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Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6

(neurodegeneration/astrocytosis/angiogenesis/acute-phase response)

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ABSTRACT **Cytokines are thought to be important mediators in physiologic and pathophysiologic processes affecting the central nervous system (CNS). To explore this hypothesis, transgenic mice were generated in which the cytokine interleukin 6 (IL-6), under the regulatory control of the glial fibrillary acidic protein gene promoter, was overexpressed in the CNS. A number of transgenic founder mice and their offspring exhibited a neurologic syndrome the severity of which correlated with the levels of cerebral IL-6 expression. Transgenic mice with high levels of IL-6 expression developed severe neurologic disease characterized by runting, tremor, ataxia, and seizure. Neuropathologic manifestations included neurodegeneration, astrocytosis, angiogenesis, and induction of acute-phase-protein production. These findings indicate that cytokines such as IL-6 can have a direct pathogenic role in inflammatory, infectious, and neurodegenerative CNS diseases.**

Cytokines are important soluble mediators of cellular communication in both physiologic and pathophysiologic states. There is accumulating but largely circumstantial evidence that cytokines may play a key role as direct effectors of both the clinical and pathologic manifestations seen in many central nervous system (CNS) diseases (1–3). However, the role of cytokines in the pathogenesis of CNS disease remains unclear. In the present study we used a transgenic approach to overexpress the cytokine interleukin 6 (IL-6) in murine astrocytes *in vivo*. IL-6 is a prototypic cytokine with a spectrum of biologic actions (4), many of which overlap with those of other cytokines, including IL-1α/β and tumor necrosis factor α. Expression of IL-6 in the brain has been documented in a wide range of CNS disorders, including AIDS dementia complex (5), viral (6) and bacterial meningitis (7), multiple sclerosis (8), Alzheimer disease (9), and trauma (10). Localized production of IL-6 in the CNS may be mediated not only by infiltrating immuno-inflammatory cells, but also by astrocytes (11) and microglia (12). Thus, IL-6 can be produced in the CNS during the host response to infection or injury and could potentially initiate or contribute to pathology.

MATERIALS AND METHODS

Construction of GFAP-IL6 Fusion Gene and Production of Transgenic Mice. An expression vector derived from the murine glial fibrillary acidic protein (GFAP) gene was used to target expression of IL-6 to astrocytes. Use of this and a similar vector was shown previously to direct the astrocyte-specific expression of β-galactosidase and α_1-antichymotrypsin (ACT), respectively, in transgenic mice (13, 14). A full-length cDNA for murine IL-6 (15) was modified by replacing the 3′ untranslated region with a 196-bp simian virus 40 (SV40) late-region fragment providing a polyadenylylation signal (16). In an initial study this construct was inserted in a previously described (13) GFAP expression vector and used for microinjection (GFAP1-IL6). Subsequently, to improve the expression efficiency *in vivo*, the IL-6 cDNA was inserted into the unique *Sal* I restriction site of a murine GFAP gene [genomic clone pSVGFA (17), obtained from N. J. Cowan, New York University Medical Center] after mutating the two ATG codons between the transcriptional start site and the *Sal* I site in exon 1 to TTG codons by recombinant PCR primer modification and inserting an SV40 late-gene splice (16) 5′ to the IL-6 cDNA (GFAP2-IL6; Fig. 1*A*).

The GFAP fusion genes with or without GFAP-lacZ (13) were microinjected into fertilized eggs of (C57BL/6J × SJL)F_1 hybrid mice, and transgenic offspring were identified by slot-blot analysis (18) of tail DNA with ^{32}P-labeled SV40 late-region fragment.

RNA Isolation and Analysis. Organs were removed and immediately frozen in liquid nitrogen, and poly(A)$^+$ RNA was isolated (19). RNA was denatured, electrophoresed in 1% agarose/2.2 M formaldehyde gels, transferred to nylon membranes, and hybridized at 45°C with ^{32}P-labeled cDNA probes: a 0.69-kb *Eco*RI–*Bgl* II fragment of IL-6 cDNA (15); a 2.7-kb *Xho* I–*Bam*HI fragment of rat GFAP cDNA (complete cDNA clone rGFA15 obtained from R. Milner, Hershey Medical School, University of Pennsylvania, Hershey); a 1.0-kb *Hin*dIII–*Kpn* I fragment of exon 28 from the murine von Willebrand factor (vWF) gene (obtained from David Ginsburg, University of Michigan, Ann Arbor); a 1.8-kb *Eco*RI fragment of EB22/3 cDNA which hybridizes with mRNA products of the *Spi-2* gene (20), the mouse homologue of the human ACT gene; and a 0.26-kb fragment of β-actin gene (21) generated by PCR and provided by M. Nerenberg (The Scripps Research Institute, La Jolla, CA).

