

Interleukin-6 (IL-6) Production by Astrocytes: Autocrine Regulation by IL-6 and the Soluble IL-6 Receptor

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In the CNS, astrocytes are a major inducible source of interleukin-6 (IL-6). Although IL-6 has beneficial effects in the CNS because of its neurotrophic properties, its overexpression is generally detrimental, adding to the pathophysiology associated with CNS disorders. Many factors have been shown to induce IL-6 expression by astrocytes, particularly the cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). However, the role of IL-6 in its own regulation in astrocytes has not been determined. In this study, we examined the influence of IL-6 alone or in combination with TNF- α or IL-1 β on IL-6 expression. IL-6 alone had no effect on IL-6 expression; however, the addition of the soluble IL-6 receptor (sIL-6R) induced IL-6 transcripts. Addition of TNF- α or IL-1 β plus IL-6/sIL-6R led to synergistic increases in IL-6 expression. This synergy also

occurred in the absence of exogenously added IL-6, attributable to TNF- α - or IL-1 β -induced endogenous IL-6 protein production. IL-6 upregulation seen in the presence of TNF- α or IL-1 β plus IL-6/sIL-6R was transcriptional, based on nuclear run-on analysis. Experiments were extended to other IL-6 family members to determine their role in IL-6 regulation in astrocytes. Oncostatin M (OSM) induced IL-6 alone and synergized with TNF- α for enhanced expression. These results demonstrate that IL-6/sIL-6R and OSM play an important role in the regulation of IL-6 expression within the CNS, particularly in conjunction with the proinflammatory cytokines TNF- α and IL-1 β .

Key words: glial cells; cytokines; interleukin-6; central nervous system; receptors; astrocytes

Interleukin-6 (IL-6) is a regulator of inflammatory and immunological responses, and belongs to a family of neurotrophic factors including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-11, and others (for review, see Taga and Kishimoto, 1997; Heinrich et al., 1998). Characteristic of this family is the pluripotency and redundancy of biological responses elicited by its members, which stem from the use of a common signal transducing receptor, gp130 (for review, see Taga and Kishimoto, 1997). IL-6 acts on target cells through a receptor complex composed of an IL-6-binding subunit, the IL-6 receptor- α (IL-6R α), and the signal transducing receptor gp130. Initiation of IL-6 signaling occurs when IL-6 binds to the IL-6R α , leading to an association with gp130. This event leads to activation of gp130-associated Janus kinases (JAKs), activation of various signaling pathways (JAK/STAT, MAPK), and subsequent gene activation (for review, see Taga and Kishimoto, 1997). The IL-6R α is found in both membrane-bound and soluble (sIL-6R α) forms (Lust et al., 1992; Müllberg et al., 1993). The sIL-6R α can complex with IL-6, bind, and signal through gp130, thus serving as an agonist of IL-6-induced responses (for review, see Rose-John and Heinrich, 1994). The sIL-6R α can be generated by shedding of the membrane-bound receptor or by mRNA alternative splicing (Lust et al., 1992; Müllberg et al., 1993).

Under physiological conditions, IL-6 levels in the CNS remain low. However, in CNS injury and inflammation, IL-6 levels become elevated (for review, see Gruol and Nelson, 1997; Benveniste, 1998). Elevated levels of IL-6, as well as the proinflammatory cytokines IL-1 β and tumor necrosis factor (TNF)- α , are detected in the CSF of patients with multiple sclerosis, Alzheimer's disease, meningitis, and stroke (for review, see Zhao and Schwartz, 1998). These cytokines mediate inflammation and contribute to the neuropathology and pathophysiology associated with the inflamed CNS (for review, see Eng et al., 1996; Benveniste, 1997). Data from transgenic mice suggest that overexpression of IL-6 leads to reactive gliosis, neurodegeneration, breakdown of the blood-brain barrier (BBB), and angiogenesis (Campbell et al., 1993). However, other reports suggest a beneficial role of IL-6 in the CNS. Occlusion of the middle cerebral artery leads to IL-6 bioactivity in the ischemic hemisphere, and injection of IL-6 into the ischemic region significantly reduces brain damage (Loddick et al., 1998). IL-6, in the presence of the sIL-6R, enhances neuronal survival in the absence of nerve growth factor in rat sympathetic neurons (März et al., 1998a). Our laboratory has shown that IL-6 plus the sIL-6R inhibits TNF- α -induced expression of vascular cell adhesion molecule-1 (VCAM-1) in astrocytes (Oh et al., 1998). These studies demonstrate the need for tight regulation of IL-6 to maintain beneficial actions and prevent IL-6-induced neuropathology.

Astrocytes are the major source of IL-6 in CNS injury and inflammation (for review, see Gruol and Nelson, 1997). *In vitro* analysis of IL-6 production by astrocytes indicates that many stimuli can upregulate its production, in particular, the cytokines TNF- α and IL-1 β (Benveniste et al., 1990, 1994). However, the role of IL-6 in its own regulation is less well defined. In this study, we have examined the role of IL-6 and the sIL-6R in IL-6 induction, with an emphasis on the ability of IL-6/sIL-6R to

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modulate TNF- α - and IL-1 β -induced IL-6 expression in astrocytes.

MATERIALS AND METHODS

Cell lines and primary human astrocyte cultures. The U373-MG astrogloma cell line was grown in DMEM supplemented with 10 mM HEPES, pH 7.2, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.1 mM nonessential amino acid mixture, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS), as described previously (Oh et al., 1998). CRT human astrogloma cells were grown in RPMI 1640 medium supplemented with 10 mM HEPES, pH 7.2, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS as described previously (Rosenman et al., 1995). Biopsy material from patients undergoing surgery to treat intractable epilepsy was used to prepare human adult astrocyte cultures as described previously (Barnum et al., 1992). Primary human adult astrocytes were obtained after 30 d in culture and were grown in DMEM, high glucose formula supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acid mixture, 0.1% gentamicin, 2.5 μ g/ml amphotericin B, and 10% FBS. Astrocytes were 87–90% GFAP positive (Barnum et al., 1992). Human astrocytes obtained from this source have many of the same functional responses as do primary cultures of rat astrocytes (Benveniste et al., 1994; Oh et al., 1998).

Reagents. Human recombinant TNF (rTNF- α) (9.0×10^7 U/mg) and human rIL-1 β (2.0×10^8 U/mg) were purchased from Genzyme (Cambridge, MA), and human rIL-6 (7.0×10^3 U/ μ g), sIL-6R α [100 active units (AU)/ μ g], rTGF- β_1 (2.5×10^4 U/ μ g), rOSM (4.4×10^3 AU/ μ g), rLIF (8.3×10^4 U/ μ g), rCNTF (10 AU/ μ g), soluble rCNTFR (3.3 AU/ μ g), and rIL-11 (6.6×10^3 AU/ μ g) were purchased from R & D Systems (Minneapolis, MN). Hyper-IL-6 (H-IL-6) was the kind gift of Professor Stefan Rose-John (Universität Mainz) and was prepared as described previously (Fischer et al., 1997). Anti-human IL-6, anti-human IL-6R, and anti-human gp130 neutralizing antibodies were obtained from R & D Systems. Purified mouse IgG₁ (anti-TNP) (used as an isotype control) was purchased from PharMingen (San Diego, CA).

