Oncostatin M and the Interleukin-6 and Soluble Interleukin-6 Receptor Complex Regulate \( \alpha_1 \)-Antichymotrypsin Expression in Human Cortical Astrocytes*

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\( \alpha_1 \)-Antichymotrypsin (ACT) is an acute phase protein expressed in the brain which specifically colocalizes with amyloid-\( \beta \) during Alzheimer's disease. We analyzed ACT synthesis in cultured human cortical astrocytes in response to various cytokines and growth factors. Oncostatin M (OSM) and interleukin (IL)-1\( \beta \) were potent stimulators of ACT mRNA expression, whereas tumor necrosis factor-\( \alpha \) had modest activity, and IL-6 and leukemia inhibitory factor (LIF) were ineffective. The finding that OSM, but not LIF or IL-6, stimulated ACT expression suggests that human astrocytes express a "specific" OSM receptor, but not IL-6 or LIF receptors. However, cotreatment of human astrocytes with soluble IL-6 receptor (sIL-6R)-IL-6 complex did result in potent stimulation of ACT expression. When the human ACT gene was cloned, two elements binding STAT1 and STAT3 (signal transducer and activator of transcription) in response to OSM or IL-6-sIL-6R complexes could be identified and characterized. Taken together, these findings indicate that OSM or IL-6-sIL-6R complexes may regulate ACT expression in human astrocytes and thus directly or indirectly contribute to the pathogenesis of Alzheimer's disease.

\( \alpha_1 \)-Antichymotrypsin (ACT)\(^{1} \) is one of the major positive human acute phase proteins produced by the liver and secreted into blood plasma (1, 2). The expression of this proteinase inhibitor in hepatic cells is enhanced by interleukin (IL)-6 and glucocorticoids and to a lesser extent by IL-1 (3, 4). Although ACT is also found in the brain, the plasma-derived inhibitor is separated from this origin by a tight blood-brain barrier consisting of endothelial cells. For this reason it is believed that astrocytes are the likely source of ACT produced within the central nervous system (5). Significantly, ACT has been identified as one of the amyloid-associated proteins found in the brains of patients with Alzheimer's disease (6, 7). The pathological feature of this disease is cerebral degeneration with neuronal cell death and deposition of abnormal proteins in the form of amyloid plaques and neurofibrillary tangles. Because the expression of ACT is enhanced dramatically in affected brain regions in Alzheimer's disease, a state of cerebral "acute phase" in response to neuronal degradation has been proposed. IL-1 and IL-6, which are produced by cells of CNS, were suggested to induce ACT expression in astrocytes (8). Indeed, the induction of ACT expression by IL-1 has been shown in human astrocyte cultures (9); however, regulation by IL-6 has not been confirmed.

To understand the control of ACT expression in the brain we used human astrocyte cultures and analyzed the pattern of its synthesis after stimulation with a variety of factors including IL-1 and cytokines of the IL-6 family. We have also cloned the 5'-flanking region of the ACT gene and performed analysis of its transcriptional activity. Our results suggest that at least one cytokine, oncostatin M (OSM), may play an important role in up-regulating ACT expression in astrocytes, whereas IL-6 requires the presence of soluble IL-6 receptor (sIL-6R).

MATERIALS AND METHODS

Cell Culture—Human cortical astrocyte cultures were established using dissociated human cerebral tissue at 16–20 weeks gestation. The protocol for obtaining postmortem fetal neural tissue complied with federal guidelines for fetal research and with the Uniformed Anatomical Gift Act. Cortical tissue was washed three times in Ca\(^+\)/Mg\(^2+\)-free Hanks' balanced salt solution and then dissociated by repeated pipetting. DNase (Sigma) was added to a final concentration of 0.05 mg/ml, and the solution was passed through a 100-\( \mu \)m nylon cell strainer (Falcon). The cells obtained were then centrifuged for 5 min at 200 \( g \), resuspended in a trypsin/EDTA solution (0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution, Life Technologies, Inc.), and incubated for 20 min at 37 °C. Modified Eagle's medium containing 1% glucose, 1 mM sodium pyruvate, 1 mM glutamine, and 10% fetal bovine serum (MEM/FBS) and DNase (final concentration of 0.05 mg/ml) were added, and the cells were again pelleted by centrifugation and resuspended in MEM/FBS. 1.6 \( \times \) 10\(^6\) cells were seeded in a T-150 tissue culture flask coated with polyethyleneimine. Cultures were maintained in an H\(_2\)O-saturated incubator with an atmosphere of 95% air and 5% CO\(_2\) at 37 °C. The culture medium was changed 1 and 4 days after plating, and the cultures were then left undisturbed for at least 1 week.

