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The Value of Neuron-specific Enolase and Cytokines from Umbilical Blood in Cytomegalovirus Infected Fetuses

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Abstract

Objective: We set out to establish a prognostic indicator for Cytomegalovirus (CMV) infection and determine the extent of CMV invasion.

Design: For five cases of fetal CMV infection treated at our facility, we measured the levels of NSE, S-100β, and GFAP, and we conducted a comprehensive analysis of cytokines to compare with the clinical course.

Results: NSE for cases 1 and 4 increased significantly from 10 to 78 ng/ml, and from 15.0 to 30.0 ng/ml, respectively. On the other hand, NSE in Case 3 showed a major decrease from 220.0 to 9.6 ng/ml, while that of Case 2 remained in the normal range and that of Case 5 fluctuated slightly from 13 to 15 ng/ml. In all cases, almost no changes in S-100β and GFAP were observed during the course of treatment. Conversely, our comprehensive analysis detected no expression of cytokines, such as TNF-α and IL-2. But IP-10, a monocytic chemotactic factor, revealed a tendency for high levels; 766.2 ± 716.1 pg/ml vs 155.0 ± 104.1 pg/ml (mean ± S.D.) (p=0.077).

Conclusion: Our results suggest that an increased NSE level with fetal CMV infection indicates nerve damage associated with CMV. Moreover, the absence of inflammatory cytokines suggests an immature cell-mediated immune response in the infected fetus.

Keywords: Inflammatory cytokines; CMV infection

Introduction

Prenatal cytomegalovirus (CMV) infection, one of many maternal infectious diseases, is seen in 0.2 to 2.0% of all pregnancies [1]. CMV infection in approximately 10% of infants infected prenatally becomes severe, and 35% of infected infants present with neurologic sequelae, such as hearing loss and developmental disabilities after birth and are often refractory to treatment [2]. Moreover, even 10 to 15% of the asymptomatic infants progressively experience hearing loss and developmental disorders [3-5]. Li shows that undifferentiated neurocytes in the ventricular zone (VZ) of the mouse brain are highly sensitive to CMV [6]. The undifferentiated neuroepithelium of the VZ ventricular wall includes neural stem cells, which mediate not only self-replication, but also differentiation of neurons and glial cells [7,8]. It has been shown that when CMV infects undifferentiated neurons, along with reduced self-renewal capacity, it also inhibits the differentiation of glial cells into neurocytes [9]. Although the in vivo infection dynamics of CMV in humans can only be conjectured on the basis of autopsy results [10], the glial cells of the ventricular wall in the brains of people infected with congenital CMV are known to be susceptible to viral infection, and it is possible that brain developmental disabilities are caused by the resulting suppression of proliferation and differentiation. Accordingly, early treatment is necessary when fetal CMV infection is detected, and if possible, prenatal treatment is preferable.

In recent years, administration of immunoglobulin injection into the fetal abdominal cavity (IFAC) has been reported as a prenatal treatment for congenital CMV [11-13]. If consistent treatment throughout the fetal and neonatal periods can be established by determining treatment result indicators, the introduction of treatment, suspension and eventual establishment of a prognosis may be possible.

In this study, we used fetal umbilical cord venous blood infected with CMV to establish the extent of fetal invasion and prognostic indicators in CMV infection.

Materials and Methods

From January 2006 to December 2010 at the National Defense Medical College, the cordcentesis was conducted on five patients with fetal CMV infection between gestational weeks 27 and 35. Diagnosis of fetal CMV infection was made when all of the following conditions were met: amniotic CMV-DNA was identified by real time PCR in cases which fetal growth restriction exceeded -1.5 S.D., fetal ascites and hepato-splenomegaly were detected by ultrasound. After giving an explanation and obtaining consent from the five patients, fetal treatment was conducted by intra-peritoneal administration of 2 g immunoglobulin per 1 kg body weight and the efficacy of treatment was evaluated by collecting approximately 5 ml venous blood.

Biochemical tests

The CMV DNA viral load was quantified using real-time polymerase chain reaction (PCR). Platelet count was performed and the levels of gamma-glutamyl transpeptidase (γGTP) and neuron specific enolase (NSE) were measured using a radioimmunoassay. S-100β and glial fibrillary acidic protein (GFAP) levels were determined by ELISA.