IL-6 production in astrocytes. U373-MG cells, CRT cells, and primary human astrocytes were resuspended in their respective media containing 10% FBS and plated at 0.5×10^6 cells/well into six-well (35 mm) plates (Costar, Cambridge, MA). The cells were allowed to reach ~90% confluency, media was aspirated, and fresh serum-free media was added to cells for 12 hr. After this time, cells were washed once with sterile PBS, and fresh serum-free media was added to each well. Astrocytes were treated with various reagents (TNF- α , IL-1 β , IL-6, sIL-6R, TGF- β , CNTF, sCNTFR, LIF, and IL-11) alone or in combination for 24 hr, then supernatants were collected, centrifuged to remove contaminating cells, and stored at -70°C until use.

Measurement of IL-6 activity. IL-6 activity in astrocyte culture supernatants was determined in a biological assay using the IL-6-dependent B cell hybridoma B9, as described previously (Norris and Benveniste, 1993). Briefly, B9 cells (6×10^3 cells/well) were plated in 96-well microtiter plates, then serial dilutions of astrocyte-conditioned medium and recombinant human IL-6 (used as a standard) were added and incubated at 37°C for 72 hr. Triplicate cultures were set up for each condition. After 72 hr, B9 cell growth was assessed using the MTT assay as described previously (Norris and Benveniste, 1993). In this assay, the amount of IL-6 in astrocyte-conditioned supernatants was determined by comparison to a recombinant human IL-6 standard in which colorimetric change versus recombinant human IL-6 concentration (picograms per milliliter) is known. The reagents used in this study to stimulate IL-6 production (TNF- α , IL-1 β , sIL-6R, TGF- β , OSM, LIF, CNTF, sCNTFR, and IL-11) do not support B9 growth when added directly to B9 cells or when added to astrocyte cultures immediately before collecting supernatants (data not shown).

RNA isolation, riboprobes, and RNase protection assay (RPA). U373-MG cells were plated at 4×10^6 cells per 100 mm² dish (Costar, Cambridge, MA). Upon confluency, cells were serum-starved for 12 hr. Total cellular RNA was isolated from resulting confluent monolayers of astrocytes that had been incubated for various times with cytokines. RNA isolation was performed as described previously (Lee et al., 1997). A pGem4Z plasmid containing a 349 bp fragment corresponding to 194–542 bp of the human IL-6 cDNA was linearized with *EcoRI*. *In vitro* transcription of this linearized plasmid with T7 RNA polymerase generated an antisense probe 408 nucleotides in length (note that this probe contains a portion of the pGem4Z plasmid). A pAMP-1 vector containing a fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (corresponding to 43–531 bp) was linearized with

NcoI, which digests within the GAPDH cDNA insert. *In vitro* transcription of this linearized plasmid with T7 RNA polymerase generates a 290 bp antisense RNA probe.

In vitro transcription of riboprobes and RPA were conducted as described previously (Lee and Benveniste, 1996). Total RNA (15 μ g) was hybridized with IL-6 (30×10^3 cpm) and GAPDH (25×10^3 cpm) riboprobes at 42°C overnight. The hybridized mixture was then treated with RNase A/T1 (1:200 dilution) at room temperature for 1 hr and analyzed by 5% denaturing (8 M urea) PAGE, and the gels were exposed to x-ray film. The protected fragments of the IL-6 and GAPDH riboprobes are 349 and 230 nucleotides in length, respectively. Quantification of protected RNA fragments was performed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values for IL-6 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition. GAPDH mRNA was used as a control gene because its levels are not affected by cytokine treatment. The RPA shown in this article is overexposed for GAPDH because the signal for IL-6 is weaker. Quantification of the original gel was performed on a PhosphorImager to arrive at accurate values.

Nuclear run-on analysis. Nuclear run-on analysis was performed as described previously (Bethea et al., 1992; Chung et al., 1992; Shrikant et al., 1994). Nuclei were isolated from confluent monolayers of astrocytes that were incubated for 30–45 min in the presence or absence of stimulus (IL-6/sIL-6R, TNF- α , IL-1 β , TNF- α + IL-6/sIL-6R, and IL-1 β + IL-6/sIL-6R). The cells (30×10^6) were collected, washed once with cold PBS, and pelleted. Nuclei were isolated by lysing the cells with 10 mM Tris, pH 7.4, 2 mM MgCl₂, 10 mM NaCl, and 0.5% Nonidet P-40, followed by centrifugation at $1000 \times g$. The nuclei were stored at -80°C in buffer containing 50 mM Tris, pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA. To perform run-on transcriptional analysis, the nuclei were thawed on ice and incubated for 30 min at 30°C in reaction buffer containing 10 mM DTT, 1 mM each of ATP, CTP and GTP, and 0.25 mCi [³²P]UTP (3000 Ci/mM) (Amersham, Arlington Heights, IL). After the reaction, DNA digestion was performed in the presence of 0.5 M NaCl, 50 mM MgCl₂, 2 mM CaCl₂, and 10 mM Tris, pH 7.4, and DNase (10 mg/ml) (Promega, Madison, WI) for 5 min at 30°C . Protein digestion was then performed in the presence of 5% SDS, 0.5 M Tris, pH 7.4, 0.125 M EDTA, and proteinase K (20 μ g/sample) for 30 min at 42°C . Nuclei were lysed and RNA was harvested as described in RNA isolation. Denatured circular plasmid DNA was immobilized on nitrocellulose paper using a Millipore (Bedford, MA) Milliblot S system. After UV cross-linking the DNA to the nitrocellulose, prehybridization was performed at 65°C for at least 3 hr in a solution of 50% formamide, 0.1% SDS, $5 \times$ SSC, $2.5 \times$ Denhardt's, 250 μ g/ml tRNA, and 50 mM Na₂PO₄, pH 6.5. For hybridization, 9×10^6 cpm of labeled and denatured RNA was used in 1 ml of hybridization solution and incubated at 65°C for 48 hr. The filters were washed twice for 30 min at 65°C in $2 \times$ SSC, 30 min at 37°C in $2 \times$ SSC with 10 μ g/ml ribonuclease A/T1, and finally for 30 min at 37°C in $2 \times$ SSC. The filters were exposed to the PhosphorImager (Molecular Dynamics) for quantification. The increase of transcriptional activation was determined by comparing the ratios of IL-6/GAPDH values obtained for each stimulus.

