Variation in the Interleukin-6 Gene Is Associated with Impaired Cognitive Development in Children Born Prematurely: A Preliminary Study

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ABSTRACT

The pro-inflammatory cytokine IL-6 may be neurocytopathogenic, and elevated levels are associated with impaired neurological outcome among children born prematurely. However, the precise mechanisms underlying this association remain unclear. The rare C (rather than G) variant at position -572 in the IL-6 gene is associated with an increased IL-6 synthetic response. If IL-6 mediates cerebral injury, we would anticipate the -572 C allele to be associated with impaired childhood development. We have examined this hypothesis, studying 113 Caucasian children born at ≤ 32 wk gestation. Cognitive and motor functions were assessed using the Griffiths Developmental Scales at 2 y and British Ability Scales (2nd Ed.) and the ABC Movement Score at 51/2 y. Performance (median, interquartile range) in all three scales was worse in the 10 carriers of the C allele than for those with GG genotype: Griffiths Developmental Quotient: C allele,

92.4 (89.9–96.6) *versus* CG 100.9 (96.7–104.8), $p = 0.002$; General Cognitive Ability: C allele, 88.0 (80.3–102.8) *versus* GG 103.0 (92.0–112.0), $p = 0.037$; Movement ABC score: C allele 8.3 (6.6–20.3) *versus* GG 4.0 (1.0–9.5), $p = 0.081$. The presence of the rare (≥ 1) IL-6 -572 C-allele (CC+GC genotypes) is associated with impaired cognitive development among children born before 32 wk gestation. These data support a role for IL-6 in the genesis of neurologic impairment in such children. (*Pediatr Res* 58: 117–120, 2005)

Abbreviations

DQ, Griffiths developmental quotient

GCA, general cognitive ability, British Ability Scales, 2nd Ed.

WMD, white matter damage

Disability is common among children born prematurely and is inversely related to gestational age at birth. As many as one half of those surviving after birth at 25 wk gestational age or less have a severe disability at 21/2 years of age, 50% of these being severe (1). Even those seemingly unaffected early on may demonstrate a range of motor, cognitive, behavioral, psychological, emotional, and educational deficits later in childhood (2–4). To date, a series of risk factors for such cognitive impairment (*e.g.* birth weight, chronic lung disease, and social factors) have been identified, although the biologic transduction mechanisms that result in cerebral injury remain occult (5).

The pro-inflammatory cytokine IL-6 may be one such biologic mediator. Perinatal concentrations of the pro-inflammatory cyto-

kines such as IL-6 correlate directly with the development of periventricular WMD and subsequent neurologic deficit (6–10). WMD, in turn, may impair gray matter development throughout childhood (11,12) and is perhaps responsible for some of the developmental deficits observed in children after premature birth. Correlating structural brain abnormality with specific functional deficits has proved difficult, however (13).

It remains unclear as to whether the association of elevated IL-6 levels with cerebral injury is innocent or causal. A genetic strategy of investigation may help elucidate the pathways that cause such injury: the association of a functional variant in the IL-6 gene with impaired neurological outcome would offer substantial evidence of causality. A candidate gene association study of this variety has advantages in the assessment of cytokine pathogenicity, where levels fluctuate rapidly and wildly, where total chronic burden of exposure cannot be measured, and where tissue generation may be more important than circulating levels themselves (14). Such a genetic variant does exist, which is applicable to such a study: a G/C substitution has been identified at position -572 in the IL-6

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gene—the rare C allele being associated with a higher IL-6 synthetic response (14).

If IL-6 plays a causal role in mediating cerebral injury and cognitive developmental progress after preterm birth, the C allele should be associated with such cognitive impairment. We have performed a pilot study to examine this hypothesis, using a well-defined cohort of preterm infants (15) with structured neurodevelopmental follow-up to 51/2 y of age.