***In Situ* Hybridization.** Brains were removed and one hemisphere was fixed overnight in ice-cold 4% paraformaldehyde in phosphate-buffered saline (pH 7.3). Paraffin-embedded sagittal sections (10 μm) were processed for *in situ* hybridization (22). ^{35}S-labeled cRNA to IL-6, used as a probe, was generated from a pBluescript SK(+/−) (Stratagene) vector that contains an *Eco*RI–*Bgl* II fragment of the murine IL-6 cDNA (15).

Preparation of Astrocyte Cultures and Determination of IL-6 Production. Astrocyte cultures were prepared (23) from

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Abbreviations: ACT, α_1-antichymotrypsin; CNS, central nervous system; GFAP, glial fibrillary acidic protein; IL, interleukin; MAP, microtubule-associated protein; vWF, von Willebrand factor.

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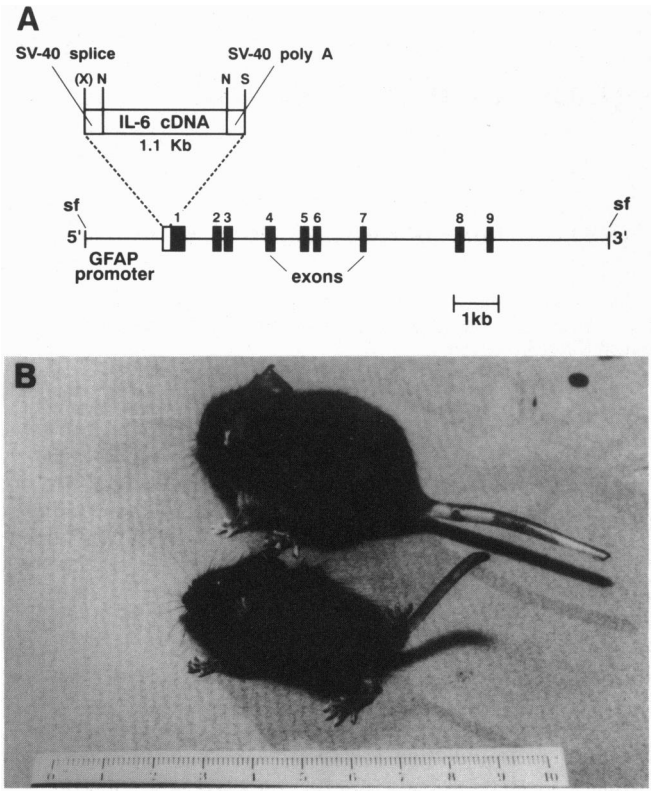

FIG. 1. Generation of GFAP-IL6 transgenic mice. (*A*) Representation of the modified GFAP2-IL6 fusion vector. N, *Not* I; S, *Sal* I; sf, *Sfi* I; x, *Xho* I. (*B*) Typical appearance of a high-expressor GFAP-IL6 mouse, exemplified here by mouse G26#15 (lower), compared with an age-and sex-matched nontransgenic littermate (upper). Note reduced size, hunched posture, ruffled fur, and splayed hindlimbs of the transgenic mouse.

cerebral cortices and cerebellum of individual neonatal (<48 hr) offspring. The purity of the astrocyte cultures was judged to be >96% by GFAP immunostain (rabbit anti-bovine GFAP; Dako).

IL-6 production was measured by bioassay with IL-6-dependent B9 cells (24). B9 cells were seeded in 96-well plates in growth medium and cultured at 10^4 cells per well (final volume, 100 μl) in the presence of serial dilutions of culture supernatants or a murine recombinant IL-6 standard (Genzyme). After 3 days the number of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (25). The minimum detection limit of this assay was 5 pg/ml for the recombinant IL-6 standard.