Statistical analysis. Significance between experimental values was determined using two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

RESULTS

Treatment of U373-MG cells with TNF- α and the sIL-6R induces IL-6 production

We were interested in determining the role of IL-6 and the sIL-6R (IL-6/sIL-6R) in its own regulation. Because of the obvious difficulty of measuring IL-6 protein production in the presence of exogenously added IL-6, an endogenous source of IL-6 was used for initial studies. Previously, our laboratory has shown that IL-6 protein secretion is upregulated by TNF- α in primary rat astrocytes (Benveniste et al., 1990). To determine whether similar IL-6 induction is seen in the human glioma cell line U373-MG, cells were treated with TNF- α (50 ng/ml) for 24 hr, and supernatants were analyzed for IL-6 protein secretion by the B9 bioassay. This concentration of TNF- α was previously determined to be optimal for IL-6 production by astrocytes (Oh et al., 1998). Constitutively, U373-MG cells express low levels of IL-6

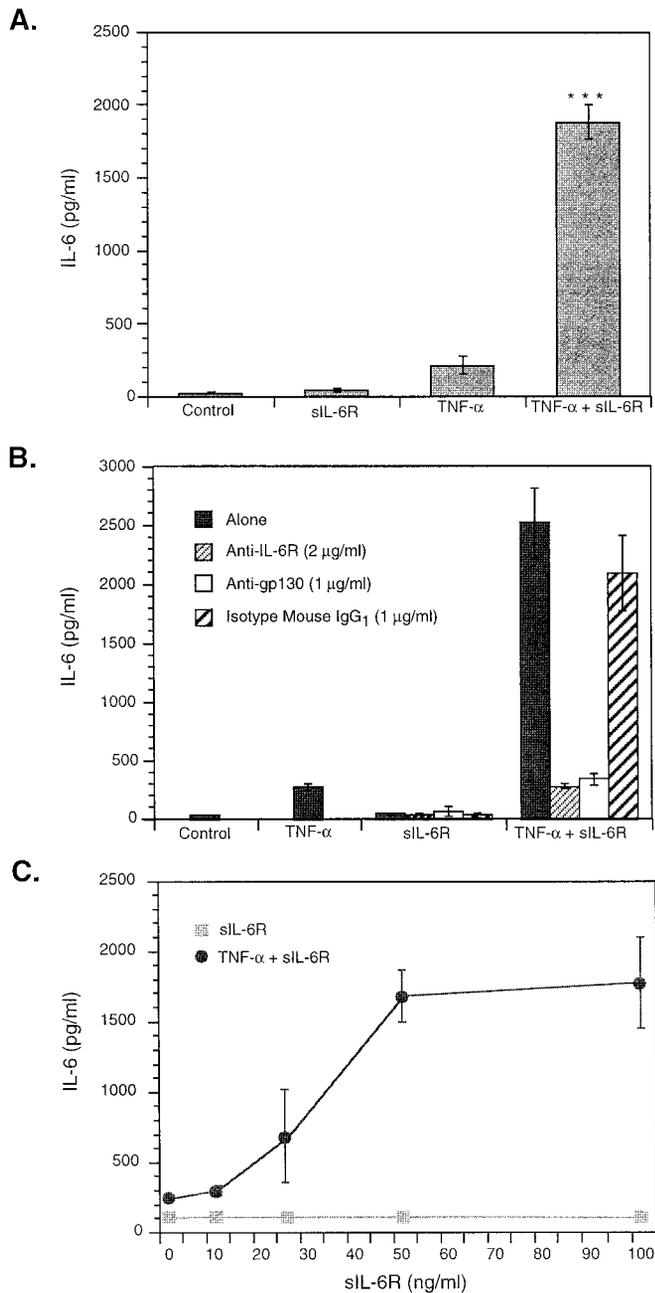


Figure 1. Stimulation of IL-6 bioactivity in U373-MG cells by TNF- α and the soluble IL-6 receptor. U373-MG cells were incubated with TNF- α (50 ng/ml) and the sIL-6R (100 ng/ml) alone and in combination for 24 hr, and supernatants harvested and then analyzed for IL-6 activity as described in Materials and Methods. $***p < 0.001$ (A). Neutralizing antibodies to the IL-6R (2 μ g/ml) were preincubated with sIL-6R for 30 min at room temperature after which it was added to U373-MG cells alone or in combination with TNF- α , or U373-MG cells were preincubated for 30 min at room temperature with neutralizing antibodies to gp130 (1 μ g/ml), then TNF- α , sIL-6R, or TNF- α + sIL-6R were added to the cells. Supernatants were analyzed for IL-6 activity (B). Cells were treated with TNF- α alone (50 ng/ml), TNF- α plus increasing concentrations of the sIL-6R (0–100 ng/ml), or sIL-6R alone (C). Results are the mean \pm SD of three experiments analyzed in triplicate.

protein, and after 24 hr of stimulation with TNF- α , IL-6 protein levels in supernatants increased by \sim 10-fold, reaching levels over 200 pg/ml (Fig. 1A). To determine whether endogenous IL-6, induced by TNF- α , could complex with exogenous sIL-6R and

Table 1. IL-6 induction in human astrocytes and CRT cells by TNF- α and the sIL-6R

Cell treatment ^a	IL-6 bioactivity (pg/ml)	
	Astrocytes	CRT
Medium	0.6 \pm 0.4 ^b	1.3 \pm 0.1 ^b
sIL-6R (100 ng/ml)	2.0 \pm 0.9	3.2 \pm 0.6
TNF- α (50 ng/ml)	221.6 \pm 37.3	244.4 \pm 76.7
TNF- α + sIL-6R	29,793.4 \pm 10,568.0 ^{c,*}	1,626.7 \pm 345.1 ^{c,**}

^aTwenty-four hour incubation period.

^bMean \pm SD of triplicate samples from two experiments analyzed in triplicate.

^cSignificance between experimental values was determined by two-way ANOVA: * $p < 0.05$; ** $p < 0.01$.

affect IL-6 secretion, U373-MG cells were treated with sIL-6R (100 ng/ml) alone or in combination with TNF- α for 24 hr, and supernatants were analyzed for IL-6 secretion. Treatment with the sIL-6R alone did not increase IL-6 expression in U373-MG cells; however, co-stimulation with TNF- α and the sIL-6R significantly increased IL-6 protein levels in the supernatants to \sim 1900 pg/ml (Fig. 1A), indicating a synergistic interaction between TNF- α and the sIL-6R. Identical experiments have been performed on human adult primary astrocytes and the human astrogloma cell line CRT (Table 1). In all cases, results are qualitatively similar, suggesting that this effect is not specific to U373-MG cells but extends to all astrocyte sources thus far tested.

Addition of neutralizing antibodies to the IL-6R and gp130 blocked induction of IL-6 in the presence of TNF- α and the sIL-6R, reducing levels to those seen on treatment with TNF- α alone (Fig. 1B). Addition of an isotype-matched antibody did not affect IL-6 expression by U373-MG cells in the presence of TNF- α and the sIL-6R (Fig. 1B). Taken together, these data show that the significant increase of IL-6 in the presence of TNF- α and the sIL-6R is specific to the sIL-6R and requires gp130. As well, induction of IL-6 by the sIL-6R in the presence of TNF- α is dose dependent, with maximal responses observed using 50–100 ng/ml of sIL-6R (Fig. 1C). The concentration of 100 ng/ml was used for all subsequent experiments.