METHODS

Patients and developmental assessment. The study was approved by the ethical committees of Southmead Hospital and United Bristol Health Care Trust. Parental consent was obtained for participation in neurodevelopmental follow-up (15). Consent was not required for the genetic component of this study, as all personal information was held separately from the genetic information and patients were identified solely by study codes. The patients (≤ 32 wk gestation, Caucasian) participated in a population-based developmental outcome study, the Avon Premature Infant Project (APIP) (15). Patients were prospectively recruited to a randomized controlled trial of Portage (an early education programme focused on the child) or social support (parent adviser scheme focused on the mother) intervention started at discharge and continued for up to 2 y, as previously described (15). Patients who were followed up at 2 and 5.5 y were identified and Guthrie spots located where possible. Genotypes were successfully determined in 113 patients (below), representing 37% of the original APIP population ($n = 308$) and 60% of all infants followed up to 5.5 y ($n = 187$).

Cognitive and motor functions were assessed using the Griffiths Developmental Scales at 2 y and British Ability Scales (2nd Ed.) (BAS) and the ABC Movement Score at 51/2 y. The Griffiths Mental Development Scales, used to assess motor and cognitive performance, was performed at 2 y corrected age (16). The Griffiths scales comprise five subscales, including personal and social, hearing and speech, locomotor, eye hand co-ordination, and performance domains, from which is derived an overall developmental quotient (DQ). A lower Griffiths DQ reflects a poorer neuro-developmental performance. A score of 70 (-2 SD) would indicate severe disability instead of the conventional 55 (-3 SD) due to secular drifts in population scores that have resulted in a higher population mean. Cognitive developmental progress at 5.5 y of age was assessed using the BAS (17). The BAS computes GCA together with visuospatial, verbal, and nonverbal subscales. The GCA is a developmental quotient equivalent to an IQ estimate, normalized at 100 ($SD \pm 15$) in which a lower score again indicates poorer conceptual ability. The Movement ABC scales (18) assesses manual dexterity, ball skills, and balance over 10 tests. Scores of each component are summed to produce a score ranging from 0 to 40, with high scores indicating more impaired motor skills and scores close to 0 normal skills. A psychologist performed the Griffiths Scales of Mental Development, a second psychologist performed the BAS, and a research nurse the Movement ABC tests. These assessments were blind to the child's neonatal course and progress.

Cranial injury. Cranial ultrasound was performed on the day of birth, and then at least weekly until discharge. Images were reviewed prospectively (N.M.). Hemorrhagic ultrasound findings were classified as subependymal, intraventricular, or intraparenchymal, intraventricular hemorrhage being graded as small or large (the latter where the clot distended the lateral ventricle). Severe hemorrhage was defined as a large intraventricular hemorrhage or an intraparenchymal hemorrhage. Cystic periventricular leukomalacia was defined as echo-lucent cysts of ≥ 2 mm diameter in the periventricular white matter; ventriculomegaly was present if the ventricular size on the last scan was $>97^{\text{th}}$ percentile (19). No children developed progressive posthemorrhagic hydrocephalus in this cohort. For analysis, cystic periventricular leukomalacia and ventriculomegaly were combined as PVL consistent with the view that most ventricular enlargement is due to WMD, either focal (cystic) or diffuse (20–22). The worst grade of findings was used in all cases.

Genotyping. DNA was extracted from routinely stored Guthrie cards and IL-6 genotypes determined as previously described (14) using the primers (5'-GGAGACGCCCTTG-AAGTAACTGC-3' and 5'-GAGTTTCCTCT-GACTCCATC-GCAG-3') to generate a 163 bp PCR fragment. Genotypes were resolved by using restriction endonuclease digestion. The rare -572 C allele lacks the digestion site for MblI. The size of the digestion products was determined by using microtiter array diagonal gel electrophoresis by two independent observers blinded to clinical details.

