Association Between Levels of TNF-α and TNF-α Promoter –308 A/A Polymorphism in Children with Kawasaki Disease

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Background and Purpose: Tumor necrosis factor-alpha (TNF-α) has been shown to play a central role in the pathogenesis of vasculitis in Kawasaki disease (KD). We investigated the serum levels of TNF-α and soluble TNF receptor 1 (STNFR1) levels, and genetic polymorphisms of the TNF-α promoter gene in children with KD to delineate the genetic basis of KD.
Methods: A total of 18 children (12 boys and 6 girls) with KD were studied, 9 of whom had the complication of coronary artery lesion (CAL) within 30 days after the onset of symptoms. Serum levels of TNF-α and STNFR1 were assayed by enzyme-linked immnosorbent assay, and DNA polymorphisms of the 5' flanking region of TNF-α promoter gene at position –308 [guanine (G) to adenine (A)] and –238 (G to A) were studied by direct nucleotide sequencing.
Results: The serum TNF-α level in KD patients was 113 ± 209.9 pg/mL (range, 2.0 to 756.9 pg/mL; median, 24.7 pg/mL; normal, < 10 pg/mL). The serum levels of STNFR1 in KD (4255 ± 2425 pg/mL) were higher than those of the control group (160 ± 116 pg/mL). Allele frequencies of –308A and –238A were 11.1% and 0% in the KD patients, and 0% and 3.1% in the control group. Neither TNF-α promoter polymorphism nor any significant risk factor for CAL was identified in KD patients. One patient, who was homozygous for –308A, showed the highest TNF-α level and elevated STNFR1 level but had no evidence of CAL. Positive correlations were found between serum levels of STNFR1 and C-reactive protein ($r = 0.731$, $p = 0.007$), and between STNFR1 and leukocyte counts at admission ($r = 0.620$, $p = 0.008$).
Conclusions: Increased serum levels of TNF-α and STNFR1 were found in KD patients but there was no correlation between these levels. The relationship between the pathogenesis of KD and TNF-α gene promoter –308G to A mutation towards cytokine production remains to be clarified.

Key words: Mucocutaneous lymph node syndrome; Tumor necrosis factor; Polymorphism (genetics)

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Kawasaki disease (KD) is an acute febrile vasculitis of childhood that is accompanied by the development of coronary artery lesions (CAL) in 15 to 29% of patients.[1] The etiology of KD and the mechanisms involved in the development of CAL are unknown. Previous studies have supported a central role of tumor necrosis factor-alpha (TNF-α) in the pathogenesis of KD.[2–4] These results, along with other studies showing the involvement of TNF-α in the pathogenesis of severe infectious disease,[5,6] suggest that the genetic propensity of the host to produce TNF-α may be related to the onset and severity of KD.

Two guanine (G) to adenine (A) transition polymorphisms at nucleotide positions –308 (G to A, termed –308A) and –238 (G to A, –238A) in the TNF-α gene promoter have been reported to be involved in

the susceptibility to and outcome of some infectious[7] and autoimmune diseases.[8] However, the relationship between TNF-α gene promoter polymorphism and TNF-α production is unclear.

Previous studies have shown that soluble TNF receptor (STNFR) is the natural homeostatic regulator of TNF-α activity, and may reflect the true biologic activity of TNF-α more closely than serum TNF-α level.[9] TNF-α exerts these biological effects by interacting with 2 distinct receptors, STNFR1 (p60 STNFR or CD 120a) and STNFR2 (p80 STNFR or CD 120b).[10] The p60 receptors are expressed in virtually all mammalian cell types, whereas the p80 receptor is expressed only in cells of the immune system and endothelial cells.[10] Furukawa et al found that the shedding of p60 STNFR into the circulation increases during acute KD and

that p60 STNFR levels in serum are an immunologic marker for the severity of vascular damage.[9]

To elucidate the genetic background of KD, we investigated a possible role of serum TNF-α level, STNFR1 level, and the existence of 2 genetic polymorphisms of TNF-α promoter gene, −308G→A and −238G→A, in the pathogenesis of KD.