Effect of TNF- α and IL-6/sIL-6R on IL-6 mRNA levels

To directly examine the role of IL-6/sIL-6R in IL-6 regulation, analysis was performed at the mRNA level. U373-MG cells were incubated in the presence of exogenous IL-6 (5 ng/ml) and the sIL-6R (100 ng/ml), and RNA was isolated at the indicated times and analyzed by RPA. Results indicate that IL-6/sIL-6R treatment has a modest effect on IL-6 mRNA, raising levels approximately fourfold above constitutive expression at 1 hr and declining thereafter (Fig. 2). IL-6 at a concentration of 5 ng/ml was determined in a dose–response experiment to be optimal for IL-6 induction in the presence of 100 ng/ml of the sIL-6R (data not shown). Similar time course analysis was performed on U373-MG cells treated with TNF- α . TNF- α -induced IL-6 mRNA also peaked at 1 hr, with levels approximately eightfold above constitutive levels and declining thereafter (Fig. 2). On treatment of U373-MG cells with TNF- α plus IL-6/sIL-6R, IL-6 mRNA levels reached \sim 12-fold at 1 hr; this reflects an additive effect of TNF- α and IL-6/sIL-6R. However, at 6 hr, samples treated with TNF- α plus IL-6/sIL-6R were significantly elevated (\sim 15-fold induction), although the levels induced by IL-6/sIL-6R or TNF- α alone were quite low (Fig. 2). These data indicate that IL-6 mRNA levels at 1 hr are increased in the presence of TNF- α plus

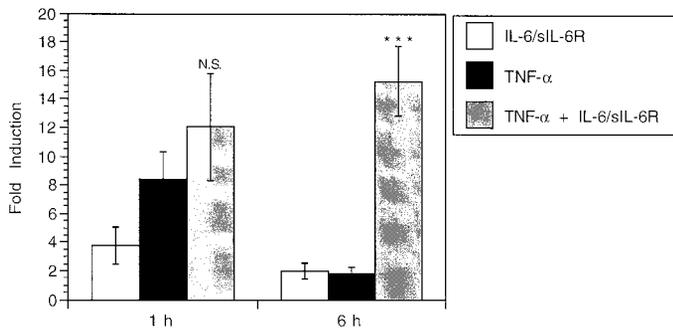


Figure 2. IL-6 mRNA induction by IL-6/sIL-6R and TNF-α alone and in combination. U373-MG cells were treated with IL-6 (5 ng/ml)/sIL-6R (100 ng/ml), TNF-α (50 ng/ml), or both, for 1 or 6 hr. RNA was extracted and examined by RPA for IL-6 and GAPDH mRNA expression. PhosphorImager quantification of the blots was performed, and data were normalized to GAPDH expression and represented as fold induction above constitutive IL-6 expression. Results are the mean ± SD of three experiments. ****p* < 0.001; N.S., not significant.

IL-6/sIL-6R over treatment with either factor alone and remain elevated at 6 hr.

IL-6 is required for the synergistic induction seen in the presence of TNF-α and sIL-6R

We speculated that endogenous IL-6, induced by TNF-α, could complex with exogenous sIL-6R and affect IL-6 secretion. On treatment of U373-MG cells with TNF-α plus the sIL-6R, high-level expression of IL-6 protein was seen (Fig. 1). However, we have not shown the absolute necessity for IL-6 in this response. To address this issue, U373-MG cells were treated with TNF-α plus sIL-6R or TNF-α plus IL-6/sIL-6R for 6 hr, and IL-6 mRNA expression was analyzed by RPA. As shown previously, IL-6/sIL-6R or TNF-α stimulation induces a modest increase in IL-6 mRNA expression at 6 hr (Fig. 3A, lanes 4 and 5). Addition of IL-6 plus TNF-α does not affect IL-6 mRNA compared with TNF-α alone (compare lanes 5 and 6). Inclusion of IL-6/sIL-6R plus TNF-α induces a synergistic increase in IL-6 mRNA expression (lane 9), and the addition of neutralizing antibody to IL-6 abrogates this response (lane 10). As well, addition of sIL-6R plus TNF-α enhances IL-6 mRNA expression over that seen with TNF-α alone (compare lanes 5 and 7), and neutralizing antibody to IL-6 blocks this effect (lane 8). Quantification is shown in Figure 3B. These results provide evidence that endogenous or exogenous IL-6 is required to complex with the sIL-6R to initiate signaling.

To further characterize this response, the ability of Hyper-IL-6 (H-IL-6) to induce IL-6 mRNA expression alone and in combination with TNF-α was compared with IL-6/sIL-6R treatment in U373-MG cells. H-IL-6 is a recombinant protein composed of the functional domains of IL-6 and the IL-6R. It has been shown to have greater bioactivity than IL-6 and sIL-6R added separately (Fischer et al., 1997). As demonstrated by RPA, H-IL-6 (10 ng/ml) is a slightly better inducer of IL-6 mRNA than IL-6 plus sIL-6R (Fig. 3C). Co-treatment of U373-MG cells with TNF-α and H-IL-6 led to ~27-fold increase over unstimulated cells, whereas treatment with TNF-α and IL-6/sIL-6R gave an ~18-fold increase (Fig. 3C).

IL-1β, but not TGF-β, synergizes with sIL-6R to enhance IL-6 expression

Inducers of IL-6 in astrocytes include the proinflammatory cytokines TNF-α and IL-1β and the anti-inflammatory cytokine