Statistical analysis. Outcomes were examined for association with IL-6 promoter polymorphism genotypes -572 CC+CG compared with GG, using SPSS for Windows v9.0 (SPSS Inc, Chicago, IL). Categorical data were analysed by chi-square or Fisher's exact test where appropriate. Mann-Whitney U was used for simple comparison of continuous data; data shown are median (interquartile range). Multiple linear regression was used *posthoc* to

control for potential confounders of outcome measures associated with genotype. A probability of $p < 0.05$ was considered statistically significant for examination of primary outcomes. Secondary analysis was performed to demonstrate any association between genotype, disability, and performance in subscales of the developmental tests. Adjustment for multiple comparison was made using the Bonferroni correction. A probability of $p < 0.005$ was considered statistically significant for secondary (subscale) analysis.

RESULTS

IL-6 genotype and follow-up to 51/2 y were available in 113 Caucasian patients. The study group [gestation, 30 wk (range, 22–32); birthweight, 1451 g (range, 670–2300); males, 68 (60%), maternal age, 27 y (16–39)] (see also Table 1) was representative of the original APIP population (15): severe intraventricular hemorrhage (IVH) occurred in 18 out of 308 (6%) patients in the APIP and in 7 patients in this study (6%), all GG genotype; PVL was found in 21 out of 308 (7%) patients in APIP and 12 out of 113 in this study group (10%). Antenatal corticosteroid use was 18% in this cohort (21 out of 113).

Genotype frequencies, CC 1 (1%), GC 9 (8%), GG 103 (91%), demonstrated Hardy-Weinberg equilibrium and were similar to UK population controls, suggesting no survival selection before enrolment (14). Median gestational age of infants with ≥ 1 C allele was lower than the comparison group (GG genotype): 29 (27–30) wk gestation and 31 (29–32) wk, respectively. All other demographic and neonatal variables were similar (Table 1).

There was no difference in the incidence of cranial ultrasound abnormalities across genotype groups: severe IVH occurred in 0 of the carriers of the C allele and in 7 (7%) of the GG genotype infants ($p = 0.91$), whereas WMD due to all PVL was observed in 1 (10%) of those with a C allele and 11 (11%) of those of GG genotype ($p = 0.99$) (Table 1). Cystic PVL occurred in 1 (10%) of those with the C allele compared with 4 (4%) in GG infants and ventriculomegaly was found on 0 (0%) and 7 (7%) occasions, respectively. Two (20%) of the infants with a C allele were born at <27 wk gestation compared with 7 (7%) of those with GG genotype ($p = 0.18$). Both of the infants <27 wk carrying a C

Table 1. Study group characteristics and genotype.

	-572 C allele ($n = 10$)	-572 GG ($n = 103$)
Male sex	6 (60%)	62 (60%)
Gestation (wk)*	29 (27–30)	31 (29–32)
Intrauterine growth restriction	1 (10%)	9 (10%)
5-min Apgar score <6	1 (10%)	9 (9%)
Prelabor rupture of membranes >24 h	2 (20%)	8 (9%)
Septicemia	4 (40%)	33 (32%)
Severe intraventricular hemorrhage	0 (0%)	7 (7%)
Periventricular leukomalacia	1 (10%)	11 (11%)
Oxygen dependence at 36 wk	0 (0%)	2 (2%)
Corticosteroids for chronic lung disease	1 (10%)	9 (9%)
Nonmanual parental occupations	8 (80%)	52 (51%)
Maternal age (y)*	27 (27–29)	26 (24–30)
Maternal car use	7 (70%)	69 (67%)
Mother educated beyond 16 y	5 (50%)	43 (42%)
Developmental support	5 (50%)	36 (35%)

Numbers in parentheses indicate interquartile range.

Continuous data comparison is by Mann-Whitney U . * $p = 0.33$.

allele had normal cranial ultrasounds throughout their clinical course and neither were disabled. The Griffiths DQ and the GCA for the two infants born at <27 wk and carrying a C allele were (DQ) 99.8 and 94.8 and (GCA) 81.0 and 92.0. No child carrying a C allele had a DQ or a GCA <70, compared with 1 infant (1%) of GG genotype who had a DQ of <70 and 3 (3%) who had a GCA <70.