Histology and Immunocytochemistry. Neuronal damage was evaluated by laser confocal immunomicroscopy (26). Vibratome-cut sections of paraformaldehyde-fixed sagittal tissue blocks were immunolabeled and analyzed (26, 27). Antibodies used were against a dendritic marker, microtubule-associated protein 2 (MAP2 monoclonal antibody; Boehringer Mannheim); a presynaptic terminal marker, synaptophysin (polyclonal antibody; Dako); and a marker for inhibitory interneurons, parvalbumin (monoclonal antibody; Sigma). In other immunocytochemical analyses, for vWF, a rabbit antibody to human factor VIII-related antigen (Dako) was used; for ACT-related protein, two antibodies were used and gave similar results: a goat anti-human ACT antibody (Atlantic Antibodies, Stillwater, MN) and a rabbit anti-human ACT antibody (MBL, Nagoya, Japan).

RESULTS

Neurologic Disease in Mice Expressing the GFAP-IL6 Transgene. Initially, a combination of GFAP1-IL6 construct with GFAP-lacZ was used to generate the bigenic mice designated G16 (Table 1). The remaining two microinjections utilized the GFAP2-IL6 construct alone and gave rise to the transgenic mice designated G26 and G36, respectively. Mice expressing these different constructs displayed similar phenotypic features (Table 1 and see below). By week 1 to week 2 postnatally, five founder pups were smaller than their normal littermates, and they progressed to develop hunched posture, piloerection, tremor, ataxia, hindlimb weakness, and seizures (Fig. 1*B*). Such mice died at an early age (3–10 weeks), typically during and perhaps as a result of seizures. An identical but less severe neurologic disorder was exhibited by transgenic offspring from the G369 founder. Interestingly, the G369 founder has remained healthy and productive for >12 months, giving normal inheritance frequency of the transgene. Hemizygous offspring of line G167, which have been maintained successfully for over a year, progressively developed tremor, ataxia, and, infrequently, seizures by 6 months of age. Homozygous offspring from this line developed by ≈1 month a less severe form of the neurologic disorder seen in the founder generation (see above).

Transgene RNA expression was investigated by Northern blot analysis and *in situ* hybridization (Fig. 2). In all five founder mice and G369 offspring, mRNA hybridizing to the IL-6 probe was expressed at high levels in brain. Lower levels of this transcript were expressed in the brains of G167 mice, with offspring homozygous for the transgene displaying higher levels of transgene mRNA than their hemizygous littermates. The size of the transgene mRNA was ≈0.9 kb in all groups of GFAP-IL6 mice with the exception of the G36 F_1 offspring, which expressed multiple transcripts hybridizing to the complementary IL-6 probe. The predominant band was at ≈1.6 kb. Expression of the transgene IL-6 mRNA was specific to brain and not observed in the peripheral organs, spleen, kidney, or liver (Fig. 2*A*). A low level of 1.3-kb mRNA was occasionally seen in spleen, kidney, and liver of transgenic and normal mice and presumably corresponded to

Table 1. Characteristics of GFAP-IL6 transgenic mice

Mouse	Status	Transgene copy number	Transgene expression	Neurologic disease	Neuronal damage	Astrocytosis	Angiogenesis
G16#0	Founder	2	++	+	ND	+	ND
G26#15	Founder	5	+++	++	ND	+++	+++
G36#1	Founder	3	+++	++	ND	+++	+++
G36#4	Founder	1	+++	+++	ND	++	+++
G36#7	Founder	10	++	++	ND	++	+++
G369	F_1 offspring	8	+++	++	++	+++	+++
G167	Heterozygote	4	+	+/−	ND	+	++
G167	Homozygote	8	++	+	++	++	+++

Mice were generated from three separate microinjections of the GFAP-IL6 fusion gene into fertilized eggs of (C57BL/6J × SJL)F_1 hybrid mice. Mice were scored in comparison with normal littermates, as having mild (+), moderate (++), or marked (+++) increase in the indicated phenotypic features. ND, not determined.

