Multiple Levels of Regulation of the Interleukin-6 System in Stroke

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Background and Purpose—Serum levels of the cytokine interleukin-6 (IL-6) rise markedly in stroke. IL-6 is a key regulator of inflammatory mechanisms that play an important part in stroke pathophysiology. The action of IL-6 is modified by its soluble receptor subunits sgp130 and sIL-6R. The purpose of this study was to investigate whether serum levels of the receptor subunits are changed after ischemic stroke and to define the role of genetic influences on IL-6 expression in acute stroke.

Methods—In 48 patients with acute stroke and 48 age- and sex-matched control subjects, serum concentrations of IL-6, sgp130, and sIL-6R were measured by enzyme-linked immunosorbent assay. Furthermore, IL-6 promoter haplotypes comprising 4 different polymorphisms (−597G→A, −572G→C, −373A(n)T(n), −174G→C) were determined by DNA sequencing and allele-specific oligonucleotide polymerase chain reaction. The effect of the common haplotypes on IL-6 gene transcription was tested by transfecting reporter fusion genes in the astrocytelike cell line U373.

Results—Whereas serum concentrations of IL-6 significantly rose (P<0.001), sgp130 levels were transiently reduced after stroke (P<0.05), and sIL-6R levels remained unchanged. IL-6 levels depended on the infarct size and the haplotype of the promoter region. The common haplotype A-G-8/12-C was associated with low IL-6 levels after stroke and a reduced induction of IL-6 transcription on stimulation with an adenosine analog in vitro.

Conclusions—The data demonstrate genetic variation in the expression of IL-6 in stroke. Induction of the inflammatory response by IL-6 might be enhanced by a transient downregulation of the potential IL-6 antagonist sgp130. (Stroke. 2003;34:1864-1870.)

Key Words: acute-phase reaction • interleukins • polymorphism

Stroke triggers an acute-phase response. Elevated serum levels of acute-phase proteins such as C-reactive protein (CRP) or fibrinogen are found in about three quarters of patients with ischemic stroke.1,2 Up to 60% of patients have a core temperature >37.5°C in the first week after symptom onset.3,4 Fever and elevated serum concentrations of CRP or fibrinogen have been linked to poor prognosis.1–3 In infection, the cytokine interleukin-6 (IL-6) is a key regulator of the acute-phase response. IL-6 elevates the temperature set point in the hypothalamus and stimulates the expression of CRP, fibrinogen, and other acute-phase proteins in the liver. Infection secondary to stroke may contribute to the observed activation of the inflammatory response in stroke patients; however, experimental studies support the notion that brain ischemia also triggers an inflammatory response. Cerebral ischemia induces the expression of IL-6 in neurons and astrocytes.5–7 In patients, IL-6 serum concentrations rise within several days. The ischemic brain appears to be a major source of IL-6 in stroke because the serum concentrations correlate with infarct size and because cerebrospinal fluid concentrations exceed the serum concentrations of IL-6.8 The correlations between IL-6 and CRP,9 IL-6 and fibrinogen, or IL-6 and body temperature10 argue for a role of IL-6 in the regulation of the inflammatory response in stroke. However, the fact that no acute-phase response is found in about one quarter of patients shows that the regulation is more complex.1,2

The expression of IL-6 is regulated mainly at the transcriptional level. The promoter of the human IL-6 gene contains several polymorphisms that influence IL-6 gene transcription in selected cell types.11 The −174 G/C polymorphism has been linked to several diseases, including Alzheimer and lacunar infarcts.12,13 However, the effect of these polymorphisms on IL-6 induction in neural cells is unknown.
Extracellular IL-6 exerts its effects through a membrane receptor that consists of the 2 subunits gp130 and IL-6R. Soluble forms of both subunits circulate in blood. IL-6R is missing in many cell types, but sIL-6R can substitute for the resident membrane receptor and thereby acts as an agonist. In contrast, sgp130 is a decoy receptor that exerts an antagonistic effect in vitro. The serum concentration of sIL-6R has been reported to be elevated in several inflammatory and atherosclerotic diseases, but to the best of our knowledge, neither sIL-6R nor sgp130 has been studied in acute stroke. Therefore, we have investigated the serum concentrations of the IL-6 receptors and the effect of polymorphisms in the IL-6 promoter on IL-6 expression in acute stroke.