Astrocyte cultures were prepared by multiple passageing of the established mixed brain cell cultures. The cells from one T-150 flask were replated into three to five T-150 flasks. Just before confluence the cells were repassaged by trypsinization. This process was repeated until the cultures were \( >89\% \) pure astrocytes as judged by immunocytochemistry analysis for glial fibrillary acidic protein (three to four passages). Human astrocytoma CCF-STTG1 cells were obtained from ATCC (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium
supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and nonessential amino acids.

**Cytokines and Cell Stimulation**—Cells were stimulated with 50 ng/ml recombinant human IL-6 (1.9 × 10^7 units/mg), 25 ng/ml OSM (4.7 × 10^7 units/mg), 5 ng/ml recombinant human IL-1 (1.2 × 10^7 units/mg) (all a gift from Immunex Corp., Seattle), 200/ml recombinant human interferon-γ (2.0 × 10^7 units/mg) (Boehringer), 25 ng/ml recombinant human epidermal growth factor (Upstate Biotechnology Inc., Lake Placid, NY), 50 ng/ml IL-6R (R&D Systems Inc., Minneapolis, MN), 10 ng/ml TNF-α, 10 units/ml recombinant human LIF (a gift of Dr. Heinz Baumann, Roswell Park Cancer Institute, Buffalo, NY), 100 ng/ml phorbol 12-myristate 13-acetate, and 1 μM dexamethasone (both from Sigma). Actinomycin D (Sigma) was used at 5 μg/ml. OSM and IL-6R in cerebrospinal fluid were measured using enzyme-linked immunosorbent assay as recommended by the supplier (R&D Systems Inc., Minneapolis, MN).

**RNA Preparation and Northern Blot Analysis**—Total RNA was prepared using the phenol extraction method (10, 11). 5′-protruding ends with Klenow enzyme using pUC19, University of Pennsylvania) labeled by random priming (13). After the hybridization, non-specifically bound radioactivity was removed by washing in 2 × SSC at room temperature followed by two washes in 2 × SSC and 1% SDS at 68 °C for 20 min.

**Synthetic Oligonucleotides**—The following oligonucleotides were used to obtain a −352 to +25 fragment of ACT promoter: 5′-ATGTCCTAGAAGATAATTACCATCCAAATG-3′ and 5′-GGTGAAGGTCCGAGAAAGCCGTCTGTG-3′; primer 5′-ATCATTAGAATCTATGTCCTGTGAG-3′; primer 5′-ATGTCCTAGAAGATAATTACCATCCAAATG-3′ and 5′-GGTGAAGGTCCGAGAAAGCCGTCTGTG-3′; and 5′-GGTGTGGACCCGAGGATTATCATGTC-3′. Mutants containing point mutations in the ACT-A and/or ACT-B elements were generated by polymerase chain reaction using the Pwo polymerase (Boehringer Mannheim) and the following primers: 5′-GGTGAAGGTCCGAGAAAGCCGTCTGTG-3′ and 5′-GGTGTGGACCCGAGGATTATCATGTC-3′. Mutants containing point mutations in the ACT-A and/or ACT-B elements were generated by polymerase chain reaction using the Pwo polymerase (Boehringer Mannheim) and the following primers: 5′-GGTGAAGGTCCGAGAAAGCCGTCTGTG-3′ and 5′-GGTGTGGACCCGAGGATTATCATGTC-3′.

**RESULTS**

**OSM, IL-1, and TNF-α Regulate the Expression of ACT in Human Astrocytes**—ACT is produced by human astrocytes with IL-1 having already been shown to induce its mRNA expression (9). We analyzed the pattern of ACT production in human astrocytes in response to other factors regulating gene expression during inflammation and the acute phase response. Human astrocytes were stimulated with IL-1, TNF-α, IL-6, OSM, LIF, phorbol 12-myristate 13-acetate, interferon-γ, and epidermal growth factor and analyzed by Northern blotting. An example of the results obtained is shown in Fig. 1A. In addition to IL-1, as reported previously, TNF-α and OSM were potent stimulators of ACT mRNA synthesis, and this was enhanced greatly by the synthetic glucocorticoid dexamethasone. Dexamethasone alone had only a small effect. In contrast, IL-6, LIF, epidermal growth factor, interferon-γ, and phorbol 12-myristate 13-acetate were ineffective (Fig. 1A and data not shown).