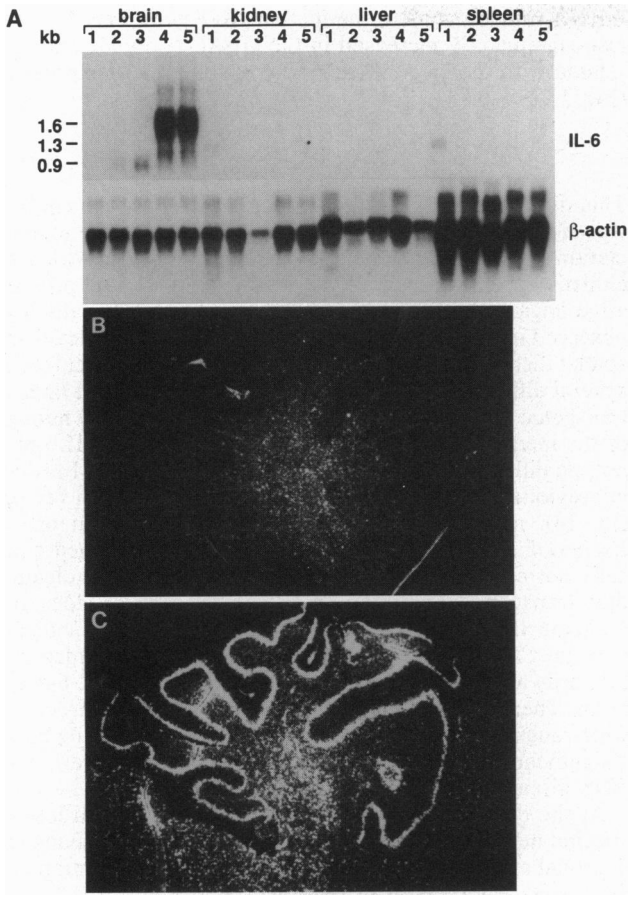

FIG. 2. Expression of the transgene IL-6 mRNA. (*A*) Northern blot analysis of poly(A)$^+$ RNA (5 μg) isolated from brain, kidney, liver, and spleen of a normal mouse (lane 1), a G167 heterozygote (lane 2), a G167 homozygote (lane 3), and G369#28 and G369#35 F_1 mice (lanes 4 and 5, respectively). Transgene-encoded mRNAs were detected with the IL-6 cDNA probe. Hybridization with a β-actin cDNA provided an indication of RNA integrity and loading within each group of lanes. (*B* and *C*) *In situ* hybridization of ^{35}S-labeled IL-6 antisense RNA probe to sagittal sections showing cerebellum from normal mouse (*B*) and transgenic mouse G36#4 (*C*). (×30.)

the endogenous IL-6 transcript (15). No IL-6 mRNA was detectable in the brains of normal mice. *In situ* hybridization revealed a similar widespread distribution of IL-6 RNA expression in the brains from different groups of GFAP-IL6 transgenic mice, with particularly high levels in thalamus and cerebellum (Fig. 2 *B* and *C*).

Evidence for CNS production of IL-6 protein in the GFAP-IL6 mice was obtained from *in vitro* studies. Supernatants derived from astrocyte cultures prepared from brain tissue of G167 hemizygous and G167 homozygous mice were found to contain significant IL-6 bioactivity (150 pg/ml and 310 pg/ml) in comparison with cultures prepared in parallel from a normal littermate (<5 pg/ml).

In the G167 line, expression of the *lacZ* reporter gene was assessed by β-galactosidase histochemical staining of frozen sections of brain tissue (13) and found to have overlapping distribution with the transgene IL-6 RNA (data not shown).

Cerebral Overexpression of IL-6 Results in Neuronal Damage. Routine histological examination of the brain in all GFAP-IL6 founder groups and the G369 F_1 offspring revealed a significant condensation of the molecular layer of the cerebellum and neovascularization which was also most evident in the cerebellum. Inflammation (encephalitis or meningitis) was never seen, although a discrete perivasculitis limited to a small number of larger blood vessels in cerebellar sulci was present in mice with high IL-6 expression. In two different groups of GFAP-IL6 mice (G369 F_1 and G167 homozygotes), laser scanning confocal microscope imaging of brain sections immunolabeled with anti-MAP-2 provided evidence for significant neuronal and dendritic changes in both hippocampal and cerebellar neurons (Fig. 3 *A–D*). In the CA1 region of the hippocampus, dendritic processes were collapsed and vacuolized and overall dendritic complexity was decreased (32% less than controls). In the cerebellum, the molecular layer was atrophic and the dendritic processes of the Purkinje cells were tortuous and dilated and showed 50% less branching than controls. Immunolabeling of adjacent sections with anti-parvalbumin revealed a marked decrease in immunoreactive cells in the hippocampal formation of transgenic mice compared with normal littermates (Fig. 3 *E* and *F*). The area most affected was the dentate gyrus, with a 90% loss of these cells.

