Sequence Analysis of the Tumor Necrosis Factor Gene in Pediatric Patients with Autoimmunity

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ABSTRACT

Tumor necrosis factor- α (TNF) is a multifunctional protein hormone that contributes to host defense and perinatal immunologic development. Dysregulated TNF production, however, occurs during the pathogenesis of autoimmune diseases and may be inherent to their development. In animal models of autoimmunity, dysregulated TNF synthesis has resulted from mutations in TNF gene regulatory sequences, specifically those sequences involved in translational control of TNF gene expression. In this study, we have determined whether mutations in the TNF translational control sequences are present in pediatric patients with type I diabetes mellitus and connective tissue diseases. Blood samples were collected from 48 patients with connective tissue diseases, 32 patients with diabetes, and 29 controls. A 250-bp fragment of the translational control sequences present in the

TNF 3'-untranslated region was amplified by the polymerase chain reaction, sequenced, and analyzed relative to the published TNF sequence. In this study, all patients and controls exhibited the normal sequence, with no insertions or deletions in the translational control motifs. We conclude that polymorphisms in the TNF 3'-untranslated region occur infrequently, if at all, in patients with diseases examined here. (*Pediatr Res* 37: 165-168, 1995)

Abbreviations

TNF, tumor necrosis factor- α
3'-UTR, 3' untranslated region
RA, rheumatoid arthritis
PCR, polymerase chain reaction

TNF is a pluripotent cytokine, encoded in the MHC, which mediates inflammatory responses (1-5). Appropriately regulated, TNF biosynthesis is critically important to host defense (6) and immune ontogeny (7, 8). However, dysregulated TNF production may lead to the development of autoimmunity, as evidenced by recent data from animal models and patients. In type I diabetes mellitus, TNF potentiates IL-1-mediated islet cell toxicity *in vitro* and enhances interferon-induced HLA class II gene expression on islet cells (9, 10). Furthermore, overexpression of TNF in the pancreatic islets of transgenic mice results in chronic lymphocytic insulinitis (11, 12). Dysregulated TNF production has also been implicated in the pathogenesis of RA. Elevated TNF levels are present in the joint fluid of RA patients (13) and may promote cartilage breakdown by chondrocytes and bone resorption by osteoclasts (14, 15).

Given these associations, several investigators have speculated that mutations within the TNF gene might account for the MHC-linked components of diabetes, RA, and other autoimmune diseases (16, 17). Indeed, interindividual variations in TNF secretory capacity have recently been correlated with HLA haplotypes, restriction fragment length polymorphisms,

and TNF microsatellites (18-20). Presumably, these genetic markers reflect the existence of TNF alleles with mutations in the regulatory regions of the TNF gene. Among these regulatory regions is the TNF 3'-UTR, which powerfully regulates TNF expression at the level of translation (3, 21, 22). Evolutionarily conserved motifs ("TTATTTAT" sequences) in the TNF 3'-UTR were shown to have two functions: to shorten the TNF mRNA half-life (23) and to suppress translation of TNF mRNA during normal conditions (24). When Keffer *et al.* (25) replaced these translational regulatory sequences with irrelevant sequences from the 3'-UTR of the β -globin gene, the resulting transgenic mice displayed aberrant TNF production and developed chronic inflammatory polyarthritis. Recently, insertional mutations were found in the TNF 3'-UTR "TTATTAT" sequences of New Zealand White mice, which are known to bear an MHC-linked gene implicated in the development of murine systemic lupus erythematosus (26). These data further suggest that polymorphisms in the TNF translational control region may be associated with the development of autoimmunity in certain controlled systems.

Inasmuch as the TNF 3'-UTR is an important regulator of TNF biosynthesis and is associated with autoimmune conditions in animal models, we undertook our study to determine whether mutations in the TNF 3'-UTR were present in pediatric patients with autoimmunity. Based on animal models and

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known associations of human TNF polymorphisms with autoimmune diseases, we chose type I diabetes mellitus and juvenile arthritis as the primary diseases for study.