**TABLE I**

| Oligonucleotides used in gel retardation assays | Name | Sequence | Ref. |
|-----------------------------------------------|------|----------|------|
| ACT-A                                         | ffl12size:8g5′-GATCCTGATTGACACCGAATAAT-3′ | This paper |
| ACT-B                                         | 3′-AAGTTGGTGGATCAAGAGGATGTA-5′          | This paper |
| ACT- (A + B)                                   | 5′-GATCTCGGAGGAAGACCGAAGAAGA-3′         | This paper |
| SIE                                           | 3′-AAGTTGGTGGATCAAGAGGATGTA-5′          | 31 |
| SPI-3 STAT                                    | 5′-GGATGTTCCCCAGAGA-3′                  | 14 |

To analyze the regulation of ACT transcription we cloned the ACT gene from a human genomic library. The 5′-flanking region of the ACT gene was fused to the bacterial reporter gene coding for CAT, and the construct obtained was transiently transfected into astrocytes. CAT gene...
expression was induced in response to OSM (Fig. 2B). Significantly, the same construct did not respond to IL-1 and TNF-α, suggesting that the elements conferring the response to these cytokines were not present in the 3.6-kilobase-long 5′-fragment of the ACT gene. The nature of these elements is not known, although activation of nuclear factor-κB has been correlated with the IL-1- and TNF-α-induced transcription of the ACT gene in human astrocytoma cells (25).

sIL-6R Restores Responsiveness of Astrocytes to IL-6—The family of IL-6 type cytokines is characterized by the use of a common receptor subunit, the signal transducing protein gp130 (26). Signaling through gp130 results in the phosphorylation of tyrosine residues of latent cytoplasmic proteins called STATs, particularly STAT1 and STAT3, followed by their dimerization, translocation to the nucleus, binding to specific elements within the promoters of target genes, and activation of transcription (26–28). Because the synthesis of ACT mRNA in astrocytes was induced by OSM but not by IL-6 (Figs. 1A and 2B), and astrocytes were reported to produce IL-6 (50 ng/ml), OSM (25 ng/ml), IL-1 (5 ng/ml), TNF-α (10 ng/ml), and/or dexamethasone (DEX) (1 μM). RNA was isolated after 18 h and subjected to Northern blot analysis using ACT cDNA as a probe. Panel B, astrocytes were stimulated with 25 ng/ml OSM and 1 μM dexamethasone or 5 ng/ml IL-1 and 1 μM dexamethasone for 12 h, and then actinomycin D (5 μg/ml) was added. After the times indicated RNA was isolated and subjected to Northern blot analysis. Lower panels show 28 S ribosomal RNA stained with ethidium bromide on a membrane. Therefore, a weak action of IL-6 and LIF suggested a small number of the corresponding surface receptors (Fig. 3B, right panel). Furthermore, when astrocytes were transfected with the ACT-CAT construct the sIL-6R restored their responsiveness to IL-6 (Fig. 3C).

Identification of STAT-binding Elements within the ACT Promoter—Because both OSM and IL-6-sIL-6R greatly enhance ACT expression in astrocytes as well as activate transcription of the −3.6-kilobase ACT-CAT construct, a series of 5′-deletion mutants was made to identify the elements conferring this responsiveness. Constructs were transiently transfected into astrocytes, and their responsiveness to either OSM, dexamethasone, or OSM together with dexamethasone was assayed. The construct containing the −156 to +25 fragment of the ACT promoter and all of the longer constructs were fully responsive to OSM (or OSM with dexamethasone), whereas dexamethasone alone had no effect (Fig. 4). However, the truncation to −103 resulted in a drastic loss of responsiveness to OSM by 80%. The responsiveness to OSM was abolished completely by further truncation to −68. The enhancing effect of dexamethasone (approximately 2-fold) was observed with all constructs, suggesting an activation of the transcriptional machinery rather than induction via a specific glucocorticoid receptor-responsive element. Because we could not detect any glucocorticoid receptor-responsive element within the ACT promoter constructs, and the glucocorticoid receptor was recently shown to directly interact with the STAT5 protein (32), it is possible that the enhancing effect of dexamethasone is mediated by an interaction of the glucocorticoid receptor with STATs.