Extensive Astrocytosis and Angiogenesis in GFAP-IL6 Mice. In all GFAP-IL6 mice studied, a dramatic increase in GFAP immunoreactivity was found, especially in the thalamus, glial limitans, Bergmann glia, and cerebellar white-matter tracts. In addition, GFAP-positive astrocytes in the GFAP-IL6 mice were clearly hypertrophied with numerous prominent processes radiating from the enlarged cell body (Fig. 4 *A* and *B*). This reactive astrocytosis was also associated with markedly elevated levels of GFAP mRNA in the transgenic mice (Fig. 4). The degree of reactive astrocytosis correlated with the level of transgene-derived IL-6 expression, which was represented at the RNA level (Fig. 5).

A striking feature in GFAP-IL6 mice evident with conventional staining of brain sections was neovascularization. This was particularly pronounced in the cerebella of older (6 months) mice of the G167 line, which developed a prolifer-

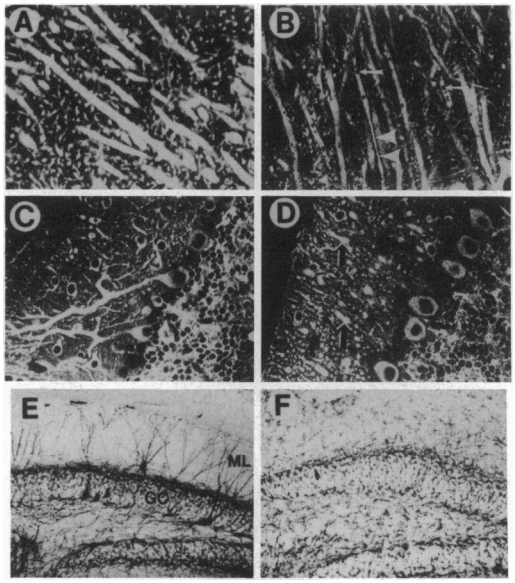

FIG. 3. Neuronal alterations in the CNS of a GFAP-IL6 mouse. Laser scanning confocal microscopy images of the CA1 region of the hippocampus, immunolabeled with an antibody against MAP2, from a normal mouse (*A*) and a G167 homozygous transgenic mouse (*B*) show collapse (arrowheads) and vacuolization (arrows) of the dendritic processes in the transgenic animal. Sections analyzed in parallel show cerebellum in the normal (*C*) and G167 (*D*) mice. Note in the transgenic mouse, the molecular layer was atrophic and the dendritic processes of the Purkinje cells were tortuous and dilated and showed reduced branching (arrows). The hippocampal region of the normal mouse (*E*) showed abundant parvalbumin-positive interneurons in the granular cell layer (GC) and in the molecular layer (ML). In contrast, in the transgenic mouse (*F*) there was a paucity of parvalbumin-immunoreactive interneurons. (*A–D*, ×360; *E* and *F*, ×95.)