Immunological tests

Using data from the three cases CMV infection and two normal cases collected during the same period, the levels of 27 cytokines were measured with a multiplex system (Bio-Rad, Hercules, CA). To compare the two groups, the Wicoxon signed rank sum test was performed using JMP 8.0 (2008; SAS Institute Inc., Cary, NC). Statistical significance was

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defined as $p < 0.05$. This study was approved by The National Defense Medical College Hospital Ethics Committee, and written consent was obtained from the patients prior to including them in the study.

Results

Biochemical tests

The changes in levels are summarized in Figure 1.

Case 1: γGTP levels gradually decreased from 701 to 621 IU/l.

Platelet count decreased from 1.4×104 to $0.7 \times 10^4/\mu l$ and NSE levels increased markedly from 10 to 78 ng/ml.

Case 2: CMV-DNA levels increased slightly from 2.5×102 to 4.4×102 copies/ml. Although the γGTP levels increased from 570 to 855 IU/l, the platelet count stayed almost the same at $1.9 \times 106/\mu l$. NSE levels did not change significantly and remained in the range of 5.9 to 5.2 ng/ml.

Case 3: CMV-DNA levels decreased from 4.9×103 to 2.4×103
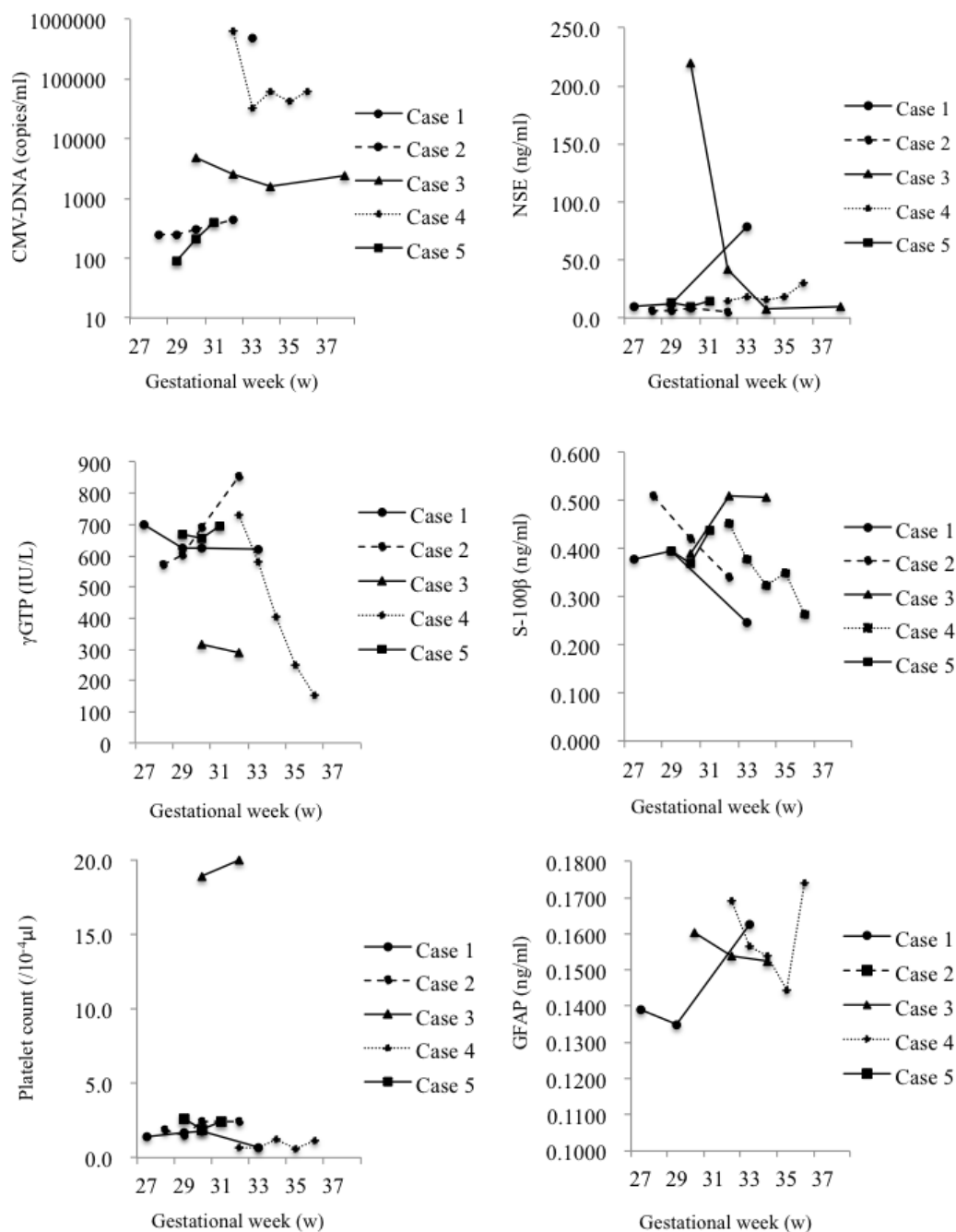

Figure 1: NSE increased significantly for cases 1 and 4, but in Case 3 showed a major decrease in Case 3. In all cases, almost no changes in S-100β and GFAP were observed during the course of treatment.