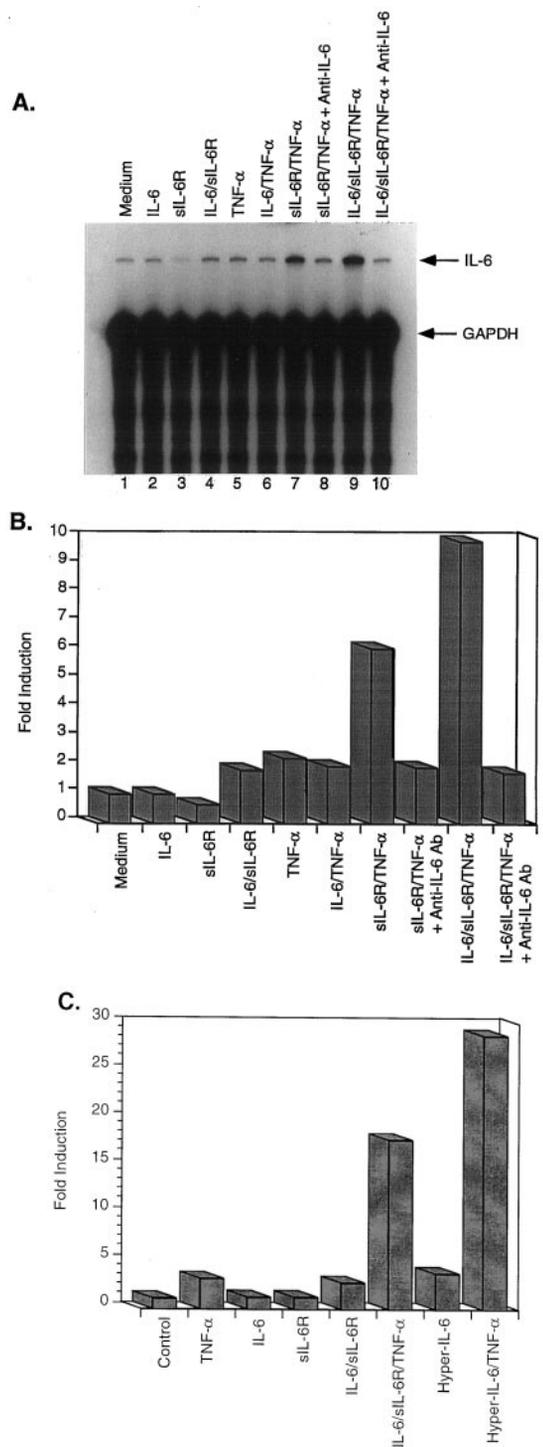


Figure 3. TNF-α, the sIL-6R, and endogenous or exogenous IL-6 induce IL-6 mRNA expression. U373-MG cells were treated with TNF-α (50 ng/ml), sIL-6R (100 ng/ml), IL-6 (5 ng/ml), or anti-IL-6 neutralizing antibody (1 μg/ml) alone and in various combinations for 6 hr. RNA was extracted and examined by RPA for IL-6 and GAPDH mRNA expression (A). PhosphorImager quantification of the blot is shown in B. Data have been normalized to GAPDH expression and are represented as fold induction above constitutive IL-6 expression (lane 1, Medium). These data are representative of two experiments. Cells were treated with TNF-α, IL-6, sIL-6R, or H-IL-6 (10 ng/ml) alone and in various combinations for 6 hr. RNA was analyzed for IL-6 mRNA as described above (C). Data have been normalized to GAPDH expression and are represented as fold induction above constitutive IL-6 expression. These data are representative of two experiments.

Table 2. IL-6 induction by TNF- α , IL-1 β , and TGF- β in the presence of the sIL-6R

Cell treatment ^a	IL-6 bioactivity (pg/ml)	
	Alone	+sIL-6R (100 ng/ml)
Medium	21.4 \pm 4.4 ^b	38.1 \pm 13.1
TNF- α (50 ng/ml)	210.6 \pm 61.4	1,885.3 \pm 116.6 ^{c,***}
TGF- β (10 ng/ml)	184.4 \pm 40.3	333.0 \pm 157.8 ^{N.S.}
IL-1 β (2 ng/ml)	23,275.6 \pm 5,334.4	1,000,667.0 \pm 25,807.9 ^{c,***}

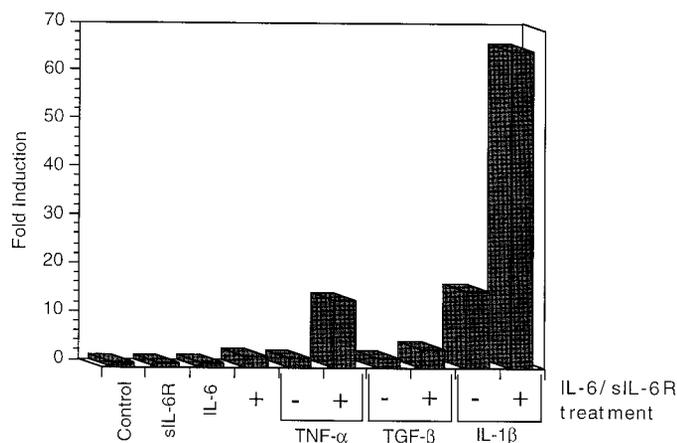
^aTwenty-four hour incubation period.^bMean \pm SD of three experiments analyzed in triplicate.^c*p* values for cytokine versus cytokine + sIL-6R treated samples: ****p* < 0.001. N.S., Not significant.

Figure 4. TNF- α and IL-1 β , but not TGF- β , synergize with IL-6/sIL-6R for IL-6 mRNA expression. U373-MG cells were treated with TNF- α (50 ng/ml), TGF- β (10 ng/ml), or IL-1 β (2 ng/ml) alone and in combination with IL-6 (5 ng/ml) and the sIL-6R (100 ng/ml) for 6 hr. RNA was extracted and examined by RPA for IL-6 and GAPDH mRNA expression. PhosphorImager quantification of the blot is shown. Data have been normalized to GAPDH expression and are represented as fold induction above constitutive IL-6 expression. These data are representative of two experiments.

TGF- β (Benveniste et al., 1990, 1994). Therefore, studies were extended to determine whether similar synergy with the sIL-6R would be seen with these other inducers of IL-6. A 24 hr incubation in the presence of IL-1 β (2 ng/ml) or TGF- β (10 ng/ml) alone or in combination with the sIL-6R revealed that IL-1 β is a potent inducer of IL-6 and, as well, that synergy does occur with this cytokine and the sIL-6R (Table 2). The levels of IL-1 β induction are specific to IL-1 β , because the addition of the IL-1 receptor antagonist (IL-1RA, 100 ng/ml) blocked IL-1 β induction (data not shown). TGF- β alone induced expression of IL-6 to levels similar to those seen for TNF- α ; however, significant increases were not seen on addition of the sIL-6R (Table 2). Analysis at the mRNA level correlated with the results obtained for IL-6 protein levels. Both TNF- α and IL-1 β synergized with IL-6/sIL-6R to increase IL-6 mRNA expression, whereas TGF- β did not (Fig. 4).

TNF- α - and IL-1 β -induced IL-6 message is not stabilized by the addition of IL-6/sIL-6R

To determine whether the synergy between TNF- α or IL-1 β and IL-6/sIL-6R on IL-6 mRNA expression was occurring at the transcriptional or post-transcriptional level, *t*_{1/2} experiments were performed to assess the stability of the IL-6 message. U373-MG

cells were incubated with TNF- α , IL-6/sIL-6R, or TNF- α plus IL-6/sIL-6R for 6 hr, then actinomycin D (ACT-D; 5 μ g/ml) was added for an additional 30–120 min. RNA was isolated at the indicated time points and analyzed for IL-6 and GAPDH mRNA levels by RPA. As shown in Figure 5A, the *t*_{1/2} of IL-6 mRNA induced by IL-6/sIL-6R was \sim 30 min, that for TNF- α -induced IL-6 was \sim 90 min, and that of TNF- α plus IL-6/sIL-6R-induced IL-6 mRNA was \sim 30 min. These results indicate that IL-6/sIL-6R does not promote stabilization of TNF- α -induced IL-6 message. Identical results were obtained when IL-6 message stability was assessed after a 1 hr stimulation with TNF- α , IL-6/sIL-6R, or TNF- α plus IL-6/sIL-6R (data not shown). Comparable experiments were performed with IL-1 β as the stimulus. We initially assessed IL-6 steady-state mRNA levels induced by IL-1 β in the absence or presence of IL-6/sIL-6R at 1 and 6 hr. As shown in Figure 5B, IL-1 β induced an approximately sevenfold induction of IL-6 mRNA at 1 hr, and inclusion of IL-6/sIL-6R increased induction to \sim 17-fold. After a 6 hr incubation, IL-1 β treatment induced a \sim 25-fold increase, and addition of IL-6/sIL-6R led to a further increase to \sim 69-fold (Fig. 5B). These findings with IL-1 β differ from those using TNF- α as the stimulus (Fig. 2); in those experiments, peak IL-6 mRNA expression was seen after a 1 hr treatment with TNF- α , with levels declining thereafter. We next assessed IL-6 message stability in the presence of IL-1 β or IL-6/sIL-6R, or both, after a 1 or 6 hr stimulation period. The *t*_{1/2} of IL-6 message induced by a 1 hr stimulation with IL-1 β was \sim 60 min, and that of IL-1 β plus IL-6/sIL-6R-induced mRNA was \sim 30 min (Fig. 5C). Thus, IL-6/sIL-6R does not enhance the stabilization of IL-1 β -induced IL-6 message. When the stimulation period with IL-1 β was extended to 6 hr, a different pattern of IL-6 mRNA stability emerged. The *t*_{1/2} of IL-1 β -induced IL-6 mRNA was $>$ 18 hr, because there was no degradation of IL-6 message during this time period (Fig. 5D). The inclusion of IL-6/sIL-6R did not affect the *t*_{1/2} of IL-1 β -induced IL-6 message (Fig. 5D). These results indicate that (1) IL-1 β stimulation for a prolonged time period (6 hr) promotes stabilization of the IL-6 message, and (2) IL-6/sIL-6R treatment likely does not influence IL-1 β -induced IL-6 gene expression at the post-transcriptional level.

