thesis), nor is there very much (approximately 10 pg/ml (Azuma *et al.*, 2000)) in the CNS of normal healthy humans (for review see Gruol and Nelson, 1997). Inappropriate IL-6 responses (too much or too little IL-6 production) can lead to CNS damage, as in cases of neurodegeneration, astrocytosis, and glutamate-induced seizures (Campbell *et al.*, 1993; Campbell *et al.*, 1994; Campbell *et al.*, 1991; Finck *et al.*, 1994; Gadient and Otten, 1997; Hirano, 1998; Suematsu *et al.*, 1989).

The sensitivity of astrocytes to TNF-α suggests a possible detection system using cultured astrocytes in a sandwich ELISA microbead-assay system that would provide much greater speed and sensitivity in comparison to existing techniques. Cells can be grown on silicon chips, which may contain antibody-covered beads in wells (Weigum *et al.*, 2001). Cerebrospinal fluid, or plasma samples flowing through the wells bring the molecule to be detected, in this case TNF-α, in contact with the cells, which would respond by secreting a much higher amount of IL-6 immediately adjacent to the capturing IL-6 antibody on the bead. A detecting antibody (anti IL-6 with a fluorophore attached) is then used to reveal the captured IL-6 and the resulting fluorescence would indicate the presence of TNF-α in the initial sample. The speed and sensitivity of this system potentially is much higher than standard ELISA due to the small well volume and immediate vicinity of the antigen producing cells to the antibodies bound on beads. However, the response time and secretion mechanism of the cultured astrocytes, and

dose-response relationship of TNF-α to IL-6 must be known for this chip-based assay system to prove practical. Based on preliminary experiments, other studies on different cytokines (Zheng and Specter, 1996), and the well-characterized secretory pathway (Kelly, 1985), it was expected that IL-6 might be detectable in culture supernatant by ELISA only after 90 to 120 minutes of stimulation, a time course similar to other late response gene products. Secretion of IL-6 may follow the well characterized pathway for secreted proteins, i.e., from mRNA translation on rough endoplasmic reticulum (ER), synthesis of pre-IL-6 into the endoplasmic reticulum, subsequent transfer to the Golgi apparatus, followed by containment in vesicles and exocytosis resulting from vesicle fusion with the plasma membrane (Kelly, 1985). However, it was also possible that IL-6 production is constitutive and that IL-6 is stored in regulated, sub-membrane, exocytotic vesicles that might be released in response to an external signal such as TNF-α binding.

To determine the secretion pathway and timing of IL-6 in cultured astroglioma cells, I used immunogold techniques in combination with ELISA to distinguish between constitutive *vs. de-novo* secretion, the secretion pathway, and the time required for new IL-6 to reach the exterior of the cell. No endogenous IL-6, or sub-membrane vesicles containing IL-6, were found prior or for up to 90 minutes after stimulation by 100 ng/ml TNF-α. However, beginning after 90 minutes of TNF-α stimulation, IL-6 was detected initially in association with the rough endoplasmic reticulum and then subsequently with electron dense vesicles which appeared to move to the cell surface and be exocytosed in significant number by 120 minutes of TNF-α stimulation. ELISA of culture supernatant confirmed this timing, with a strong increase in IL-6 levels occurring between 90 and 120 minutes post-TNF-α stimulation.

MATERIALS AND METHODS

A. Maintenance and Stimulation of Glioma Cell Cultures

U87MG human glioma cells (ATCC HTB-14) were seeded from frozen stock cultures maintained in fetal bovine serum (FBS, Sigma, St. Louis, MO) with 10% dimethyl sulfoxide (DMSO, Sigma). The number of viable cells was determined by trypan blue staining and a hemacytometer (Fisher Scientific, Pittsburgh, PA), and cells were diluted to an initial seeding density of $6.0x10^4$ cells/cm^2.

The cell line was grown and maintained in minimum essential medium (MEM, Sigma-Aldrich, St. Louis, MO) that contained 10% FBS, 2 mM L-glutamine and 10,000 U penicillin/10,000 mg/ml streptomycin. Cells were kept in a 37°C, humidified atmosphere with 95% air and 5% CO_2. The cells were allowed to grow to near confluency, which took 6 days. Experimental cultures were stimulated by replacing normal culture medium with 5 ml of medium containing 100 ng/ml human TNF-α (Sigma-Aldrich, St. Louis, MO).

B. Transmission Electron Microscopy (TEM)

For electron microscopy, cells were cultured in 25 cm^2 flasks. One flask served as the control (no TNF-α) and cells in the remainder were incubated in TNF-α containing media for 5, 15, 30, 90, or 240 minutes. The control flask is referred to as the "0" time point in the results section below. The entire experiment was repeated three times.

At the appropriate time, the medium was removed and the cells were washed briefly in phosphate-buffered saline (PBS) solution (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3

mM Na_2HPO_4, 1.4 mM KH_2PO_4). The cells were detached by incubation in PBS containing 0.25% Trypsin and 0.3% EDTA for 5 minutes. The cell suspension was then poured into a sterile 15 ml conical centrifuge tube and centrifuged into a pellet at 700 *g* in a clinical centrifuge for 30 seconds. They were then fixed at room temperature for 1 hour in a 4% formaldehyde (PFA) solution in PBS freshly made from paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA). The fixed pellet was post-fixed in 1% osmium tetroxide in 0.05 M Na cacodylate (pH 7.5), washed, then processed for embedment in Spurr's epoxy resin (Spurr, 1969) using standard procedures. The resulting blocks were trimmed and sectioned using a Reichert Ultracut S at a thickness of 70 nm and mounted on 300 hexagonal mesh nickel grids for immuno-staining and viewing.