	Gestational week	PDGF-bla {peal}	IL-lill(pginal)	1L-1 ra (pgital)	IL-2 (pginal)	IL-4 (p1itn1)	IL-5 {pgitn1)	IL-6 (p6/tn1)	IL-7 (ps/snl)	IL-8 (p6/tn1)	1L-9 (pWral)	IL-10 (pgirn1)	IL-12 (pgical)	LL-13 (pg.rail)	1L-15 (pginal)
CMV 1	27-5	50.9	n.d.	74.5	n.d.	n.d.	n.d.	n.d.	n.d.	26.0	n.d.	n.i	n.i	n.d.	n.d_
	29-2	28.9	n.d.	153.3	n.d.	ad.	n.d.	19.1	n.d.	33.6	n.d.	n.i	n.i	n.d.	n.d_
	334	617	n.d.	26.5	n.d.	ad.	n.d.	n.d.	nit	23.6	n.d.	rid_	n.i	rid_	rid_
CMV 2	28-3	98_5	n.d.	n.d.	n.d.	ad.	n.d.	n.d.	nit	n.d.	n.d.	rid_	n.i	rid_	rid_
	30-6	2617	n.d.	9.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.i	n.d.	n.d_
	32-6	106.6	n.d.	n.d.	n.d.	ad.	n.d.	n.d.	n.d.	n.d.	n.d.	n.i	n.i	n.d.	n.d_
CV1V 3	30-2	201.1	n.d.	n.d.	n.d.	ad.	n.d.	n.d.	n.d.	n.d.	n.d.	rid_	n.i	rid_	rid_
	32-0	1079A	n.d.	11.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.i	n.d.	n.d_
	34-1	124.5	n.d.	8.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.i	n.i	n.d.	n.d.	n.d_
Normal 1	33-6	1712	n.d.	60.6	n.d.	n.d.	n.d.	n.d.	n.d.	651.0	n.d.	n.d.	12.4	n.d.	n.d_
Nornaal 2	34-1	258_6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40.9	rid_	rid_	n.d.	rid_	nil_

	Gestational week	1L-17 (pgintl)	Eotaxin (pglinl)	FOE (pginal)	G-CSF (pVial) GM-CSF (pWrial)	IFNI (pWrial)	IP-I0 (pgind)	MCP-1 (pgind)	M1P-1a (pglin.1)	IVI1P- 1 b (pgiffil) RANTES (pgiffil)	TNF-ct (pgical)	VEGF (pg1m1)
CM V 1	27-5	n_d_	27.2	58.2	n.d.	134.5	9.1	1377.1	401.9	rid_	305.6	1061.1	rid_	18.4
	29-2	n.d_	15.9	61.3	n.d.	70.4	n.d.	1284.7	540.1	n.d.	339.1	458.4	n.d.	10.8
	33-1	n.d.	n.d.	25.7	n.d.	74.0	5.1	2258.0	57.8	n.d.	330.1	453.1	n.d.	n.d.
C_W2	28-3	n.d.	n.d.	67.6	n.d.	63.9	0.7	456.9	394.8	n.d.	185.4	1639.1	n.d.	142
	30-6	n.d.	rut	98.0	n.d.	64.6	7.1	532.1	452.3	n.d.	259_7	2035_1	n.d.	16.4
	32-6	nil	n_d.	54.4	n.d.	71.8	19.8	269.1	241.3	n.d.	156.1	17653.7	n.d.	10.0
CM V 3	30-2	nisi	n.d.	44.7	n.d.	116.7	10.9	162.9	246.6	n.d.	126.0	18343.7	n.d.	222
	32-0	n.d_	n.d_	53.9	n.d.	107.5	31.4	265.1	186.7	rid.	1892	n.d.	n.d.	18.3
	34-1	mil_	n_d_	38.9	n.d.	85.7	14.6	269.7	9115	n.d.	126.5	34900	n.d.	12.3
Normal 1	33-6	15	n.d_	122.4	n.d.	159.4	71.0	228.6	185.6	n.d.	426.6	n.d.	n.d.	245.4
Normal 2	34-1	n_d.	n_d_	55.2	n.d.	97.5	19.8	61.4	270.11	n.d.	81.2	7761.6	n.d.	29.4

n_d_ I not detected.