Enhancement of TNF- α - and IL-1 β -induced IL-6 expression by IL-6/sIL-6R is transcriptional

Our results examining the increase in IL-6 mRNA expression after a 1 hr stimulation with TNF- α , TNF- α plus IL-6/sIL-6R, IL-1 β , or IL-1 β plus IL-6/sIL-6R indicate that the effect is not post-transcriptional (Fig. 5A,C), suggesting an influence at the transcriptional level. Nuclear run-on analysis was performed on U373-MG cells treated with the above-mentioned factors alone and in combination for 30–45 min. The nuclei were isolated, and the RNA transcripts that had been initiated were allowed to complete in the presence of [³²P]UTP. Labeled RNA transcripts were then hybridized to slot-blotted cDNA encoding human IL-6, human GAPDH, or DNA vector as a negative control. The levels of IL-6 transcription were normalized to that of GAPDH. As shown in Figure 6, IL-6/sIL-6R treatment induced IL-6 transcription by \sim 5.8-fold, TNF- α by \sim 6.7-fold, and IL-1 β by \sim 13.3-fold. Synergistic increases in IL-6 transcription were observed in the presence of TNF- α plus IL-6/sIL-6R (\sim 36.8-fold induction) or IL-1 β plus IL-6/sIL-6R (\sim 45.9-fold induction). These results support the hypothesis that enhancement of TNF- α - and IL-1 β -induced IL-6 gene expression by IL-6/sIL-6R is transcriptional.

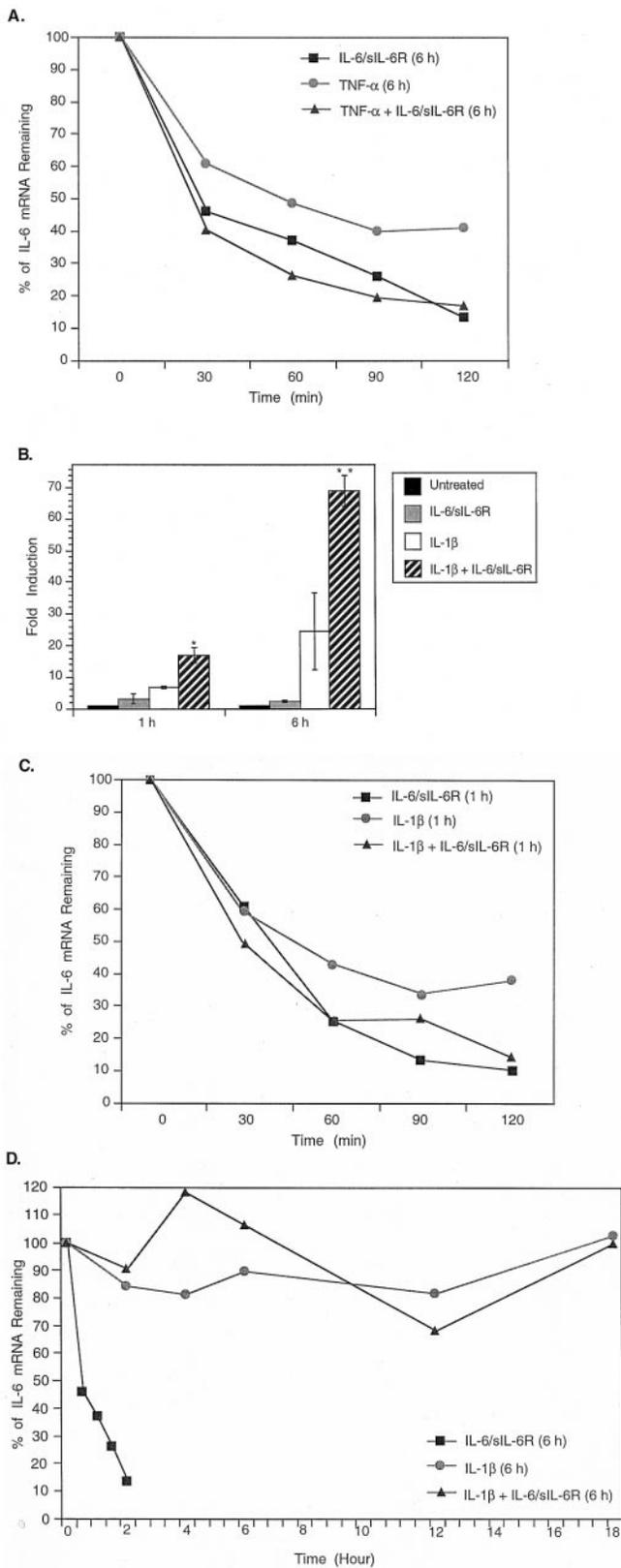


Figure 5. IL-6/sIL-6R does not affect TNF- α - or IL-1 β -induced IL-6 mRNA stability. U373-MG cells were incubated with TNF- α , IL-6/sIL-6R, and TNF- α plus IL-6/sIL-6R for 6 hr, then actinomycin D (ACT-D; 5 μ g/ml) was added, cells were harvested at the indicated times, and RNA was subjected to RPA for IL-6 and GAPDH mRNA. IL-6 mRNA at time 0 (before the addition of ACT-D) was plotted as 100%. The average of two experiments is presented (A). Cells were incubated with medium,

Regulation of IL-6 by other members of the IL-6 cytokine family

Members of the IL-6 family are known to elicit redundant responses (for review, see Taga and Kishimoto, 1997); therefore, we were interested in determining whether OSM, CNTF plus the soluble CNTF receptor, LIF, or IL-11 had the capacity to regulate IL-6 expression in U373-MG cells. Cells were treated for 24 hr with the above-mentioned cytokines alone or in combination with TNF- α , and supernatants were isolated and analyzed for IL-6 bioactivity as described previously. OSM and LIF alone were able to induce IL-6 bioactivity. In combination with TNF- α , OSM induced high levels of IL-6, similar to levels seen on treatment with TNF- α and the sIL-6R (Fig. 7). LIF did not function in a synergistic manner with TNF- α for enhanced IL-6 expression, whereas CNTF/sCNTFR and IL-11 had no significant effect either alone or with TNF- α (Fig. 7). Comparable results were obtained using human adult astrocytes and CRT astrogloma cells (data not shown).