The distributions of the developmental scales in this cohort were normal—Griffiths DQ: mean DQ, 101 (SD 11), median DQ, 101 (interquartile range, 94–110); GCA: mean GCA, 100 (SD 16), median GCA, 102 (interquartile range, 91–112); Movement ABC: mean summative score, 7 (SD 7), median summative score, 5 (interquartile range, 1–10). C allele carriage was associated with worse DQ at 2 y and GCA at 51/2y but not worse motor performance (2 y locomotor subscale DQ and Movement ABC)—DQ: C allele, 92.4 (89.9–96.6) *versus* 102.4 (94.4–110.6) for those with GG genotype ($p = 0.002$); GCA: C allele, 88.0 (80.3–102.8) *versus* GG 103.0 (92.0–112.0) ($p = 0.037$); Movement ABC summative score: C allele, 8.3 (6.6–20.3) *versus* 4.0 (1.0–9.5) ($p = 0.081$) (Table 1).

Gestational age itself was not predictive ($p < 0.05$) of a worse performance in the Griffiths or BAS in APIP or in this cohort, despite such description elsewhere (23). However, such trends exist within the original APIP data [effect size on DQ: +0.6 for each week of gestation, 95% confidence interval (CI) –0.3 to 1.6, $p = 0.20$] or GCA (effect size on GCA: +1.0 for each week of gestation, 95% CI –0.1 to 2.1, $p = 0.07$). Multiple linear regression (controlling for genotype and gestation for these reasons) demonstrated only an association between the C allele and a lower DQ or GCA (effect size of C allele on Griffiths DQ: –9.2, 95% CI –16.8 to –1.7, $p = 0.017$; effect size of C allele on GCA: –10.6, 95% CI –21.2 to –0.1, $p = 0.047$) but not gestational age (effect size on DQ: 0.0 for each week of gestation, 95% CI –1.1 to 1.0, $p = 0.97$; effect size on GCA: 0.5 for each week of gestation, 95% CI –1.0 to 2.0, $p = 0.55$). In the infants born at ≤ 30 wk gestation [$n = 56$ (8 C allele, 48 GG genotype)], developmental scores tended to be lower in infants with a C allele—Griffiths DQ: C allele, 92.4 (89.4–95.5) *versus* 102.4 (95.9–110.6) for those with GG genotype ($p = 0.002$); DQ adjusted for social variables (15): C allele, 93.3 (91.4–99.6) *versus* GG genotype 99.9 (94.8–105.0) ($p = 0.036$); GCA: C allele, 96.0 (81.0–106.6) *versus* GG genotype 103.5 (92.3–112.0) ($p = 0.22$).

DISCUSSION

We have demonstrated an association between IL-6 –572 C allele carriage and worse cognitive development after preterm birth. The numbers of patients carrying the relatively rare –572 C allele was small (10) and further confirmatory studies are clearly required, as are haplotype analyses in larger cohorts (24).

The lack of association between C allele carriage and ultrasound changes consistent with cystic PVL and ventriculomegaly may be due to the rarity of the C allele and the small number of patients with ultrasonographic evidence of white matter injury. The resolution of cranial ultrasonography is clearly inadequate to define all white matter injury (25), of which perhaps 50% of very preterm infants suffer to some extent. It is possible, therefore, that the link between the IL-6 –572 C allele and worse developmental performance at 2 and 5.5 y is due to IL-6-mediated white matter damage affecting subsequent gray matter development and its ongoing wiring

into childhood (11). Certainly, preterm infants with MRI evidence of white matter injury have reduced cortical gray matter volume (12). The mechanisms that injure the developing gray matter are, however, poorly elucidated and the same mechanisms that injure the white matter may directly affect gray matter. Nothing is known about constitutive IL-6 gene expression or IL-6 gene expression in response to ischemic, infective, or inflammatory signals within different areas of the preterm brain. It is therefore possible that gray matter injury and impaired cognitive development may occur independently of white matter damage and that different responses may be mediated by different IL-6 promoter polymorphisms.