Table 1: Anasysis of cytokine in umbilical vein for congenital CMV infected fetuses. Ahuost no inflammatory cytokines, such as 1L-2, IL-6,1L- 8, 1L-10, and TNF-a, were detected in either group.

copies/ml. There were almost no changes in γGTP (315 to 289 IU/l) levels or platelet count (1.9×10^5 to 2.0×10^5/μl). NSE levels showed a major decrease from 220.0 to 9.6 ng/ml.

Case 4: CMV-DNA decreased to ~10% from 6.3×10^5 to 6.0×10^4 copies/ml. γGTP levels decreased markedly, but remained in the normal range from 731 to 154 IU/l. The change in platelet count from 0.7×10^4 to 1.1×10^4/μl was less significant. NSE levels increased from 15 to 30 ng/ml.

Case 5: CMV-DNA levels increased slightly from 9.1×10^1 to 4.0×10^2 copies/ml. γGTP levels increased from 668 to 693 IU/l, and platelet count decreased from 2.6×10^4 to 2.4×10^4/μl, which was not significant. NSE levels did not change significantly and remained in the range of 13 to 15 ng/ml.

Only slight changes of S-100β and GFAP levels were observed in the patients during the course of treatment.

Immunological tests

Table 1 and Figure 2 show the comprehensive analysis of cytokines, such as IL-2, IL-6, IL- 8, IL-10 and TNF-α in umbilical cord venous blood. Almost no inflammatory cytokines were detected in either group. Conversely, the values of the monocyte chemocyte factor known
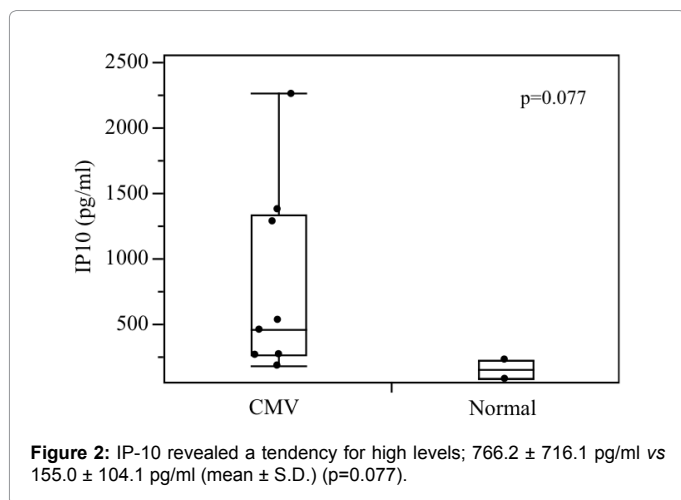
as gamma interferon-inducible protein 10 (IP10) showed a trend for higher (766.2 ± 716.1 pg/ml vs 155.0 ± 104.1 pg/ml, mean ± S.D., p=0.077), and tended to decrease in line with fetal therapy.

Discussion

Extent of fetal CMV invasion

Cytomegalic inclusion disease (CID) is defined as a CMV infection that results in underdevelopment and microcephaly, including at least one of the following: jaundice, hepato-splenomegaly, thrombocytopenia, petechiae or seizures [14-17]. Although CMV infection is diagnosed when detected in amniotic fluid by PCR, there is no relationship between the CMV gene dosage in the amniotic fluid and neonatal prognosis [18]. The CMV-DNA level in umbilical cord venous blood was used to diagnose the presence of the viral infection; however, it did not appear to be a suitable indicator of therapeutic effect.

On the other hand, because impaired liver function is inevitable with fetal CMV infection, γGTP was proposed as a possible indicator of fetal treatment efficacy. γGTP contributes to the movement of amino acids through the cell membrane and exists *in vivo* mostly as a membrane-bound enzyme. In humans, γGTP is most active in the kidney, and it is also distributed in the pancreas, liver, small intestine and testes. The microsomal fraction of hepatocytes is produced in the

Figure 2: IP-10 revealed a tendency for high levels; 766.2 ± 716.1 pg/ml *vs* 155.0 ± 104.1 pg/ml (mean ± S.D.) (p=0.077).

liver, and although their function is to move to the membrane of cells in structures, such as the bile canaliculi, liver and bile system disorders cause enzyme deviation and cause them to enter the circulatory system. As a result, γGTP is generally used as an indicator of liver function.