Subjects and Methods

Subjects

In this study, 48 patients treated for acute ischemic stroke in our clinic were included prospectively over the course of 7 months. All patients were admitted within 24 hours after onset of symptoms. Patients were not included in the study if symptoms lasted <24 hours, if they had a history of a malignant tumor or stroke within the last year, if they were currently receiving anti-inflammatory or immunosuppressive medication, or if they presented with signs or symptoms of acute or chronic infection. Patients were excluded from the study if they developed signs of infection (4 patients), if intensive care therapy became necessary (4 patients), or if they were discharged before day 3 or 7 (17 patients). The control group was recruited simultaneously. Control subjects were sex and age matched and were electively admitted to the Department of Ophthalmology for cataract surgery or glaucoma therapy. During hospitalization, stroke patients were treated with anticoagulants (heparin or phenprocoumon, 17 patients), antiplatelet drugs (20 patients), statins (18 patients), or antihypertensive agents (31 patients). All subjects gave informed consent. The study was approved by the local ethics committee.

Imaging and Clinical Examinations

The neurological status of stroke patients was assessed by the National Institutes of Health Stroke Scale (NIHSS) on days 1 and 7 and later than day 90 after symptom onset. Neurological improvement or deterioration was defined as a change of NIHSS of at least 4 between days 7 and 90 or later compared with day 1. The origin of stroke was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria. The aural temperature of stroke was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.

Laboratory Investigations

In stroke patients, blood samples were taken between 7 and 8 AM on day 1 (<24 hour after onset of symptoms) or day 2 (between 24 and 48 hours after onset), day 3, day 7, and after 90 days. In the control group, blood was taken on admission before surgery. CRP was determined by an ultrasensitive turbidimetric test that uses latex beads (Quantex CRP Ultrasensitive, Instrumentation Laboratory). The threshold of detectability was 1.0 mg/L. Fibrinogen was measured by functional coagulation testing (derived fibrinogen) with Recombiplastin (Instrumentation Laboratory) as reagent. For cytokine determination, samples were immediately put on ice and centrifuged within 30 minutes. Serum was then stored at −80°C until the enzyme-linked immunosorbent assays (ELISAs) were performed. For determination of IL-6, sIL-6R, and sgp130, commercial ELISA kits (R&D) were used.

Haplotypic Determination

The primers IL-6-00501 (5′-AGT GGG CTG AAG CAG GTG AAG AAA-3′) and IL-6-11028 (5′-CTG ATT GGA AAC CTG ATT AAC ATT GT-3′) were used for amplification of the IL-6 promoter region, which was sequenced with standard techniques. Eight different IL-6 promoter haplotypes have been described by Terry et al in whites (haplotypes A through G and X, with X corresponding to a deletion of a G residue immediately 5′ to the AT run in haplotype D). On the basis of this work, we interpreted the sequence data from the promoter region of 34 patients and 21 control subjects. If homozygosity was observed for all 4 polymorphic positions (−597G→A, −572G→C, −373A(nT)n, −174G→C) or if 3 of them, the 2 haplotypes were unequivocally established. When ≥2 of the 4 promoter polymorphisms were found to be heterozygous, the combination of individual genotypes made it possible to deduce 2 haplotypes on the basis of the 8 haplotypes described by Terry et al.

Some of these deduced haplotypes were confirmed by allele-specific oligonucleotide polymerase chain reaction (PCR) with the following primers: AAGTAACTGACGAAATTTGAGGG, AAGTAACTGACGAAATTTGAGGA, GCAATGTGACGTCCTTTAGCATC, and GCAATGTGACGTCTTTAGCATC. Sequence analysis of the allele-specific PCR products consistently confirmed the deduced haplotypes on the basis of the 8 known, naturally occurring haplotypes.