METHODS

Patient selection. The experimental protocol was approved by the Institutional Review Board for Human Research at the University of Texas Southwestern Medical Center. Pediatric patients with or without autoimmune syndromes were recruited from the outpatient and inpatient populations at Children's Medical Center, Parkland Memorial Hospital, and Texas Scottish Rite Hospital, Dallas.

Genomic DNA purification. A 500- μ L blood sample was collected in Ca-EDTA specimen tubes from each patient enrolled in the study. Cellular elements were pelleted by centrifugation for 10 s at $10\,000 \times g$; pellets were washed twice for 10 s in 500 μ L of a solution consisting of 10 mM Tris, 1 mM EDTA, pH 8. Pellets were then resuspended in 100 μ L of a solution containing 0.5% Tween 20, 50 mM KCl, 10 mM Tris, pH 8.3, and 2.5 mM MgCl₂. Fifteen μ g of Proteinase K (Sigma Chemical Co., St. Louis, MO) were then added and incubated for 1 h at 60°C. The samples were then heated to 100°C for 10 min; a 10- μ L aliquot of this solution was next subjected to PCR amplification.

PCR. A 250-bp segment of the TNF 3' UTR was amplified using Vent polymerase (New England Biolabs, Boston, MA) and primers with the sequences 5'-CCCGAGCTCTTAGGC-CTTCCTCTCTCCAGA containing an upstream *Sac*I site and 5'-CCCGGATCCGTTTCAGCTCCGTTTTCACGGA containing a downstream *Bam*HI site. The designed restriction sites of the oligonucleotide primers allowed for ligation of amplified fragments into the vector pGEM 3Z (Promega Biotec, Madison, WI) for sequencing. Clones containing the 250-bp insert were then sequenced by the Sanger dideoxynucleotide chain termination method using M13 universal primers that match vector sequence flanking the fragment cloning site.

RESULTS

A total of 109 patients and volunteers were enrolled into the study, 80 of whom were diagnosed with connective tissue diseases or type I diabetes. The clinical diagnoses supplied by the patients' attending physicians are listed in Table 1. A 250-bp segment of the TNF 3'UTR containing the known TNF translational regulatory sequences (Fig. 1) was amplified by

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2954 TTAGGCCTTCTCTCTCCAGATGTTCCAGACTTCCTTGAGACACGGA
GCCAGCCCTCCCATGGAGCCAGCTCCCTCTATTTATGTTGCACTT
GTGATTTATTTATTTATTTATTTATTTATTTATTTATTTACAGATGAATGT
ATTTATTTGGGAGACCGGGGTATCTGGGGGACCCAATGTAGGAGCT
CCTTGGCTCAGACATGTTTCCGTGAAAACGGAGCTGAAC 3185
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Figure 1. Amplified segment of the human TNF 3'-UTR. Octameric "TTATTTAT" translational control sequences are underlined.

PCR and subcloned; a minimum of four clones from each PCR were sequenced. Two additional PCR amplifications followed by subcloning and sequencing were done on samples that initially appeared to display mutations to eliminate PCR or sequencing artifact. In this study, all patients and controls exhibited the normal, wild-type sequence, with no evident deletions or insertions in the "TTATTTAT" sequences or other TA-rich motifs.

DISCUSSION

Clinical and experimental evidence suggest that dysregulated TNF biosynthesis is important in the pathogenesis of certain autoimmune conditions. In patients with RA, TNF levels are elevated in both the systemic circulation and the synovial fluid obtained from diseased joints (13, 27). *In situ* hybridization and immunochemistry have localized TNF-producing cells to the cartilage-pannus junction, which is the leading edge of tissue destruction in affected joints (28, 29). In experimental models, instillation of TNF and IL-1 into the synovial joint space causes leukocyte infiltration, prostaglandin secretion, cartilage destruction, and bone resorption (30). These and other data have led to clinical trials of MAb directed against TNF in patients with autoimmune arthritis; a recent open label phase I/II trial demonstrated significant therapy-associated improvements in Ritchie articular index, swollen joint count, and biochemical parameters of inflammation (31).