C. Immuno-staining for TEM

The resin sections were immunogold stained using a protocol adapted from Hayat (2002), as follows: sections were first treated with a 0.01M sodium citrate solution at 95° C for 15 minutes using a Progene (Techne, Minneapolis, MN) thermocycler for temperature control. The sections were then washed three times in PBS and blocked with 10% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. Another PBS wash was done and then the sections were incubated with primary antibody (anti-IL-6 mAb, see Table 1) overnight at 4° C. The resin sections were treated with a high salt wash of 0.75 M NaCl and then washed again in PBS three times. Secondary antibody (see Table 1) was next applied and allowed to incubate at room temperature for 2 hours. The resin sections were again treated to a high salt wash and three washes in PBS. The cells were rinsed briefly in distilled water and dried and stored. Negative controls were performed by omitting the primary antibody.

D. ELISA

After the cells had grown to confluency, the growth medium was replaced with 5ml of either culture medium (control) or stimulation medium (culture medium containing 100 ng/ml human TNF-α) in each of the four flasks. There was one control and three experimental flasks. Immediately after the control and experimental media were replaced, 350 μl samples were removed from each flask. The samples were replaced by an equal volume of the same medium to maintain a constant volume in the control and experimental flasks. The samples were then stored in 500 μl at -80°C until needed. This procedure was repeated every 15 minutes over 240 minutes for a total of 17 samples per flask.

ELISA was performed using the QuantiGlo ® Human IL-6 Immunoassay ELISA Kit from R&D Systems (QuantiGlo Q6000, Minneapolis, MN). The kit employs a "sandwich" immuno- capturing and detection system and uses horseradish peroxidase with a luminol/hydrogen peroxide substrate that results in chemiluminescence that was quantitated using a Spectramax Gemini (Molecular Devices, Sunnyvale, CA) plate-reading luminometer. Prior to assaying the samples, a standard curve was generated using positive control standards. Each 350μl sample was assayed in triplicate by placing 100 μl in each of three wells of the anti-IL-6 coated 96-well plate provided. Relative luminosity was recorded from each well, converted to picogram/ml of IL-6 using the standard curve, and averaged by time point. Single factor ANOVA was applied to the experimental data and the Student's T test was used to compare the two data sets showing the initial release of IL-6 into the culture medium. $P \leq 0.05$ was taken to indicate statistical significance.

E. Analysis of Immunogold Labeled Cells

Ultra thin sections were examined using a JEOL 1200 EXII transmission electron microscope. Cells were photographed and the developed negatives were then digitized using an Epson 2450 flatbed high-resolution scanner driven by a Macintosh G5 computer (Apple Computer, Ins., Cupertino, CA). These images were processed and enhanced using Adobe PhotoShop CS (Adobe, Seattle, WA). The location and number of gold particles were then recorded. Representative micrographs are presented in the results section.

Table 1. Antibodies and chemicals used for immunogold techniques.

	Species/ Isotype	**Dilution**	**Secondary antibody conjugated to**	**Supplier**
Anti-Human-Interleukin-6 monoclonal antibody	Mouse IgG	2.0 μg/ml in PBS		Sigma Chem. Co., St. Louis, MO
Anti-Mouse IgG	Goat IgG	1:50 in PBS	10nm Gold	Sigma Chem. Co., St. Louis, MO
*Human Tumor Necrosis Factor -α	Human	100ng/ml in sterilized PBS		R&D Systems Minneapolis, MN

RESULTS

Ultrastructural analysis of the U87MG human glioma cells prior to and during the first 90 minutes of TNF-α stimulation revealed morphology typical of astroglioma cells grown in culture (Glass *et al.*, 2002; Hahn *et al.*, 2000) (Figure 1). Well-defined nuclei were apparent, and the cytoplasm contained abundant ER and other endosomal vesicles. Mitochondria were not well defined and sparse in occurrence, and well-developed Golgi apparati were not observed. Some vesicles had electron dense cores suggesting high protein content (Figure 1, C and D). Cells exposed to TNF–α for longer than 90 minutes showed increasing numbers of dense vesicles, and apparent movement from perinuclear areas to the submembrane space, strongly suggesting exocytosis beginning between 90 and 120 minutes after the onset of TNF-α stimulation (Figure 2).

Immuno-labeling with anti-IL-6 of thin sections from cells not exposed to TNF-α did not reveal labeling above non-specific background levels. Although small groups of gold particles were occasionally found near the nucleus and the ER, this was rare. Sections of cells exposed to TNF-α for 30 minutes showed infrequent labeling on or near ER close to the nucleus (Figure 1). After 90 minutes of exposure to TNF-α, prominent labeling of ER and vesicles apparently derived from the ER was observed throughout cells (Figure 2). Cells subjected to TNF-α stimulation for 240 minutes showed intense, discrete labeling of vesicles near the plasma membrane, along patches of the membrane itself, and scattered clumps on the resin external to but near the membrane (Figure 3).

Similar results were observed by ELISA of IL-6 levels in culture supernatant, which showed an abrupt, significant ($P = 9.27x10^{-9}$ by ANOVA) rise in IL-6 levels between 90 and 105 minutes after exposure to TNF-α. The concentration for these two points was 0.54 and 3.2 pg/ml, respectively. Previous to 90 minutes the amount of IL-6 in the media was measured at less than 0.003 pg/ml. The Student's T-test comparison of the 90 and 105-minute samples revealed a significant ($P = 0.0001$) 6 fold increase in IL-6 concentration in the 105-minute sample. After 90 minutes, IL-6 based luminosity increased linearly with time until approximately 150 minutes of TNF-α exposure reaching a level approximately four to five times the control, or background level, whereupon a plateau was reached. This difference in luminosity correlates to an increase in IL-6 concentration of approximately a thousand fold. As with luminosity, the differences in the amount of IL-6 in the media could not be determined prior to 90 minutes. No decrease in IL-6 concentration was observed in the samples after 2 hours of stimulation (Figure 4).