Cell Culture and Transfection

The human astrocytoma cell line U373 was cultured in Earle’s modified Eagle medium (PAA) containing 10% fetal calf serum, penicillin (50 IU/mL), streptomycin (50 μg/mL), and 1% nonessential amino acids (Gibco BRL). One day before transfection, cells were plated on a 24-well plate and transfected with DEAE dextran. Wells were washed with phosphate-buffered saline, and then 1 μg of the first reporter plus 0.1 μg pRL-SV40 as a second reporter (Promega) were added in Earle’s modified Eagle medium containing 75 μg/mL DEAE-dextran but no serum for 4 hours. Then cells were shocked by the addition of 10% dimethyl sulfoxide in phosphate-buffered saline for 1 minute. After incubation for 42 hours in full medium, cells were stimulated with 10 μmol/L 2-chloroadenosine (RBI, Biotrend). Cells were harvested, and the activities of firefly and renilla luciferase were measured with the Dual Luciferase Reporter Assay (Promega). The reporter fusion genes containing haplotypes of the human IL-6 promoter and the coding sequence of firefly luciferase have been described before.

Statistical Analysis

Correlation was quantified by use of Spearman’s correlation. Data are presented as median and percentiles (10th, 25th, 75th, and 90th in box plots) because not all parameters were normally distributed. We used the Mann-Whitney U test and the Wilcoxon test to analyze differences between groups and time points, respectively. Analysis of variance (ANOVA) was performed to test for differences between groups. Allele frequencies were compared by the exact χ² test for small sample size. A linear regression model was used to adjust the log₁₀-transformed IL-6 concentrations for log₁₀-transformed infarct volume and to compare IL-6 and IL-6 receptor concentrations in patients and control subjects, with age, sex, and IMT of the common carotid artery as covariates.

Results

Clinical and demographic data of our study population are summarized in Table 1. Although we did not include patients with clinical signs of infection, the acute-phase proteins fibrinogen (Figure 1A) and CRP (Figure 1B) were increased in stroke patients, as has been reported before. In our group of patients, 5 had a body temperature >37.4°C on day 3 compared with no control subjects (P<0.025); the average body temperature did not differ from that of the control group (Figure 1C). IL-6 serum concentrations were markedly ele-
...vated after stroke (P<0.001) (Figure 1D). In contrast, the serum concentration of sgp130 was significantly reduced the first week after stroke (P<0.05) but not at follow-up 3 months later (Figure 1E). IL-6 and sgp130 remained different after adjustment for age, sex, and IMT, a marker of atherosclerosis (IL-6, P<0.001; sgp130, P=0.003). Serum concentration of sIL-6R did not differ between control subjects and patients with acute stroke (Figure 1F). There were no significant correlations between IL-6 serum levels and its receptors in stroke patients.

The kinetics of the increase in IL-6 and CRP differed: IL-6 serum concentration had already increased in the first 24 hours after stroke and before the peak of the acute-phase protein CRP on day 3 (Figure 1B and 1D). On days 3 and 7, serum concentration of IL-6 closely correlated with CRP and fibrinogen concentrations (IL-6 day 1 and CRP day 3, r=0.35, P=0.03; IL-6 day 3 and CRP day 3, r=0.36, P=0.04; IL-6 day 7 and CRP day 7, r=0.6, P=0.005; IL-6 day 1 and fibrinogen day 3, r=0.35, P=0.04; IL-6 day 7 and fibrinogen day 7, r=0.5, P=0.02) but not with leukocyte count or body temperature (data not shown). CRP concentrations were significantly higher in patients on days 3 and 7 compared with control subjects but not on day 1, possibly because of the limited sensitivity of the CRP assay (Figure 1B).

Serum concentration of IL-6 correlated with infarct volume (r=0.4, P=0.003) and clinical status of stroke patients as expressed by NIHSS on day 1, on day 7, and after day 90 (r=0.4, P=0.003; r=0.4, P=0.02; r=0.5, P=0.012, respectively). The concentrations of the 2 receptor subunits did not correlate with infarct size or NIHSS (data not shown), nor was there any obvious difference in infarct volume (1-way...
ANOVA, \( P=0.2 \) or CRP, IL-6, and IL-6 receptor concentrations in the etiological subgroups. We found no correlation of temperature, CRP, fibrinogen, IL-6, and IL-6 soluble receptors on day 1 and neurological deterioration or improvement.