DISCUSSION

IL-6 has been shown to have beneficial potential in the CNS because of its neurotrophic and neuroprotective effects (Satoh et al., 1988; Hama et al., 1989; Gadiant and Otten, 1997; Loddick et al., 1998; März et al., 1998b), and anti-inflammatory actions through the inhibition of VCAM-1, intercellular adhesion molecule-1, and TNF- α expression by CNS cells (Benveniste et al., 1995; Shrikant et al., 1995; Oh et al., 1998). However, other reports suggest that IL-6 is detrimental and adds to the pathophysiology associated with CNS disorders (Campbell et al., 1993; Klein et al., 1997). Collectively, these studies illustrate the pleiotropic nature of IL-6 in the CNS and stress the need for an understanding of IL-6 regulation in the CNS. Previously, we have shown that human astrocytes express low levels of the IL-6R and require the addition of the sIL-6R for IL-6-mediated responses (Oh et al., 1998). Herein, we have shown that treatment of astrocytes with IL-6 and the sIL-6R leads to modest increases in IL-6 mRNA expression. However, co-treatment with either TNF- α or IL-1 β plus IL-6/sIL-6R leads to synergistic increases in IL-6 gene expression.

We speculated that TNF- α stimulation of astrocytes would provide a source of endogenous IL-6 that would then interact with the sIL-6R and possibly influence IL-6 expression. The addition of TNF- α and the sIL-6R led to synergistic increases in IL-6 expression by U373-MG cells, primary human astrocytes, and CRT cells (Fig. 1, Table 1). These results suggest that the sIL-6R plays a pivotal role in determining the levels of IL-6 expressed by astrocytes in the CNS and furthermore influences IL-6 function in the CNS. This is supported by several reports that CNS cells that are normally slightly responsive or unresponsive to IL-6 become responsive on addition of the sIL-6R. In the absence of nerve growth factor, IL-6 weakly enhances sympa-

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IL-1 β (2 ng/ml) or IL-6/sIL-6R, or both, for 1 or 6 hr. PhosphorImager quantification of the blots was performed, and data were normalized to GAPDH expression and represented as fold induction above constitutive IL-6 expression. Results are the mean \pm SD of two experiments. * p < 0.05; ** p < 0.01 (B). U373-MG cells were incubated with IL-1 β , IL-6/sIL-6R, and IL-1 β plus IL-6/sIL-6R for 1 hr (C) or 6 hr (D), then ACT-D was added, cells were harvested at the indicated times, and RNA was analyzed for IL-6 and GAPDH mRNA. IL-6 mRNA at time 0 (before the addition of ACT-D) was plotted as 100%. The average of two experiments is presented.

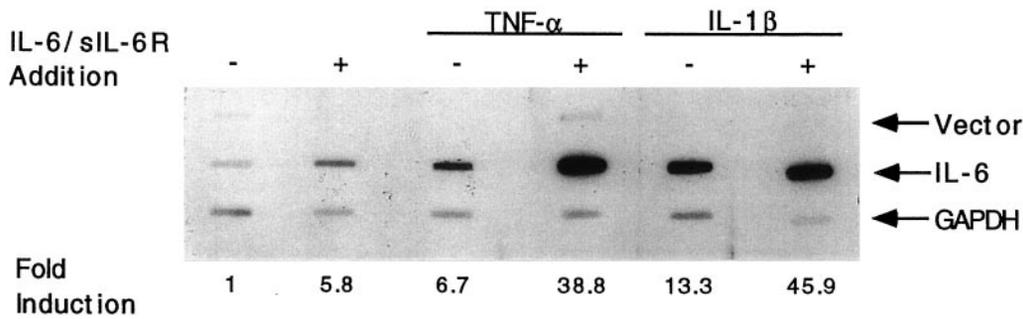


Figure 6. IL-6 transcription in the presence of TNF- α , IL-1 β , and IL-6/sIL-6R. U373-MG cells were incubated in the presence of medium, TNF- α , IL-1 β , and IL-6/sIL-6R alone and in combination for 30–45 min. Labeled transcripts were prepared as described in Materials and Methods and hybridized to filters containing 5 μ g of nonspecific DNA, human IL-6 cDNA, or human GAPDH cDNA. The blots were exposed to the PhosphorImager, and IL-6 values were normalized to the value of GAPDH for each sample. These data are representative of two experiments.

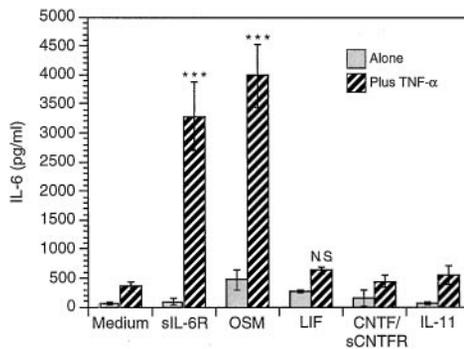


Figure 7. Stimulation of IL-6 bioactivity in U373-MG cells by IL-6 family members. U373-MG cells were incubated with sIL-6R (100 ng/ml), OSM (10 ng/ml), LIF (50 ng/ml), CNTF (100 ng/ml) plus sCNTFR (100 ng/ml), and IL-11 (50 ng/ml) alone and in combination with TNF- α (50 ng/ml) for 24 hr, and supernatants were harvested and then analyzed for IL-6 activity as described in Materials and Methods. Results are the mean \pm SD of three experiments analyzed in triplicate. *** p < 0.001; N.S., not significant.

thetic neuron survival, and the addition of the sIL-6R enhances neuronal survival (März et al., 1998a). In human astrocytes, IL-6 alone has no effect on α_1 -antichymotrypsin expression, whereas addition of the sIL-6R leads to expression (Kordula et al., 1998). In the presence of the sIL-6R, IL-6 inhibits TNF- α -induced VCAM-1 expression in astrocytes (Oh et al., 1998). These findings suggest that the sIL-6R is a functionally relevant CNS molecule. In this regard, sIL-6R is detectable in the CSF of normal individuals (0.8–1.67 ng/ml) (Frieling et al., 1994; Rose-John and Heinrich, 1994), although the source of sIL-6R in the CNS is unknown. A likely source of CNS sIL-6R is Purkinje neurons, which exhibit intense immunostaining for the IL-6R (Nelson et al., 1999). As well, IL-6R immunoreactivity has been detected in hypoglossal nerve cell bodies in the brainstem and in dorsal root ganglion neurons (Hirota et al., 1996; Thier et al., 1999). Other potential CNS sources of sIL-6R, particularly under inflammatory conditions, are activated macrophages and T-cells (Müllberg et al., 1993; Banning et al., 1998; Jones et al., 1998). Preliminary results from our laboratory indicate that sIL-6R, in the range of 300–900 pg/ml, can be detected from human brain extracts (our unpublished observation). Circulating levels of sIL-6R are considerably higher (\sim 70 ng/ml) and may increase CNS levels on disruption of the BBB, thereby providing an endogenous CNS source of sIL-6R sufficient to elicit functional responses.