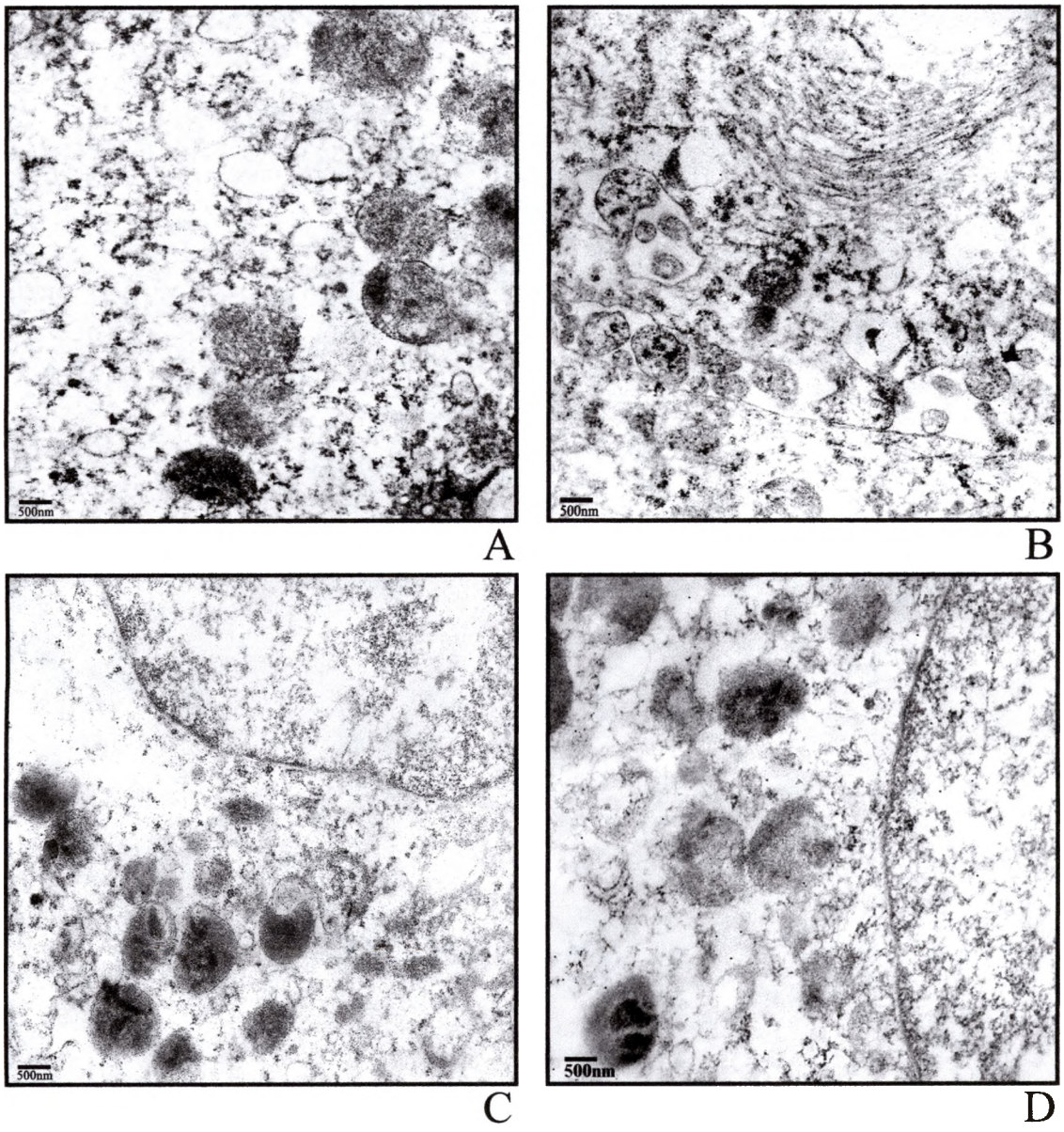
500nm
A
500nm
B
500nm
C
500nm
D

Figure 1

Image of U87MG astroglioma cells unstimulated with human TNF-α (A), and stimulated with 100ng/ml TNF-α for 5 minutes (B), 15 minutes (C), and 30 minutes (D). The cells were immunogold stained for interleukin-6. There is no IL-6 specific labeling on any of these images. There may also be a portion of a Golgi apparatus in the top right corner of B; however, this cannot be positively identified.