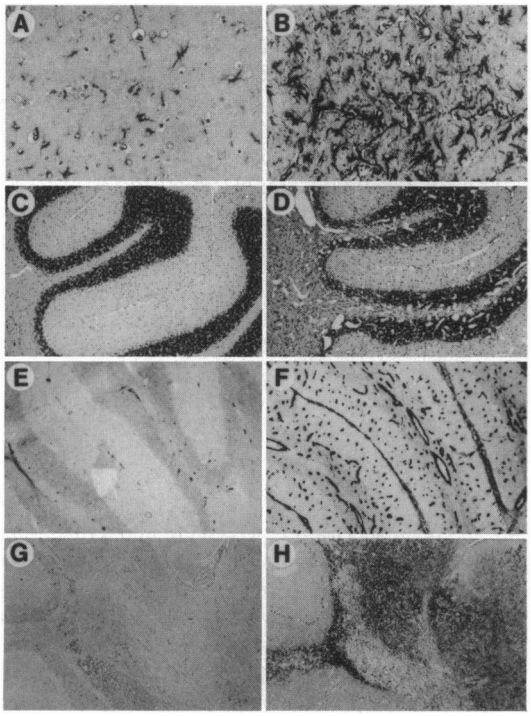

FIG. 4. Extensive neuropathology in GFAP-IL6 mice. Histology and immunocytochemistry of brain sections from normal (*Left*) and GFAP-IL6 (*Right*) mice. Immunolabeling with anti-GFAP (*A* and *B*) shows astrocytosis in transgenic mouse G36#1. Proliferative angiopathy in the cerebellum of a 6-month-old G167 heterozygous mouse is revealed by hematoxylin and eosin staining (*C* and *D*) and immunolabeling with anti-vWF (*E* and *F*). Immunolabeling with anti-ACT (*G* and *H*) demonstrates increased levels of the acute-phase protein in the cerebellum of transgenic mouse G36#1. (*A* and *B*, ×240; *C–H*, ×60.)

ative angiopathy giving rise to a distinctive "Swiss cheese" appearance (Fig. 4 *C* and *D*). Immunolabeling of brain sections with anti-vWF (an endothelial cell-specific marker) confirmed that the numerous spongiform-like structures were lined with endothelial cells and therefore represented blood vessels (Fig. 4 *E* and *F*). Expression of vWF mRNA was significantly increased in GFAP-IL6 mice and also correlated with IL-6 expression (Fig. 5).

Enhanced Expression of Acute-Phase Response Genes in GFAP-IL6 Mice. Immunolabeling of brain sections with anti-ACT revealed increased staining in hippocampus and cerebellar white-matter tracts of GFAP-IL6 mice (Fig. 4 *G* and *H*). Notably, expression of the *Spi-2* gene product, EB22/5.3

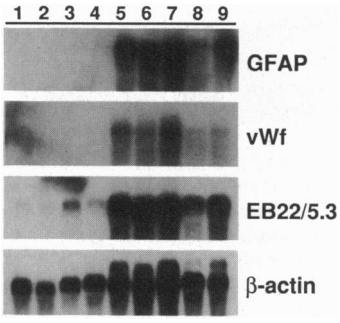

FIG. 5. Northern blot analysis of poly(A)$^+$ RNA (5 μg) from brain of normal mouse (lanes 1–4), G369#2 (lane 5), G369#3 (lane 6), and G369#5 (lane 7) F_1 mice, G167 heterozygous mouse (lane 8), and G167 homozygous mouse (lane 9). The membrane was hybridized sequentially with ^{32}P-labeled cDNA probes complementary to GFAP, vWF, EB22/5.3, and β-actin mRNAs.

mRNA (homologous to the human ACT gene; ref. 20), was also significantly increased in the GFAP-IL6 mice and correlated with the level of transgene-encoded IL-6 expression (Fig. 5).

DISCUSSION

This study demonstrated that cerebral overexpression of IL-6 was sufficient to cause profound neurologic disease in association with extensive neuropathologic changes, which included neuronal damage, reactive astrocytosis, and proliferative angiopathy. The clinical features and neuropathology observed in GFAP-IL6 mice (*i*) correlated with the level and spatial distribution of IL-6 RNA expression, (*ii*) occurred in several different founder mice as well as in different lines of transgenic offspring, and (*iii*) were unrelated to copy number of the integrated transgene. Therefore, the GFAP-IL6 phenotype did not result from an insertional mutation. Further, in previous studies using GFAP genomic expression vectors (13, 14), mice with either low or high expression of the *Escherichia coli lacZ* or human ACT genes remained clinically normal and did not exhibit neuropathology, indicating that transgene expression in astrocytes *per se* does not replicate the GFAP-IL6 phenotype. Our findings indicate that the CNS disease observed in the GFAP-IL6 mice was primarily a consequence of the overexpression of IL-6 in the brain. Therefore, cytokines such as IL-6 can have direct and wide-ranging pathogenic actions in the CNS and could make a significant contribution to the pathogenesis of a variety of CNS diseases (1–10).