Addition of TNF- α plus IL-6/sIL-6R led to an increase in the overall level of IL-6 mRNA as well as the duration of expression (Fig. 2). To determine the basis of this increase, mRNA stability assays were performed. As shown in Figure 5A, the $t_{1/2}$ of TNF- α -induced IL-6 message was \sim 90 min, and the inclusion of IL-6/sIL-6R did not affect the $t_{1/2}$ of the IL-6 message. Therefore, the synergistic effect of TNF- α and IL-6/sIL-6R on IL-6 gene expression is likely caused by transcriptional regulation. Nuclear run-on analysis indicated that both TNF- α and IL-6/sIL-6R induce IL-6 transcription, which is enhanced in the presence of both stimuli (Fig. 6).

IL-1 β alone is a more potent inducer of IL-6 mRNA and protein than TNF- α (Fig. 4, Table 2). Our results provide the explanation for this: enhanced IL-1 β induced IL-6 transcription and stabilization of the IL-6 message. Nuclear run-on analysis revealed that IL-1 β induced IL-6 gene transcription to a greater extent than TNF- α (6.7-fold for TNF- α vs 13.3-fold for IL-1 β) (Fig. 6). As well, IL-1 β induces a more stable IL-6 message than TNF- α (Fig. 5A,D). Interestingly, IL-1 β stimulation of cells for 1 hr induced an IL-6 message with a $t_{1/2}$ of \sim 60 min, whereas a 6 hr stimulation with IL-1 β resulted in an IL-6 message that did not undergo any appreciable decay for up to 18 hr (Fig. 5C,D). Thus, it appears that there is a time dependence for IL-1 β -mediated stabilization of the IL-6 message. In this regard, there are several reports of IL-1 β stabilization of IL-6 message. In murine mast cells, IL-1 β alone has no effect on IL-6 expression, but it enhances expression induced by *c-kit* ligand and IL-10 (Lu-Kuo et al., 1996). In fibroblasts, TNF- α and IL-1 β synergistically stimulate IL-6 expression that is caused in part by stabilization of the IL-6 message (Elias and Lentz, 1990). Interestingly, in those two studies, IL-1 β alone did not stabilize the IL-6 message, which is what we have observed in the U373-MG cells. Thus, the ability of IL-1 β to stabilize the IL-6 message may occur in cell type-specific manner.

IL-1 β induction of IL-6 protein expression was significantly enhanced in the presence of sIL-6R (Table 2), and elevated levels of IL-6 mRNA were detected in cells treated with IL-1 β plus IL-6/sIL-6R over treatment with either stimulus alone (Fig. 5B). The synergistic effect of IL-1 β plus IL-6/sIL-6R appears to be caused by increased transcription of the IL-6 gene in the presence of both stimuli (Fig. 6) because IL-6/sIL-6R does not affect the $t_{1/2}$ of IL-6 message induced by IL-1 β at either 1 or 6 hr (Fig. 5C,D). IL-6/sIL-6R has been shown to induce its own synthesis in osteoblasts by transcriptional mechanisms (Franchimont et al.,

1997), and our study also documents an effect of IL-6/sIL-6R on IL-6 transcription.

Co-treatment with TGF- β plus the sIL-6R did not lead to synergistic increases in IL-6 mRNA and protein expression (Fig. 4, Table 2). The difference in the ability of TNF- α and IL-1 β , versus TGF- β , to synergize with IL-6/sIL-6R may be dependent on transcriptional regulation. TNF- α and IL-1 β regulate IL-6 transcription by similar mechanisms that involve use of NF- κ B and CCAAT enhancer binding protein (C/EBP) binding sites (Akira et al., 1990; Sparacio et al., 1992; Matsusaka et al., 1993; Miyazawa et al., 1998). Treatment of U373-MG cells with TNF- α and IL-1 β , but not TGF- β , leads to NF- κ B interactions with the NF- κ B response element in the IL-6 promoter, as determined using electrophoretic mobility shift assays (data not shown). We hypothesize that synergy between TNF- α or IL-1 β and IL-6/sIL-6R requires NF- κ B activation as well as the activation of an undetermined IL-6 inducible transcription factor, possibly C/EBP β . C/EBP β has been shown to function in the positive regulation of the IL-6 promoter as well as other IL-6 inducible promoters (Miyazawa et al., 1998). Maximal IL-6 transcription as seen by TNF- α or IL-1 β plus IL-6/sIL-6R may involve the interactions of NF- κ B and C/EBP β , as has been shown for IL-8 and serum amyloid A gene expression (Stein and Baldwin, 1993; Kunsch et al., 1994; Ray et al., 1995).

Most members of the IL-6 cytokine family act through ligand-specific receptors that interact with gp130 on binding ligand. OSM differs from this model in that OSM interacts first with gp130 (Gearing et al., 1992; Sporeno et al., 1994), then recruits either the LIFR β (Thoma et al., 1994), a receptor used by various members of this family, or the OSMR, a receptor specific to OSM (Mosley et al., 1996). Other members of the IL-6 cytokine family were tested for effects on IL-6 expression; OSM induced IL-6 protein expression and strongly synergized with TNF- α for enhanced IL-6 expression. LIF alone induced a modest level of IL-6 protein but did not synergize with TNF- α (Fig. 7). On the basis of the ability of OSM to significantly influence IL-6 expression, we propose that OSM along with IL-6 may affect the function of astrocytes. To our knowledge, no studies have been conducted to determine OSM expression in the brain. However, preliminary data indicate that human astrocytes, on stimulation, can produce OSM protein (our unpublished observation). This suggests a potential CNS source of this cytokine.

In summary, data presented herein support a positive autoregulatory role of IL-6 and the sIL-6R in astrocytes for IL-6 regulation. These findings may be relevant to inflammatory conditions in the brain, where cytokines such as TNF- α and IL-1 play an essential role in this process through the induction of chemokines and adhesion molecules, recruitment of immune cells into the CNS parenchyma, and ultimately, activation of immune cells and endogenous glial cells (for review, see Benveniste, 1997). In this context of elevated CNS expression of TNF- α , IL-1 β , and IL-6, and the presence of the sIL-6R, a cytokine circuitry is established by which high levels of expression of IL-6 can occur, which may lead to some of the neuropathology associated with CNS inflammation. The ultimate biological effect of IL-6 in the CNS will depend on the availability of IL-6 receptors, both membrane bound and soluble forms, as well as the levels of soluble gp130, which can neutralize IL-6/sIL-6R complexes, thereby acting as an antagonist (for review, see Heinrich et al., 1998). Whether soluble gp130 exists in the CNS is not currently known; however, the expression of this component of the IL-6 receptor signaling complex will impact on IL-6 biological effects in the CNS.

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