Data from animal models and from humans also implicate dysregulated TNF biosynthesis and secretion in the pathogenesis of type I diabetes mellitus, although the precise mechanisms of TNF action are debatable. TNF may be produced by pancreatic islet cells (32) or by lymphocytes that infiltrate the islets during the course of acute insulinitis (33). TNF may be directly toxic to islet cells or may be indirectly injurious by enhancing IL-1 toxicity, up-regulating MHC class II gene expression, or inducing islet secretion of IL-6 (9, 10, 34–38). In contrast to local proinflammatory properties of TNF secreted within the islets, it is well established that nonobese diabetic mice profoundly underproduce TNF in response to provocative stimuli (26); in these animals and in the diabetes-prone BB rat, long-term administration of TNF significantly reduces the severity and incidence of diabetes (39, 40).

Theoretically, the association between abnormal patterns of TNF production and the development of autoimmunity may be

Table 1. Clinical diagnoses of patients undergoing TNF sequence analysis

Connective tissue disease	
Juvenile arthritis	32
Systemic lupus erythematosus	5
Dermatomyositis	6
Other	5
Noninflammatory arthritis	13
Type I diabetes mellitus	32
Septic shock	4
Postsurgical	8
Normal volunteers	4
Total patients	109

explained by three hypotheses: 1) TNF secretion may be a nonetiologic epiphenomenon. This explanation is the least likely given the accumulation of current data. 2) TNF may be an inflammatory effector of an otherwise unrelated genetic susceptibility. 3) Abnormalities in TNF regulation may directly lead to disease pathogenesis.

If primary dysregulation of TNF production were causative of autoimmunity, then traditional MHC-disease associations may reflect mutations within the TNF locus itself. Consistent with this hypothesis are provocative data recently reported by several investigators. TNF secretory capacity in humans was shown to correlate with several genetic markers including HLA haplotypes (41), TNF- β *NcoI* restriction fragment length polymorphisms (42), and TNF microsatellites (18). Diabetes-associated haplotypes carry an allele that correlates with high TNF secretory capacity, with DR3⁺ and DR4⁺ patients being the highest TNF responders. Furthermore, DR3/4 heterozygous diabetic patients have a significant increase in the frequency of TNFa2 microsatellite compared with DR3/4 individuals without diabetes who possess the TNFa6 microsatellite allele (18).

The search for mutations in the TNF gene that might account for these allelic associations is currently under way. Wilson *et al.* (43) reported an allelic polymorphism at position -308 of the TNF promoter, which is associated with the autoimmune-prone human HLA haplotype A1,B8,DR3. However, no functional significance to this polymorphism has yet been established (44). In addition, a polymorphic variation in the putative "Y box" of the TNF promoter has been described by D'Alfonso and Richiardi (45). Recently, attention has also been directed to the TNF 3'-UTR and in particular the octameric "TTATTTAT" motifs therein, which govern TNF mRNA stability (23) and translational efficiency (3). Substitution of the TNF 3'-UTR by a β -globin 3'-UTR resulted in transgenic mice that displayed inflammatory polyarthritis; this arthritis was eliminated by administration of MAb directed at TNF from the time of the animal's birth (25). Most recently, Jacob and Tashman (26) and Beutler and Brown (46) have simultaneously reported insertional mutations in the "TTATT-TAT" sequences of New Zealand White mice, which are known to carry an MHC-linked gene implicated in the development of murine lupus. In addition, Jacob (16) demonstrated that the presence of this insertion in New Zealand White mice and three other murine strains closely correlated with a decreased TNF secretory capacity to provocative stimulation.

Our study is the first to specifically examine the 3'-UTR in humans with autoimmunity. We initially chose patients with diabetes and juvenile arthritis because of the widespread evidence suggesting TNF's pathogenic importance in these conditions. Sequence analysis of the TNF 3'-UTR in 109 patients and controls indicated that polymorphisms in this region occur infrequently, if at all, in the patient populations we examined. It remains possible that such mutations may occur in other autoimmune diseases not examined in this report, or in a very small percentage of patients with diabetes and arthritis. It is also possible that such mutations do not exist at all in humans. Additional studies should be done to distinguish among these possibilities.

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