To search for genetic influences on IL-6 expression, we determined 4 polymorphisms in the IL-6 promoter region in 34 stroke patients and 21 control subjects. The frequencies of the 8 haplotypes of IL-6 were comparable, as has been described previously.\(^{\text{11}}\) The frequency distribution between patients and control subjects did not differ in general \( (P=0.19) \), although an exploratory analysis for specific haplotypes indicated a different frequency of haplotype C between control subjects and patients (Table 2).

The haplotype found most frequently was F. IL-6 serum concentrations were significantly lower in patients with haplotype F than in patients with other haplotypes in a multivariate ANOVA adjusted for infarct size \( (P=0.019; \text{Figure 2A}) \). Control subjects and patients with haplotype F had lower body temperature and CRP values, which was significant in control subjects (Figure 2B and 2C).

In cerebral ischemia, astrocytes express IL-6,\(^{\text{5}}\) and adenosine is considered a major stimulus of IL-6 transcription.\(^{\text{21}}\) Therefore, we investigated the effect of the various haplotypes of the IL-6 promoter on the induction of IL-6 transcription in the human astrocyteliike cell line U373. Basal transcription did not differ between the 8 common haplotype variations of the IL-6 promoter (data not shown). Induction by the stable adenosine analog 2-chloroadenosine significantly depended on the haplotype of the IL-6 promoter, with haplotype F mediating the lowest induction of IL-6 transcription (Figure 3).

**Discussion**

Activation of the inflammatory response plays a pivotal role in acute stroke. An increase in serum levels of CRP and fibrinogen and in body temperature predicts poor prognosis. Moreover, activation of the acute-phase response has been involved in the pathogenesis of ischemic stroke, suggesting that intervention might be beneficial. Therefore, it is important to understand the regulation of the acute-phase response in stroke.

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<th>Table 2. Haplotype Frequency in Control Subjects and Patients</th>
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Values are absolute numbers of haplotypes. Percentages are given in parentheses.

**Figure 2.** Haplotype of the IL-6 promoter influences IL-6 serum concentration, temperature, and CRP. A, Serum concentration of IL-6 in 34 stroke patients on day 1 adjusted to infarct volume was lower in the presence of haplotype F \( (P=0.019, \text{multivariate ANOVA}) \). B, C, Body temperature and CRP serum concentration were decreased in the presence of haplotype F in 21 control subjects \( (*P=0.02, +P=0.01) \).
The effects of IL-6 are modulated by the 2 soluble receptor subunits sIL-6R and sgp130. In vitro, sIL-6R promotes the effect of IL-6 as a coactivator, but sgp130 captures the soluble complex of IL-6/sIL-6R and prevents it from binding to the membrane-bound gp130 that mediates cellular effects. We report for the first time that sIL-6R levels are expressed in the ischemic brain and modulates body temperature. The 4 polymorphisms, however, exert a cooperative effect on IL-6 gene transcription. The −174G polymorphism is present in haplotypes B, D, E, C, X, and G. Its effect on IL-6 transcription depends heavily on the haplotypic promoter context (Figure 3). Therefore, our results are not directly comparable with previous work. However, given the rarity of haplotype A (Table 2), haplotype F should be present in most individuals with the −174C polymorphism. Previous population genetic studies reported both equal and lower IL-6 levels in healthy control subjects with −174C. In other medical conditions, the −174C polymorphism was associated with equal and higher serum concentrations of IL-6.