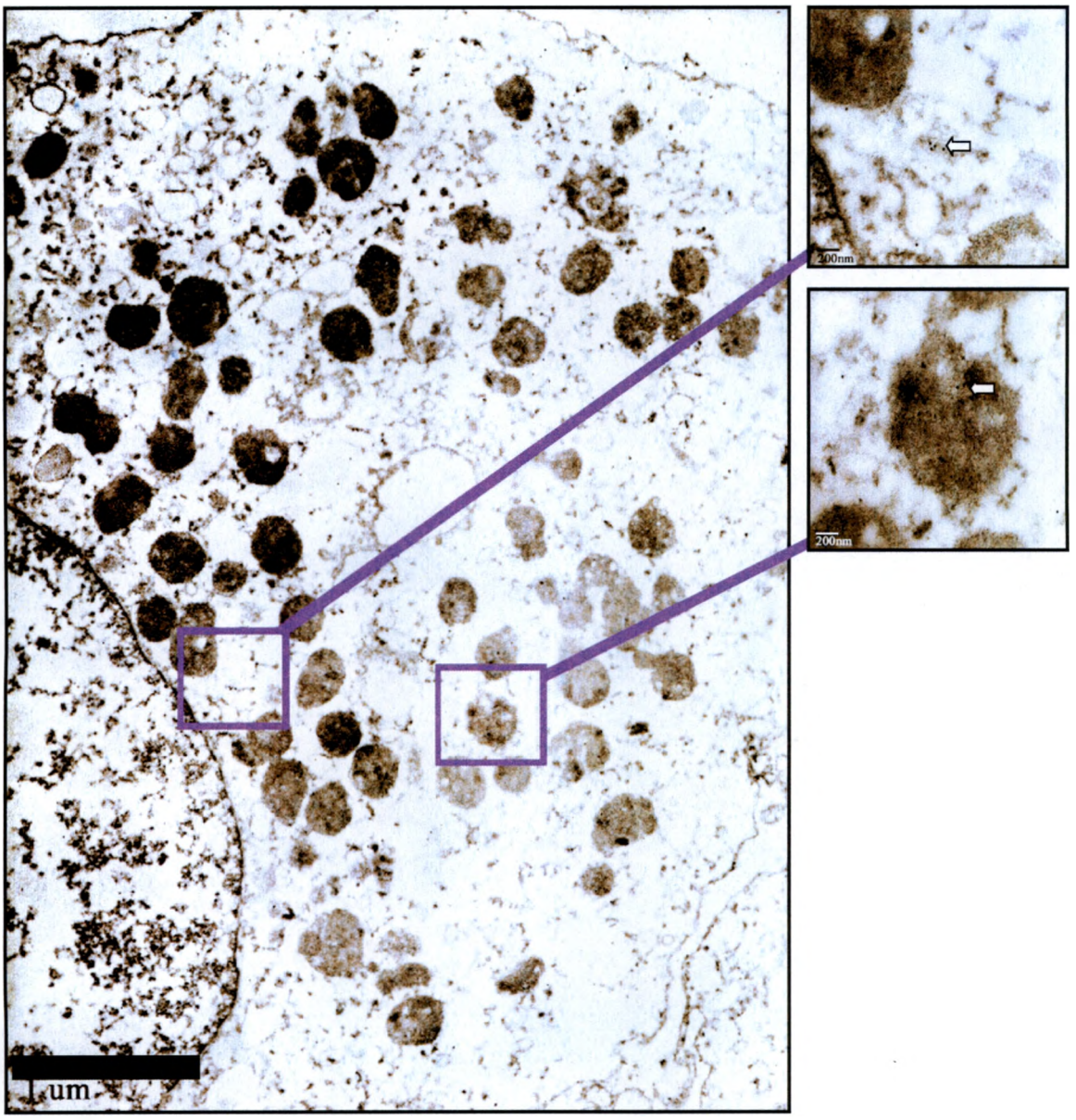
200nm
200nm
1um

Figure 2

Images of U87MG cells stimulated with 100ng/ml human TNF-α for 90 minutes. Increased numbers of electron dense vesicles are apparent. Specific labeling of vesicles and associated ER was also present in these sections.