Two possible binding sites for STAT proteins were localized by gel mobility shift assay (Fig. 5A). The sequence from −156 to +25 was shown. STAT binding sites are boxed. Panel B, human astrocytes were transfected with 5 μg of plasmid pACT-3573CAT and 3 μg of β-galactosidase expression vector pCH110 as an internal control for transfection efficiency. One day after transfection cells were stimulated with 5 ng/ml IL-1, 25 ng/ml OSM, 10 ng/ml TNF-α, and 50 ng/ml IL-6 all together with 1 μM dexamethasone. Cells were cultured for another 24 h and harvested. CAT activities were normalized to β-galactosidase activities and expressed as fold induction to control cultures defined as 1.0.
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A and B had the same drastic effect on promoter activity, reducing the responsiveness by 80%, whereas the promoter with both mutated sites was no longer induced by OSM (Fig. 6). These results indicate that both sites contribute equally to the activation of ACT transcription by OSM.

Finally, multiple copies of the identified STAT-binding elements were linked to a thymidine kinase (tk) promoter driving the transcription of the CAT gene, and transient transfections of human astrocytes were performed. The fragments containing site A or both sites (A and B) conferred responsiveness onto the thymidine kinase promoter, whereas site B alone was ineffective (Fig. 7).

Taken together these results show that two functional STAT binding elements mediate OSM-induced expression of the ACT gene in human astrocytes.

DISCUSSION

The role of inflammatory cytokines, including TNF-α, IL-1, and IL-6, within the CNS has been extensively studied over the last several years. Specifically, IL-1 and IL-6 have attracted much attention and have been suggested to mediate a cerebral acute phase response and induce the expression of cerebral acute phase proteins in astrocytes, including ACT (8). In addition, IL-6, which is the major regulator of liver acute phase proteins, has also been suggested to participate in the pathogenesis of Alzheimer’s disease (33, 34). Our results, however, clearly show that IL-6 failed to activate STAT factors or to induce ACT expression in cultured human astrocytes (Fig. 3).

The lack of response of these cells to IL-6 results from the absence of the α subunit of the IL-6R (gp80), because sIL-6R restored both activation of STAT proteins and induction of ACT expression (Fig. 3). Significantly, LIF, another IL-6 type cytokine, neither activated STAT proteins nor induced ACT expression in human astrocytes. We did not address specifically the question as to which of the components involved in LIF signaling was missing in astrocytes. However, considering that astrocytes produce both IL-6 and LIF (35, 36), we speculate that LIF-R is a likely candidate. Contrary to our present observations with human astrocytes, IL-6 has been reported to up-regulate nerve growth factor expression in mouse astrocytes (37).

We have analyzed the response of rat astrocytes to IL-6 in terms of STAT activation and regulation of gene expression. IL-6 activated STAT3 poorly in these cells and weakly induced the expression of the serine proteinase inhibitor-3 gene, a rat homolog of the ACT gene. As in human astrocytes, IL-6/sIL-6R complexes improved significantly both activation of STAT factors and induction of serine proteinase inhibitor-3 gene transcription in these cells. These results suggest that both sets of astrocytes express only small amounts of IL-6Rs or lack the presence of IL-6Rs on their cell surfaces. Also, human astrocytoma cells CCF-STTG1 and U373 barely respond to IL-6 (Fig. 2 and data not shown). Thus, IL-6 alone cannot trigger the activation of specific proteins, including ACT, in human astrocytes.

In contrast to IL-6 and LIF, OSM emerges as a potent stimulator of ACT expression in astrocytes (Figs. 1 and 3). These three cytokines possess overlapping activities because of their signaling through receptors containing the shared signal transducer gp130 (26). Binding of IL-6 to the IL-6R results in the homodimerization of gp130, whereas LIF triggers heterodimerization of gp130 with LIF-R (26). OSM can signal via two receptors: LIF-R-gp130 (also a receptor for LIF) and OSM-Rgp130 (specific OSM receptor) (26, 38–40). Because LIF failed to activate STAT proteins and induce the expression of ACT, we conclude that signaling via the LIF-R-gp130 system is