Methods

Subjects

We recruited 18 patients with KD who were admitted to National Taiwan University Hospital during the period from July 1, 2000 to June 30, 2001. Diagnosis of KD was based on the criteria of the Japanese Kawasaki Disease Research Committee.[11] After admission to the hospital, the patients received intravenous infusions of immunoglobulin at a dosage of 1 g/kg/day for 2 consecutive days. The day of onset of fever was considered the first day of illness. Blood samples were collected for determining serum levels of TNF-α, STNFR1 and DNA polymorphism on days 5 through 25 (mean ± SD, 6.7 ± 4.8) of illness and before treatment, which was defined as the acute stage. Two-dimensional echocardiography was done after admission and 30 days after the onset of KD to detect CAL. Coronary arteries with diameters of 4 mm or greater were regarded as having CAL, according to the criteria of the Japanese Kawasaki Disease Research Committee.[11] Sixteen healthy unrelated Taiwanese children served as control subjects during the study.

DNA analysis

Informed consent for genetic analysis was obtained from the parents of all 18 patients. Genomic DNA was isolated from peripheral blood leukocytes.[12] The frequency of −308A in the patients was determined as described previously.[13] Primers TNF1A

(5'-AAGGAAACAGACCACAGACCTG-3') and TNF1B (5'-GGATACCCCTCACACTCCCC-3') were used to amplify a 167 bp DNA fragment in the 5' flanking region of the TNF-α gene from position −371 to −205 at an annealing temperature of 62° C. The presence of polymorphisms at position −308 and −238 in the products was analyzed using direct sequencing.

Assay for TNF-α and STNFR1

TNF-α levels in sera were determined with a TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, San Diego, USA). STNFR1 levels in sera were determined with a soluble STNFR1 ELISA kit (R&D Systems, Minneapolis, USA).

Statistical analysis

Statistical analysis was performed using Mann-Whitney U test, Pearson's correlation, and Kruskal-Wallis test. All values are expressed as mean ± 1 standard deviation (range). A value of $p < 0.05$ was considered significant. The following potential risk factors for patients demonstrating a CAL were investigated: age; gender; duration of fever before intravenous immunoglobulin treatment; STNFR1 level; TNF-α level; TNF-α gene promoter genotype; and laboratory data at the acute stage including C-reactive protein (CRP), leukocyte count, and platelet count.

Results

A total of 18 patients (12 boys and 6 girls), aged 2 months to 7.5 years (mean, 2.1 years) were included. Serum TNF-α levels were measured during the acute stage before treatment in 17 of the 18 patients.

During the 30 days after the onset of KD, 9 patients were found to have CAL. The clinical laboratory data are summarized in Table 1. The serum TNF-α level in KD patients was 113 ± 209.9 pg/mL (range, 2.0 to

Table 1. Demographic and clinical characteristics of Kawasaki disease patients with and without coronary artery lesions (CAL).

Variable*	Total (n = 18)	CAL (n = 9)	No CAL (n = 9)
Age (years)	2.1 ± 2.1 (0.2–7.5)	1.5 ± 1.6 (0.2–5.0)	2.7 ± 2.5 (0.6–7.5)
Gender (male/female)	12/6	5/4	7/2
Fever duration before IVIG use (days)	6.7 ± 4.8 (5–25)	7.7 ± 6.7 (5–25)	5.7 ± 1.4 (5–8)
Time to fever subsidance (days)	9.7 ± 5.2 (6–27)	10.9 ± 7.2 (6–28)	8.5 ± 1.8 (6–11)
CRP (mg/dL)	10.5 ± 7.7 (1.3–27.9)	7.5 ± 4.2 (2.4–12.0)	13.5 ± 9.6 (1.3–27.9)
WBC count (cells/μL)	15,495 ± 6170 (5990–27,920)	17,320 ± 6039 (6350–27,920)	13,441 ± 6020 (5990–22,900)
Platelet count (1000/μL)	332 ± 71 (204–441)	342 ± 67 (236–441)	319 ± 79 (204–401)
AST (U/L)	38 ± 24 (14–99)	32 ± 21 (14–64)	43 ± 27 (20–99)
TNF-α level (pg/mL)	113 ± 209.9 (2.0–756.9)	59.7 ± 122.0 (2.0–358.9)	160.9 ± 264 (3.0–756.9)
STNFR1 level (pg/mL)	4255 ± 2425 (1075–10,485)	4477 ± 2910 (1075–10,485)	4032 ± 1980 (1775–7020)
Allele frequency for TNF-α −308A (%)	11.1	5.6	16.7
Allele frequency for TNF-α −238A (%)	0	0	0