In summary, our data suggest 2 further levels of regulation of the important acute-phase response in stroke. The genetic control of IL-6 expression and the acute reduction in the potential IL-6 antagonist sgp130 may modulate the acute-phase response and outcome of stroke.
Acknowledgments

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References

have normal levels of inflammatory markers after stroke, implying that ischemic stroke itself does not induce a full-blown acute-phase response.\textsuperscript{3,5}

To respond to these questions, Acalovschi et al\textsuperscript{1} analyze the IL-6 system and the acute-phase response after stroke, showing that there is a genetically determined difference in the degree of IL-6 response to stroke between individuals. The immune haplotype A-G-8/12-C was associated with lower levels of IL-6 after stroke and with a reduced induction of stimulated IL-6 transcription in human astrocytoma cells. However, they did not find any differences, as we could have expected, in the acute-phase response when comparing different haplotypes, as suggested by levels of C-reactive protein, an IL-6 hepatic induced acute-phase reactant. What can we learn from such a study?

The answer lies at many levels and ultimately may point to the futility of our present approach to the genetics of complex disorders. We have much work to do before we will know how to apply these data in practice. It is clear that genes do not commonly operate in isolation from the environment in the development of complex multifactorial disorders. This being so, it is possible that all genetic markers are either neutral or relatively protective in the absence of an environmental influence, and studies clearly need to take this in account. Furthermore, the response to environmental factors is variable among individuals, and we need to identify the genes and alleles that show variations in response to pattern.

The phenotype of ischemic stroke is incredibly complex, with variable environmental interactions. In the candidate gene philosophy approach, some genes are more important than others. This view is difficult to sustain, and we may need to study dozens of genes and hundreds of polymorphisms. For IL-6, in which there is wide interindividual variation in levels, and despite convincing evidence of a significant heritable component, the currently identified polymorphisms account for a very limited proportion of this variation.\textsuperscript{6} It is therefore highly unlikely that a polymorphic variant accounting for such a relatively small variation in levels would be related to disease.\textsuperscript{7}

Additionally, any polymorphisms identified within the genes encoding these proteins may interact to either augment or attenuate the influence of another, thereby confounding the difficulty in interpretation of the results of gene-disease associations.\textsuperscript{8} One clear solution to this is to account for the total genetic variance in the gene of interest by evaluation of all polymorphic variants and to analyze the association of common haplotypes with plasma levels and presence of disease. An additional important factor to consider is that we tend to focus on proteins that contribute to inflammation and forget that there are many that module inflammatory response. An enhanced inflammatory response might also depend on the failure to turn off inflammation when it is no longer useful to the organism. Therefore, genetic variation in all of the genes encoding these proteins and the proteins involved in their regulation may influence an individual’s inflammatory response. In this context, the inability to identify significant and consistent genetic contribution of 1 genotype in particular reflects the underlying genetic complex.

Stroke prognosis is a clearly modifiable, and although a single polymorphism or cluster of polymorphisms within a single candidate gene will not be particularly informative in determining the overall risk, analysis of the functional effects of polymorphisms will provide invaluable information regarding protein function and regulation. This information is likely to be fundamental in identification of novel targets for the development of new agents. In addition, knowledge of an individual’s genetic profile may help in defining individual drug regimens to elicit maximum therapeutic benefits. Finally, we should begin to turn our attention toward the transcriptional regulation for factors that regulate the whole system rather than just individual proteins. Careful clinical identification of very homogeneous subgroups of patients according to their history, risk factors, and inflammation markers will be the first step of this search. The amount of inflammatory response produced after stroke may represent a further marker of individual susceptibility. Once the profile of the patients is drawn, the causes, genetic or acquired, of the hyperresponsiveness can be sought. We should continue our work in this area with optimism but use genetic markers as tools for investigation rather than as the answer in itself. We must develop better skills in postgenomic functional analysis of polymorphisms to inform us as to how we can better understand the systems we study and to develop novel therapeutics and markers for therapeutic responses. Finally, we should approach molecular epidemiology in this area with caution, understanding the many pitfalls that lie in the path of attempting to nail a single polymorphism to this complex disorders. Only international, multicenter, prospective, well-designed studies will provide the currently lacking information and will gradually improve our knowledge in the field before implementing the use of genetic polymorphisms in the stroke medicine daily practice.

**Mario Di Napoli, MD, Guest Editor**

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