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2 T. Kordula, M. Bugno, D. Bagarozzi, Jr., and J. Travis, unpublished observations.
The response to OSM on the other hand is triggered through a specific OSM receptor composed of OSM-R and gp130. In contrast to human astrocytes, human astrocytoma cells CCF-STGG1, known to contain mRNA for gp130, LIF-R, and OSM-R (40), can respond to LIF (Fig. 2). Thus, these cells contain functional LIF receptors in addition to functional specific OSM receptors. It should be noted that the other well known example of signaling utilizing the specific OSM receptor but not the LIF-R-gp130 complex is the control of growth of the acquired immunodeficiency syndrome-associated Kaposi’s sarcoma cells by OSM (38). Thus, astrocytes, in parallel with Kaposi’s sarcoma cells, express a
specific OSM receptor that is being utilized to activate STAT proteins and induce expression of specific genes, including ACT. Recently, a specific OSM receptor has been shown to activate STAT5B efficiently (41, 42); however, ACT expression is not up-regulated by STAT5B overexpression.\(^3\)

The activation of ACT expression by IL-6-sIL-6R and OSM raises the question as to whether these factors can be found in the CNS. The expression of IL-6 by astrocytes, microglia, and also by astrocytoma cells is well documented (36, 43). Furthermore, IL-6 can be detected readily in cerebrospinal fluid of patients during viral and bacterial meningitis (37, 44), HIV (human immunodeficiency virus)-induced encephalopathy (45), Parkinson’s disease (46), multiple sclerosis (47), after trauma (48), and in Alzheimer’s disease (33, 34, 49). The sIL-6R that is necessary for IL-6 action on human astrocytes was detected in the cerebrospinal fluid of normal patients (1.6 ng/ml) (50), and its level was elevated to 6.6 ng/ml in the cerebrospinal fluid of a patient with Crow-Fukase syndrome (51). We measured the levels of sIL-6R in the cerebrospinal fluid of both normal individuals and patients with Alzheimer’s disease using a sensitive enzyme-linked immunosorbent assay. The amount of sIL-6R was not changed significantly in patients with Alzheimer’s disease (0.8 ng/ml for both groups, \(n = 8\)). However, it is worth mentioning that the concentration of sIL-6R in the cerebrospinal fluid is approximately 1,000-fold higher than the concentration of IL-6. These results suggest that the level of sIL-6R in cerebrospinal fluid probably does not change during Alzheimer’s disease; however, the amount of sIL-6R might still be elevated in certain regions of the brain (i.e., plaques). Clearly, the source of sIL-6R within the CNS needs to be defined. The amount of this receptor protein in plasma is much higher than in cerebrospinal fluid (77 ng/ml) (50) because blood-brain barriers prevent plasma proteins from entering the CNS. However, a blood-brain barrier breakdown has been shown to occur in response to overexpression of IL-6 in astrocytes (52, 53). Thus, the expression of IL-6 by reactive astrocytes might potentially result in diffusion of plasma proteins into the CNS (including sIL-6R and ACT). In the light of these findings the up-regulation of OSM could conceivably contribute indirectly to a cerebral acute phase response by enhancing the levels of sIL-6R in the CNS and the formation of functional IL-6-sIL-6R complexes.

We could not detect OSM in the cerebrospinal fluid of either controls or patients with Alzheimer’s disease using a sensitive enzyme-linked immunosorbent assay (detection limit 2 pg/ml).

However, plasma levels of OSM are also very low (below 20 pg/ml) (54). This cytokine is produced by activated T-cells, and these cells readily migrate into the CNS (for review, see Ref. 55). In addition, human microglia can produce OSM and are also a likely source of this cytokine within the CNS.\(^4\)

The activation of ACT transcription by OSM (or IL-6-sIL-6R) could be correlated with the binding of STAT3 and STAT1 complexes to two elements within the ACT promoter (Fig. 5). These elements bind STAT complexes with different affinities, although both contribute equally to the transcriptional activity of the ACT promoter (Fig. 6). In vitro, the distal site is bound with high affinity by STAT3 and STAT1, whereas the proximal site requires the presence of the distal element. Moreover, the distal site is a true enhancer capable of conferring responsiveness onto a heterologous promoter. The element containing both distal and proximal sites is qualitatively as good as the enhancer element containing only the high affinity distal site (Fig. 7). This observation indicates that the binding of STATs to promoter elements in vitro does not always correlate with the activation of transcription via STAT elements in vivo.

Taken together these studies indicate that the increase of IL-6 alone cannot account for the enhanced ACT expression and that astrocytic responses to IL-6 require the formation of functional IL-6-sIL-6R complexes. Indeed, a role for OSM must now be considered because it is a potent stimulator of ACT expression in cultured human astrocytes.

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\(^3\) H. Baumann, unpublished observations.

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