* Except for gender and allele frequencies, data are mean ± SD (range).
IVIG = intravenous immunoglobulin; CRP = C-reactive protein; WBC = white blood cell; AST = aspartate aminotransferase; TNF-α = tumor necrosis factor-alpha; STNFR1 = soluble tumor necrosis factor receptor 1.

Table 2. Relationship between tumor necrosis factor (TNF)-α promoter −308 genotype and TNF-α levels in patients with Kawasaki disease.

TNF-α promoter −308 genotype	TNF-α level (pg/mL)
G/G [n = 14; mean ± SD (range)]	81 ±141 (2–449)
G/A [n = 2; mean ± SD (range)]	15 ±13 (6–25)
A/A (n = 1)	757

756.9 pg/mL; median, 24.7 pg/mL; normal, < 10 pg/mL). Levels of STNFR1 in serum increased during the acute stage of KD (4255 ± 2425 pg/mL, range, 1075 to 10,485 pg/mL), as compared with controls (160 ± 116 pg/mL; range, 70 to 363 pg/mL) [$p < 0.001$]. During the acute stage, levels of TNF-α and STNFR1 were not significantly different in patients with KD with and without CAL ($p = 0.931$ and 0.236, respectively). No risk factor was found to predict CAL in KD patients. A positive correlation was found between serum levels of STNFR1 and CRP (n = 12; r = 0.731, $p = 0.007$); and between STNFR1 and leukocyte counts at admission (n = 17; r = 0.620, $p = 0.008$) [Fig. A and B]. One patient, who was homozygous for −308A, showed elevated TNF-α levels and elevated STNFR1 levels, but did not have CAL. The relationship between TNF-α levels and polymorphisms of −308A are listed in Table 2 ($p = 0.199$). However, no positive correlation was found between levels of STNFR1 and levels of TNF-α (Fig. C), platelet count at acute stage, or polymorphisms of −308A.

The allele frequencies of −308A and −238A in the 18 KD patients were 11.1% and 0%, respectively (controls, 0% and 3.1%). The genotype of −308A/A, A/G, and G/G was found in 5.6%, 11.1%, and 83.3% of KD patients, respectively (control G/G, 100%). Allele frequencies in any polymorphic positions tested in KD individuals were not significantly different from those in healthy Taiwanese subjects.