At the cellular level, GFAP-IL6 mice exhibited a loss of specific neuronal subpopulations and a significant change in neuronal morphology, including extensive vacuolization and decreased arborization of neuronal dendrites in the hippocampus. Similar dendritic pathology has been described in Creutzfeldt–Jakob disease (28), scrapie (29), and human immunodeficiency virus encephalitis (26). The finding of similar neuronal pathology across such a diverse spectrum of CNS diseases suggests either a common mechanism for induction of toxicity, involving perhaps cytokines such as IL-6, or that neurons have a limited ability to respond to unrelated toxic factors. A loss of parvalbumin-positive neurons was also observed in the hippocampus of GFAP-IL6 mice; a decrease in this subset has been reported in human immunodeficiency virus encephalitis (27). Parvalbumin is enriched in inhibitory interneurons that contain γ-aminobutyrate (30). Therefore, loss of parvalbumin reactivity in the GFAP-IL6 mice could indicate metabolic derangement and/or death of these cells. Damage to inhibitory neurons in the hippocampus could result in increased excitatory input (31) and underlie the seizures in the GFAP-IL6 mice. The molecular basis for the neurodegenerative changes seen in GFAP-IL6 mice is not known, but the changes could conceivably result directly from IL-6 itself, from an indirect pathway activated by IL-6, or from a combination of these.

Reactive astrocytosis was a prominent feature in GFAP-IL6 mice. Induction of astrocytosis is commonly seen following inflammation or injury in the CNS (32, 33) and may be mediated in part by cytokines. Consistent with this, intracerebral injection of IL-1 (34) or interferon γ (35) has been shown to promote reactive astrocytosis. We speculate that the reactive astrocytosis in the GFAP-IL6 mice may be induced directly in an autocrine/paracrine fashion by IL-6 produced by the transgenic astrocytes. This speculation is supported by the observation that IL-6 can stimulate astrocyte growth (36). Alternatively, the astrocyte response may be secondary to induction of other factors or injury mediated by IL-6. Astrocytes play a crucial role in the development and support of neurons (37). Therefore, the chronic perturbation of these glial cells, as is seen in the GFAP-IL6 mice,

might contribute specifically to the morphologic and functional impairment of surrounding neurons.

The angiogenic response in the CNS of GFAP-IL6 mice was particularly striking, giving rise to a proliferative angiopathy which caused considerable distortion of surrounding neuronal tissue. A close association between IL-6 gene expression and physiological angiogenic processes (38) and the ability of IL-6 to stimulate motility of endothelial cells (39) implicate this cytokine in angiogenesis. Additionally, reactive astrocytes in the GFAP-IL6 mice may be a further source of other angiogenic factors (40). How such extensive angiogenesis might impact upon neuronal biology is unknown. Aside from the gross structural imposition, angiogenesis may be accompanied by changes in perfusion and/or vascular permeability that could perturb both glial and neuronal cell homeostasis.

A prominent biologic action of IL-6 is induction of acute-phase protein synthesis and secretion. Cerebral expression of murine ACT-related (EB22/5.3) gene product in GFAP-IL6 mice was considerably increased over that in normal mice. The cellular origin of the EB22/5.3 gene expression in the mouse brain is not known. However, given its homology with the human ACT gene, the astrocyte may be a primary source (41). In the human brain, ACT production is elevated in a number of neurodegenerative diseases, including Alzheimer disease, where it is speculated to play a role in amyloidogenesis (41). By analogy with their human counterpart, murine ACT-related proteins may function in the brain to inhibit the potentially detrimental activity of proteases released locally by reactive astrocytes or microglia.

In conclusion, our findings indicate that overexpression of IL-6 in the CNS of transgenic mice is sufficient to induce a range of structural and functional neurologic impairments. These alterations overlap with findings in autoimmune, infectious, degenerative, and trauma-induced CNS diseases of humans in which cytokines such as IL-6 are produced. It will be of interest to compare the GFAP-IL6 mice with similar models expressing other cytokines to assess the relative neuropathogenetic potential of these host factors and to help identify potential targets for therapeutic interventions.

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