Moreover, because fetal platelet production occurs in not only bone marrow but also liver, a decrease of fetal platelet count is believed to indicate impaired liver function. Because fetal therapy using immunoglobulin is not considered a radical therapy in the treatment of CMV, restoration of normal levels is difficult, even when treatment is administered. However, the associated risk of intra-cerebral hemorrhage, which contributes greatly to selecting a delivery method, is reason enough to measure platelet count; this is consistent with fetal thrombocytopenia being considered an indirect indicator of liver function impairment.

Benoist et al. [19] reported that prognosis correlates strongly with ultrasound findings and platelet values, rather than with the levels of AST, ALT and γGTP, and the amount of CMV virus in infected infants. Rivera et al. [20] reported a correlation between symptoms at birth and ALT elevation with a neonatal platelet count <100,000/μl. Liesnard et al. [21] indicated that in 29 cases of CMV-infected infants, thrombocytopenia was observed in two cases, both of which had shown ultrasound anomalies *in utero*. Azam et al. [22] reported that in eight cases of terminated pregnancies, thrombocytopenia was observed in four cases, three of which had shown ultrasound anomalies in utero.

In addition to these reports, measuring the γGTP level and platelet count in umbilical cord venous blood to determine the extent of CMV invasion and treatment efficacy among infants displaying fetal CID can also be potentially useful for establishing a treatment plan.

Search for fetal nerve damage markers due to CMV

We tried to establish objective indicators of the extent of CMV nerve damage during fetal life. Reports on adult patients with brain trauma indicate the benefits of measuring serum S-100β, NSE and GFAP values for determining the extent of early nerve damage, as well as the benefits of tracking expected prognosis [23]. In addition, elevated serum S-100β and NSE levels have been reported in pathological conditions, such as stroke and myocardial infarction [24-28]. Moritz et al. [29] reported that only serum S-100β levels with subarachnoid hemorrhaging in adults are associated with post-disorder prognosis. Conversely, with normal-pressure hydrocephalus and dementia, cerebral infarction causes elevated cerebrospinal fluid GFAP levels [30-32]. Furthermore, Herrmann et al. [33] reported that serum GFAP values, following

cerebral infarction correlate with the volume of the infarct area and neurological damage. While S-100β is a family of calcium-binding proteins found in astroglial, oligodendroglial and Schwann cells, it is also secreted by cutaneous and intramuscular nerve tissue [34,35]. Conversely, enolase is a glycolytic enzyme with three subunits (α, β and γ) and five isozymes (αα, ββ, γγ, αβ, and αγ). Because the γγ and αγ types of enolase exist in the cytoplasm of nerve cells, they are called NSE. Normal levels of serum NSE are <10 ng/ml. GFAP, on the other hand, is a protein constituting the microstructures of glial cells.

Accordingly, we assumed that GFAP, S-100β and NSE might exert an influence on CMV infection. Umbilical blood levels of S-100β and GFAP showed no change before and after treatment. On the other hand, NSE values were abnormally elevated prior to treatment and tended to decrease after treatment. Congenital CMV infection is a disease causing neurodevelopmental disorders and the association of nerve-related proteins, such as S-100β, NSE and GFAP with long-term infant neurological outcome should be further analyzed.

Influence of CMV infection on the fetal immune system

Almost no inflammatory cytokines were detected in either group, suggesting that the cell-mediated immune response is not yet mature at around 30 weeks of gestation. CMV has high genetic homology and is a MHC class I histocompatibility antigen, which may mitigate or inhibit the response of killer T cells and natural killer cells in infected cells [36]. In cases of CMV infection, the insufficient activation of T cells and natural killer cells and the insufficient production of more specific antibodies may result in a more severe fetal infection. Therefore, if CID symptoms start to appear, early treatment, including fetal treatment, is desirable. Furthermore, although IP-10 levels tended to be high in CMV infection, we believe that treatment with immunoglobulin potentially mitigated the inflammation due to CMV because a decrease in IP-10 levels with fetal treatment was observed.

Conclusion

The increase of NSE level with fetal CMV infection indicates nerve damage associated with CMV. Moreover, the absence of inflammatory cytokines shows an immature cell-mediated immune response in the infected fetus.

Disclosure

All authors have no financial and other relationships that lead to any conflicts of interest.

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