Discussion

This study demonstrated that serum levels of TNF-α in KD patients were higher than normal, as has been previously reported.[2–4] Polymorphisms in the promoter/enhancer region of the TNF-α gene have been shown to be candidates for regulating TNF-α production, because a genetic disposition towards overproduction of TNF-α in response to bacterial products was observed in the peripheral blood mononuclear cells of children with a history of KD and CAL.[14] In this study, the allele frequency for −308A and −238A was not different from that in healthy controls. One patient, who was homozygous for −308A, showed the highest TNF-α level and elevated STNFR1 level but not CAL. The homozygous presence of the
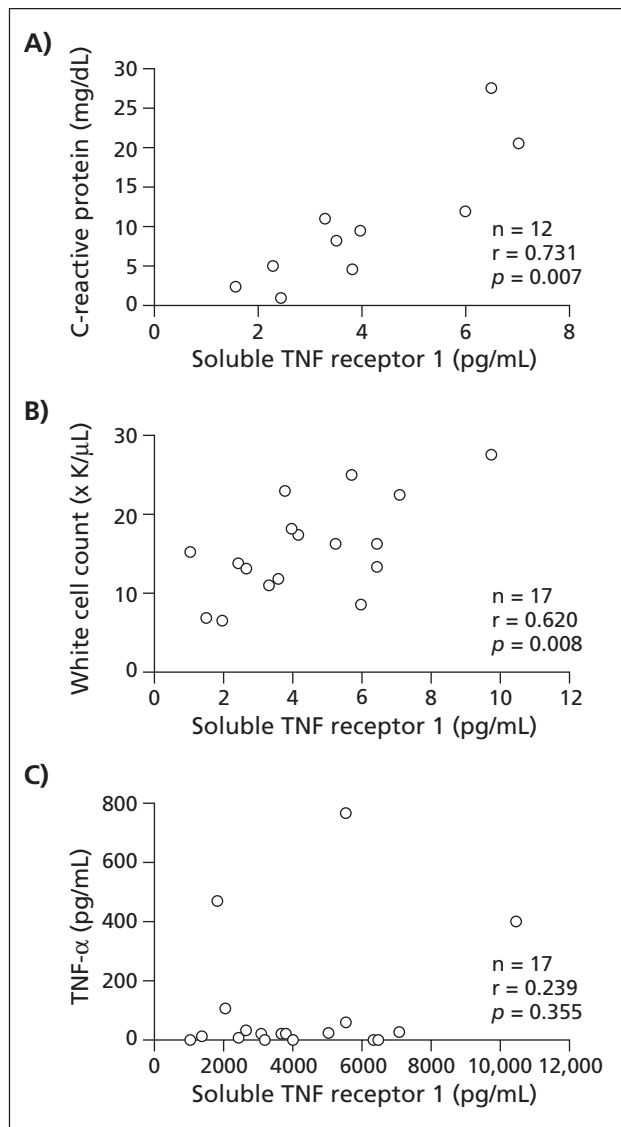
Fig. Pattern of soluble tumor necrosis factor receptor 1 (STNFR1) level, TNF-α, white cell count, and C-reactive protein. Positive correlations were found between: A) serum levels of STNFR1 and C-reactive protein (r = 0.731, p = 0.007); and B) STNFR1 and leukocyte counts at admission (r = 0.620, $p = 0.008$); but not between C) STNFR1 level and TNF-α levels.

−308A mutation has been found to increase TNF-α production 6- to 7-fold.[15,16] It appears that while the −308 polymorphism may not influence susceptibility to autoimmune disease, it may influence the severity of infectious disease.[17] Since in our study, only 1 case had the homozygous −308A mutation, further studies are needed to clarify the relationship between the pathogenesis of KD and genetic disposition towards cytokine production in response to bacterial products.

The results of this study as well as previous studies have shown that STNFR levels increase in patients with KD, severe meningococcemia, and rheumatic disease.[9,18,19] We also demonstrated that these patients

have elevated TNF-α levels. No correlation, however, was found between levels of TNF-α and STNFR1 in this study. Since previous studies have shown that STNFRs are the natural homeostatic regulators of TNF-α action, increased levels of STNFR1 in KD in the absence of a comparable increase of TNF-α levels in this study could be explained as suggesting a non-representative role of TNF-α in the pathogenesis of KD or that it is a consequence and not the cause of the inflammatory process. Further study is needed to test such hypotheses.

In this study, 2 positive correlations were found, between serum levels of STNFR1 and CRP levels, and between STNFR1 and leukocyte counts during the acute stage of KD, but there was no significant difference in serum CRP levels or leukocyte counts between patients with and without CAL. There are no known laboratory or clinical parameters which can accurately predict the development of CAL.[9] Although Furukawa et al suggested that p60 STNFR levels in serum are an immunologic marker for the severity of vascular damage such as CAL,[9] and Kamizono et al observed that the peripheral blood mononuclear cells of KD patients with coronary artery lesions produced slightly higher levels of TNF-α in response to the bacterial products,[14] our study found no difference in serum STNFR1 levels or TNF-α between patients with CAL and those without CAL. Possible reasons for this finding might be the small case number or the instability of TNF-α despite our extremely careful handling of the samples.

In conclusion, the results of this study do not suggest that STNFR1 levels in serum might be a better predictor of the development of CAL than other acute-phase reactants, although further confirmation of this finding is needed due to the small case number. The roles of overproduction of TNF-α or STNFR1 in the pathogenesis of CAL in KD patients need to be further clarified.

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