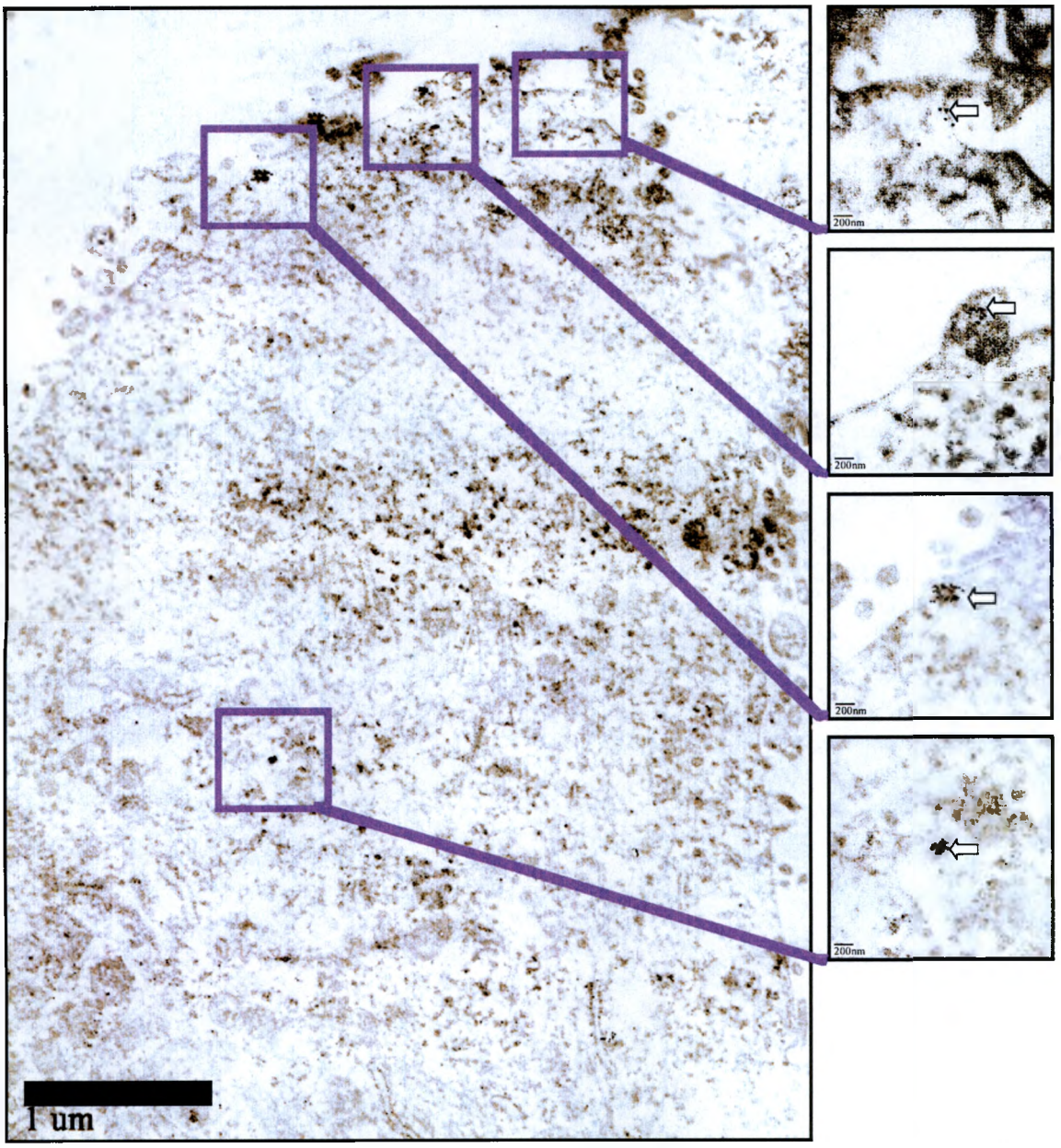
1 um
200nm
200nm
200nm
200nm

Figure 3

Representative image of a U87MG cell stimulated with 100ng/ml human TNF-α for 240 minutes. The cell has become highly vesiculated, making correlation of labeling to specific intercellular structures difficult. The labeling does appear to be more associated with vesicles and locations near the plasma membrane and labeling is greatly increased.

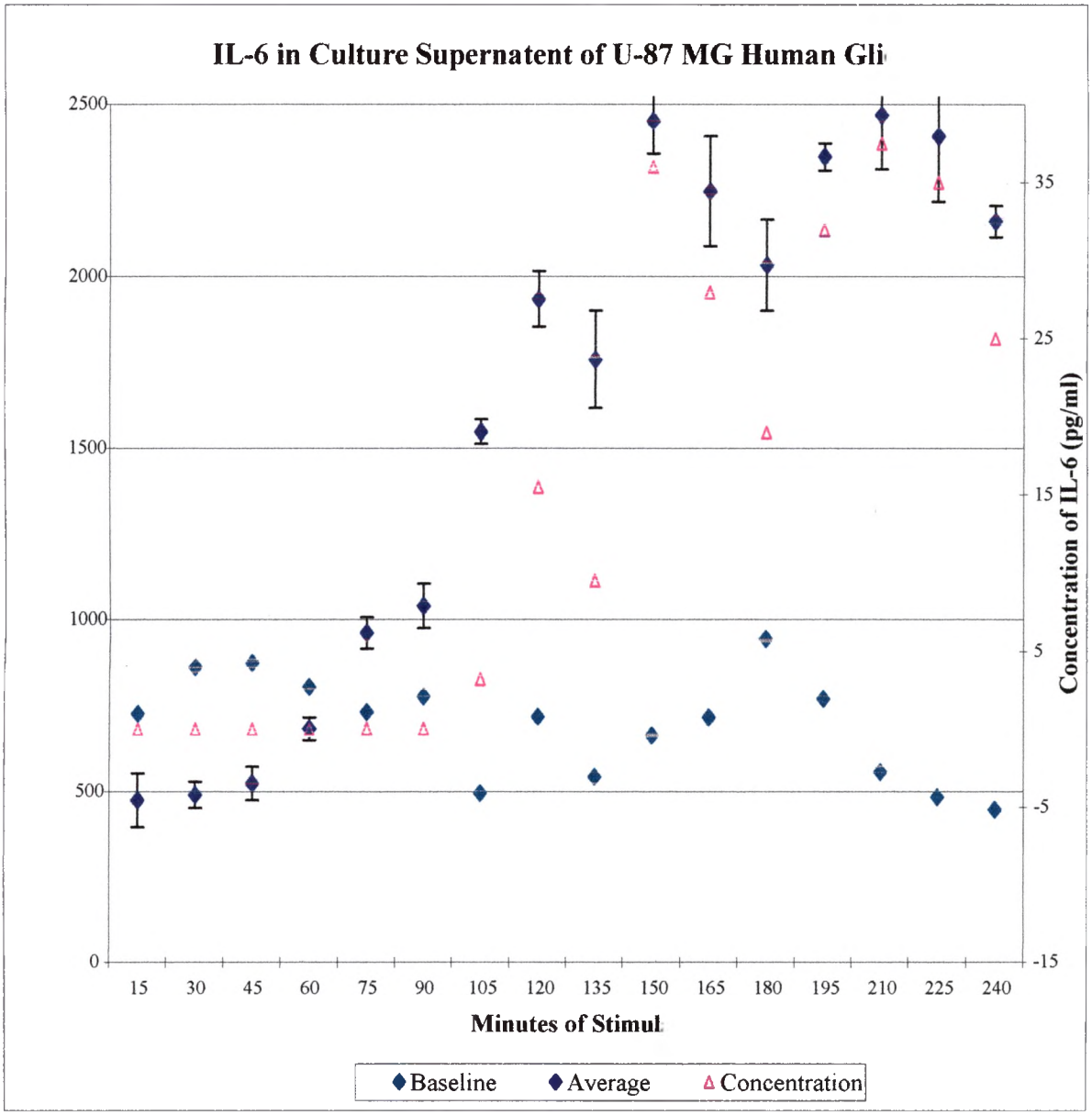
IL-6 in Culture Supernatent of U-87 MG Human Gli
2500
2000
1500
1000
500
0
35
25
15
5
-5
-15
Concentration of IL-6 (pg/ml)
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
Minutes of Stimul
Baseline
Average
Concentration

Figure 4

This graph depicts the luminescence and concentration values for the ELISA in which samples were obtained every fifteen minutes. As seen, the control flask or baseline remains at approximately the same level throughout the entire experiment. However, the experimental (shown as the average of all three runs) shows a marked and significant (ANOVA $p= 9.27x10^{-9}$) increase in luminosity after 90 minutes (two-tailed t-test $p = 0.0001$). The concentration of IL-6 in the media was determined by plotting the luminosity of the samples against a standard. As expected, the luminosity and IL-6 concentration curves closely follow one another.