Despite the association between C allele carriage and worse DQ and GCA, we did not demonstrate a statistically significant link between genotype and motor performance in the BAS or in the locomotor subscale of the Griffiths scales. There was, however, a clear trend toward worse overall performance in the ABC movement tests among C allele carriers ($p = 0.08$), which requires further exploration.

We also found an association between C allele carriage and lower gestational age in this cohort. It is possible that the –572 C allele is a risk factor for preterm birth, as other polymorphisms of pro-inflammatory cytokine and signalling molecules are also associated with preterm delivery (26,27). The greater degree of immaturity in those carrying a C allele does not appear to explain fully the impact upon cognitive outcome, however, as gestational age was not predictive of a lower DQ or GCA. Given the small numbers of patients within this cohort studied, the lack of association between gestational age and outcomes could be the result of a type 11 error. Thus, impaired cerebral development, or perhaps more correctly developmental performance, could also (or in addition) be driven by the C allele predisposing to preterm birth. However, the size of any effects of gestational age upon DQ and GCA appear to be small in comparison to that of genotype. Neither did CC+CG infants have more adverse risk factors than their GG counterparts (Table 1). Furthermore, none of those infants carrying the –572 C allele had severe IVH. Nor was there any difference in social or parental factors or developmental support (Table 2), and nor was this association driven by the poor performance of disabled infants carrying a –572 C allele (Table 2).

We have used a candidate gene association approach to investigate the role of IL-6 in cognitive development after premature birth. These data are consistent with IL-6 expression modulating cognitive development in the preterm infant. It is unlikely that basal IL-6 expression itself is responsible for this effect alone, but is due to the complex interactions of the many pro-inflammatory events to which the brain of a preterm infant is exposed, potentiated by an individual infant's pro-inflammatory response. This response may in turn be mediated by such polymorphism as the IL-6 –572 G to C substitution. Such environmental stimuli to IL-6 expression may include maternal infection, intrauterine infection, delivery mode, hypoxia, septicemia, and chronic lung disease (6,7,9,10,28–33). Because of the small numbers of patients in this preliminary study, use of two-by-four tables to try to dissect the gene-environment interactions, perhaps the gold standard for large epidemiologic genetic study (34), is not possible here. Because of the rarity of the C allele, the numbers of infants are necessarily small in this group, however, if robust, such find-

Table 2. Comparison of genotypes and developmental outcomes.

	–572 C allele (n = 10)	–572 GG (n = 103)	p
Griffiths Developmental Quotient	92.4 (89.9–96.6)	102.4 (94.4–110.6)	0.002
Griffiths DQ adjusted for social factors*	95.9 (91.7–100.4)	100.9 (96.7–104.8)	0.026
Subscale			
Locomotor	94.9 (90.6–97.4)	96.4 (92.8–100.0)	0.21
Personal-social	99.5 (94.2–109.5)	103.6 (97.5–116.9)	0.14
Hearing-speech	92.0 (80.5–96.5)	100.5 (89.6–117.6)	0.025
Eye-hand coordination	87.8 (85.4–92.2)	96.0 (97.5–103.9)	0.005
Performance	94.1 (89.3–101.5)	106.4 (98.9–116.7)	0.002
Any disability at 2 y	1 (10%)	11 (11%)	0.97
General cognitive ability	88.0 (80.3–102.8)	103.0 (92.0–112.0)	0.037
Verbal reasoning	98.0 (86.3–107.3)	105.0 (94.0–114.0)	0.21
Pictorial reasoning	90.0 (80.5–113.3)	101.0 (92.0–112.0)	0.12
Spacial reasoning	85.0 (77.0–95.0)	100.0 (93.0–107.0)	0.013
Movement ABC (summative score)	8.3 (6.6–20.3)	4.0 (1.0–9.5)	0.081

Continuous data comparison is by Mann-Whitney U.

Data given as median (interquartile range).

* (APIP, 15)

ings might contribute to risk analysis among preterm infants and suggest a potential role for antiinflammatory strategies in management of the preterm child (35).

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