DISCUSSION

The results of this study indicate that neither production nor secretion of IL-6 from TNF-α stimulated U-87 MG human glioblastoma cells is constitutive, and furthermore, that IL-6 secretion may follow the well-characterized pathway beginning on the rough ER and culminating with exocytosis (Kelly, 1985). IL-6 was not detectable by immunogold or ELISA prior to 90 minutes of TNF-α exposure, and after 90 minutes labeling placed IL-6 in the ER at 90 minutes and vesicles at 240 minutes. This time course and pathway is expected for the normal protein secretion pathway. However, because of excessive vesiculation in the cytoplasm after 90 or more minutes of TNF-α stimulation, it was not possible to confirm if Golgi apparati were present and what their involvement might be. The apparent lack of Golgi could be due to the small size or swollen appearance of the apparatus making it unrecognizable. Plasma B-cells are known to have a small but present Golgi apparatus (Wiest *et al.*, 1990).

Thus the intracellular pathway of secretion could not be completely characterized due to the morphological changes in the cells after stimulation. The longer the cells were incubated in the presence of TNF-α, the more vesiculated and less distinct the features of the cytoplasm became. It has been shown by others that the rough ER becomes increasingly elaborate when cells are mobilized for secretion (Shohat *et al.*, 1973; Wiest *et al.*, 1990). This could have contributed to the highly vesiculated cytoplasm observed here. In addition, formation of electron dense vesicles has been observed characteristic of cells that secrete proteins (Kelly, 1985). Although there are electron dense vesicles

apparent in the unstimulated and stimulated from 5-30 minute cells, the number of these vesicles and their density increased by 90 minutes, as seen in Figures 1 and 2. This observation, plus the immunogold labeling of these vesicles, supports the interpretation that these vesicles contain newly synthesized IL-6 destined for exocytosis.

In a similar study by Zheng and Specter (1996), lipopolysaccharide (LPS) was used to stimulate macrophages to produce TNF-α. The mRNA for TNF-α was detectable after 1 hour and peaked at 3 hours. TNF-α protein was then detectable starting at 2 hours. The timing of this system is thus similar to what was observed here with TNF-α stimulated IL-6 production.

Previous studies have shown that the downstream signaling cascade activated by TNF-α includes mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB (for review see Chen and Goeddel, 2002). Although stimulation of astrocytes by TNF-α causes secretion of IL-6, MAPK has been shown to inhibit IκB, the inhibitor of NF-κB (Schwenger *et al.*, 1998). MAPK has also been shown to inhibit the activation of STAT3, a step of IL-6 stimulation in astrocytes (Sengupta *et al.*, 1998). Stimulation of astrocytes by IL-6 or another cytokine that stimulates the activation of STAT3 precedes upregulation of GFAP and thus astrogliosis (Sriram *et al.*, 2004). This series of events suggests, therefore, that initially stimulation of astrocytes by TNF-α promotes astrogliosis and subsequently leads to apoptosis (for review see Baldwin, 1996).

This study was primarily aimed at determining the timing and pathway of secretion, and provided essentially no information about the sensitivity of the U8-7MG cells to TNF-α under the conditions used. Others (Benveniste *et al.* 1990) have demonstrated that primary cultured astrocytes will respond to lower concentrations (down to 5 ng/ml) of TNF–α by secreting IL-6, however, the maximal amount of IL-6 production was

obtained with 100ng/ml TNF–α. The levels of TNF–α in a non-injured, normal CNS are undetectable into the picogram range, while patients with neurological diseases such as multiple sclerosis (MS) show TNF–α concentrations of approximately 10 pg/ml (Drulovic *et al.*, 1997). Blood serum levels of TNF–α were much greater at about 100 pg/ml in normal healthy individuals and at least three times that in individuals with MS (Hohnoki *et al.*, 1998). Shown in this study, commercially available chemiluminescent ELISA kits display sensitivity in the pg/ml range for IL-6 and the same for TNF–α. However, current ELISA protocols require 8 hours minimum and at least 100 μl of sample to complete. With respect to the use of cultured astrocytes as injury detectors; that is, detection of TNF-α released from microglia by looking at IL-6 released in response, 1.75 hours elapses before detectable concentrations of IL-6 appear in the medium. Using the cell chip as described in the introduction could allow subsequent detection and measurement of the concentrations of TNF–α via IL-6 less than an hour later using only a few microliters of sample fluid. Thus it may be quite feasible that earlier and more sensitive detection is possible with the cell chip.

This system, as it is currently set up, provides a way to examine the TNF-α/IL-6 signaling mechanism(s) as it applies in injury response. Due to the versatility of this chip, this signaling method is not the only one that can be used with the cell chip. The cells chosen for this study, U-87 MG human glioblastoma cells, are a good choice for this system. They are human, durable, adherent, contact inhibited, and produce gap junctions. The U-87 MG cells are also known to respond to TNF-α and produce GFAP and IL-6 (Chen *et al.*, 1993; Ito *et al.*, 1989).

Other future studies could explore the interaction of microglia and astrocytes *in situ* and *in vitro* in a co-culture system using well known markers (IL-6, GFAP) to explore the

interactions of these cells in the nervous system, both in development and in regeneration scenarios. Additionally, a study on the dose response of astrocytes to TNF-α and to determine the lower limit of detection for this system is also necessary.

REFERENCES

Aloisi, F. 2001. Immune function of microglia. Glia 36:165-79.

Azuma, H., T. Moriyama, H. Ikeda, M. Oshima, A. Okuno, and S. Sekiguchi. 2000. Analysis of soluble interleukin 6 receptor in cerebrospinal fluid in inflammatory and non-inflammatory conditions. Cytokine 12:160-4.

Baldwin, A. S., Jr. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 14:649-83.

Bauer, J., U. Ganter, T. Geiger, U. Jacobshagen, T. Hirano, T. Matsuda, T. Kishimoto, T. Andus, G. Acs, W. Gerok, and et al. 1988. Regulation of interleukin-6 expression in cultured human blood monocytes and monocyte-derived macrophages. Blood 72:1134-40.

Benveniste, E. N., and D. J. Benos. 1995. TNF-alpha- and IFN-gamma-mediated signal transduction pathways: effects on glial cell gene expression and function. Faseb J 9:1577-84.

Benveniste, E. N., S. M. Sparacio, J. G. Norris, H. E. Grenett, and G. M. Fuller. 1990. Induction and regulation of interleukin-6 gene expression in rat astrocytes. J Neuroimmunol 30:201-12.

Campbell, I. L., C. R. Abraham, E. Masliah, P. Kemper, J. D. Inglis, M. B. Oldstone, and L. Mucke. 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci U S A 90:10061-5.

Campbell, I. L., M. V. Hobbs, J. Dockter, M. B. Oldstone, and J. Allison. 1994. Islet inflammation and hyperplasia induced by the pancreatic islet-specific overexpression of interleukin-6 in transgenic mice. Am J Pathol 145:157-66.

Campbell, I. L., T. W. Kay, L. Oxbrow, and L. C. Harrison. 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. J Clin Invest 87:739-42.

Chen, G., and D. V. Goeddel. 2002. TNF-R1 signaling: a beautiful pathway. Science 296:1634-5.

Chen, T. C., D. R. Hinton, M. L. Apuzzo, and F. M. Hofman. 1993. Differential effects of tumor necrosis factor-alpha on proliferation, cell surface antigen expression, and cytokine interactions in malignant gliomas. Neurosurgery 32:85-94.

Drulovic, J., M. Mostarica-Stojkovic, Z. Levic, N. Stojsavljevic, V. Pravica, and S. Mesaros. 1997. Interleukin-12 and tumor necrosis factor-alpha levels in cerebrospinal fluid of multiple sclerosis patients. J Neurol Sci 147:145-50.

Eng, L. F., and R. S. Ghirnikar. 1994. GFAP and astrogliosis. Brain Pathol 4:229-37.

Eskes, C., L. Juillerat-Jeanneret, G. Leuba, P. Honegger, and F. Monnet-Tschudi. 2003. Involvement of microglia-neuron interactions in the tumor necrosis factor-alpha release, microglial activation, and neurodegeneration induced by trimethyltin. J Neurosci Res 71:583-90.

Finck, B. K., B. Chan, and D. Wofsy. 1994. Interleukin 6 promotes murine lupus in NZB/NZW F1 mice. J Clin Invest 94:585-91.

Forloni, G., F. Mangiarotti, N. Angeretti, E. Lucca, and M. G. De Simoni. 1997. Beta-amyloid fragment potentiates IL-6 and TNF-alpha secretion by LPS in astrocytes but not in microglia. Cytokine 9:759-62.

Gadient, R. A., and U. H. Otten. 1997. Interleukin-6 (IL-6)--a molecule with both beneficial and destructive potentials. Prog Neurobiol 52:379-90.

Glass, T. L., T. D. Raabe, D. M. Garcia, and J. R. Koke. 2002. Phosphorylated neurofilaments and SNAP-25 in cultured SH-SY5Y neuroblastoma cells. Brain Res 934:43-8.

Griffin, W. S., J. G. Sheng, M. C. Royston, S. M. Gentleman, J. E. McKenzie, D. I.

Graham, G. W. Roberts, and R. E. Mrak. 1998. Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. Brain Pathol 8:65-72.

Gross, V., T. Andus, J. Castell, D. Vom Berg, P. C. Heinrich, and W. Gerok. 1989. O- and N-glycosylation lead to different molecular mass forms of human monocyte interleukin-6. FEBS Lett 247:323-6.

Gruol, D. L., and T. E. Nelson. 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Mol Neurobiol 15:307-39.

Hahn, M., T. Glass, and J. Koke. 2000. Extracellular matrix effects on a neuroblastoma cell line. Cytobios 102:7-19.

Hayat, M. A. 2002. Microscopy, Immunohistochemistry, and Antigen Retrieval Methods, 1 edition. Kluwer Academic / Plenum Publishers, New York, Boston, Dordrecht, London, Moscow.

Hirano, T. 1998. Interleukin 6 and its receptor: ten years later. Int Rev Immunol 16:249-84.

Hohnoki, K., A. Inoue, and C. S. Koh. 1998. Elevated serum levels of IFN-gamma, IL-4 and TNF-alpha/unelevated serum levels of IL-10 in patients with demyelinating diseases during the acute stage. J Neuroimmunol 87:27-32.

Ito, M., T. Nagashima, and T. Hoshino. 1989. Quantitation and distribution analysis of glial fibrillary acidic protein in human glioma cells in culture. J Neuropathol Exp Neurol 48:560-7.

Jean Harry, G., K. Tyler, C. L. d'Hellencourt, H. A. Tilson, and W. E. Maier. 2002. Morphological alterations and elevations in tumor necrosis factor-alpha, interleukin (IL)-1alpha, and IL-6 in mixed glia cultures following exposure to trimethyltin: modulation by proinflammatory cytokine recombinant proteins and neutralizing antibodies. Toxicol Appl Pharmacol 180:205-18.

Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. Science 230:25-32.

Maeda, Y., M. Matsumoto, O. Hori, K. Kuwabara, S. Ogawa, S. D. Yan, T. Ohtsuki, T. Kinoshita, T. Kamada, and D. M. Stern. 1994. Hypoxia/reoxygenation-mediated induction of astrocyte interleukin 6: a paracrine mechanism potentially enhancing neuron survival. J Exp Med 180:2297-308.

Marz, P., J. G. Cheng, R. A. Gadient, P. H. Patterson, T. Stoyan, U. Otten, and S. Rose-John. 1998. Sympathetic neurons can produce and respond to interleukin 6. Proc Natl Acad Sci U S A 95:3251-6.

Marz, P., K. Heese, B. Dimitriades-Schmutz, S. Rose-John, and U. Otten. 1999a. Role of interleukin-6 and soluble IL-6 receptor in region-specific induction of astrocytic differentiation and neurotrophin expression. Glia 26:191-200.

Marz, P., U. Otten, and S. Rose-John. 1999b. Neural activities of IL-6-type cytokines often depend on soluble cytokine receptors. European Journal of Neuroscience 11:2995-3004.

May, L. T., J. Ghrayeb, U. Santhanam, S. B. Tatter, Z. Sthoeger, D. C. Helfgott, N. Chiorazzi, G. Grieninger, and P. B. Sehgal. 1988. Synthesis and secretion of multiple forms of beta 2-interferon/B-cell differentiation factor 2/hepatocyte-stimulating factor by human fibroblasts and monocytes. J Biol Chem 263:7760-6.

Muraguchi, A., T. Hirano, B. Tang, T. Matsuda, Y. Horii, K. Nakajima, and T. Kishimoto. 1988. The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J Exp Med 167:332-44.

Murphy, G. M., Y. L. Lee, X. C. Jia, A. C. H. Yu, A. Majewska, Y. L. Song, K. Schmidt, and L. F. Eng. 1995. Tumor-Necrosis-Factor-Alpha and Basic Fibroblast Growth-Factor Decrease Glial Fibrillary Acidic Protein and Its Encoding Messenger-Rna in Astrocyte Cultures and Glioblastoma Cells. Journal of Neurochemistry 65:2716-2724.

Norris, J. G., L. P. Tang, S. M. Sparacio, and E. N. Benveniste. 1994. Signal transduction pathways mediating astrocyte IL-6 induction by IL-1 beta and tumor necrosis

factor-alpha. J Immunol 152:841-50.

Parish, C. L., D. I. Finkelstein, W. Tripanichkul, A. R. Satoskar, J. Drago, and M. K. Horne. 2002. The role of interleukin-1, interleukin-6, and glia in inducing growth of neuronal terminal arbors in mice. J Neurosci 22:8034-41.

Penkowa, M., A. Molinero, J. Carrasco, and J. Hidalgo. 2001. Interleukin-6 deficiency reduces the brain inflammatory response and increases oxidative stress and neurodegeneration after kainic acid-induced seizures. Neuroscience 102:805-18.

Ransom, B., T. Behar, and M. Nedergaard. 2003. New roles for astrocytes (stars at last). Trends Neurosci 26:520-2.

Sallmann, S., E. Juttler, S. Prinz, N. Petersen, U. Knopf, T. Weiser, and M. Schwaninger. 2000. Induction of interleukin-6 by depolarization of neurons. J Neurosci 20:8637-42.

Schwenger, P., D. Alpert, E. Y. Skolnik, and J. Vilcek. 1998. Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IkappaB alpha phosphorylation and degradation. Mol Cell Biol 18:78-84.

Sengupta, T. K., E. S. Talbot, P. A. Scherle, and L. B. Ivashkiv. 1998. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. Proc Natl Acad Sci U S A 95:11107-12.

Shohat, M., G. Janossy, and R. R. Dourmashkin. 1973. Development of rough endoplasmic reticulum in mouse splenic lymphocytes stimulated by mitogens. Eur J Immunol 3:680-7.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31-43.

Sriram, K., S. A. Benkovic, M. A. Hebert, D. B. Miller, and J. P. O'Callaghan. 2004. Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-

4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis in vivo? J Biol Chem 279:19936-47.

Suematsu, S., T. Matsuda, K. Aozasa, S. Akira, N. Nakano, S. Ohno, J. Miyazaki, K. Yamamura, T. Hirano, and T. Kishimoto. 1989. IgG1 plasmacytosis in interleukin 6 transgenic mice. Proc Natl Acad Sci U S A 86:7547-51.

Takai, Y., G. G. Wong, S. C. Clark, S. J. Burakoff, and S. H. Herrmann. 1988. B cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes. J Immunol 140:508-12.

Van Wagoner, N. J., and E. N. Benveniste. 1999. Interleukin-6 expression and regulation in astrocytes. J Neuroimmunol 100:124-39.

Vanden Berghe, W., L. Vermeulen, G. De Wilde, K. De Bosscher, E. Boone, and G. Haegeman. 2000. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. Biochem Pharmacol 60:1185-95.

Weigum, S., N. Christodoulides, J. McDevitt, and J. Koke. 2001. Use of Cultured Glioma Cells As Sensors in Chip-Based Assay for Astrogliosis. Molecular Biology of the Cell 12:2834a.

Wiest, D. L., J. K. Burkhardt, S. Hester, M. Hortsch, D. I. Meyer, and Y. Argon. 1990. Membrane biogenesis during B cell differentiation: most endoplasmic reticulum proteins are expressed coordinately. J Cell Biol 110:1501-11.

Zheng, Z. M., and S. Specter. 1996. Dynamic production of tumour necrosis factor-alpha (TNF-alpha) messenger RNA, intracellular and extracellular TNF-alpha by murine macrophages and possible association with protein tyrosine phosphorylation of STAT1 alpha and ERK2 as an early signal. Immunology 87:544-50.

Vita

Carlene F. Worthington was born in Las Vegas, NV on December 26, 1978, the daughter of Michelle L. Worthington and Everet S. Worthington. In 1992 she started high school at Cheyenne and the summer of 1993 started college at Community College of Southern Nevada. In January of 1994, she moved to Reno. There, she attended Proctor R. Hug High School and Truckee Meadows Community College. She graduated high school in 1995 with 20 college credits and continued her college career. In 1998 she moved to Austin, TX and continued college in 1999, first at Austin Community College and then in the summer at Texas State University-San Marcos. She graduated with a B.S. in Biology in December of 2001 with 190 credits and an M.S. in Biology out of Dr. Joseph R. Koke's cell biology laboratory in August of 2004 with 40 credits. She has plans on continuing her education with a doctorate in genetics. She also plans on continuing to enjoy life and playing Magic© for as long as she is capable of it.

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