Cutting Edge: A Soluble Form of CTLA-4 in Patients with Autoimmune Thyroid Disease

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We have recently identified a novel transcript of the CTLA-4 gene that may represent a native soluble form of CTLA-4 (sCTLA-4). To determine whether sCTLA-4 was expressed in humans, we applied a sensitive enzyme immunoassay on serum from patients with autoimmune thyroid disease (ATD). Eleven of 20 patients with ATD had circulating levels of sCTLA-4 ranging from 28 to 78 ng/ml, whereas only 1 of 30 apparently healthy volunteers had a level greater than 4 ng/ml. sCTLA-4 immunoreactivity was inhibited by its binding to B7.1, suggesting that sCTLA-4 is a functional receptor. Immunoprecipitation analysis of serum from patients with ATD revealed a polypeptide consistent with the predicted size of sCTLA-4. We conclude that a native soluble form of CTLA-4 is derived from an alternate transcript of the CTLA-4 gene, and its level in plasma is elevated among a population of patients with ATD. The Journal of Immunology, 2000, 164: 5015–5018.

Cytotoxic T lymphocyte associated gene-4 (CTLA-4) was initially described as a classical type I glycoprotein on the surface of activated T cells (1). CTLA-4 is a member of the Ig gene superfamily and along with its homologue, CD28, is a B7 binding protein (2, 3). The emerging notion of the function of CTLA-4 in man, mouse, and rat that lacked transmembrane encoding regions to the GeneBank Sequence Database (accession nos. U90273, U90270, and U90271, respectively). Recently, Magistrelli et al. (18) described the same transcript and detected immunoreactive material in human serum that is consistent with the presence of a native soluble form of CTLA-4 (sCTLA-4). Initial experiments designed to identify the sCTLA-4 polypeptide by ELISA and Western blotting in normal human serum were unsuccessful in our laboratory. Because of the well-known association between CTLA-4 polymorphisms and ATD, we speculated that a reasonable starting point in the search for expression of the native sCTLA-4 would be in patients with Graves’ disease and Hashimoto’s thyroiditis. To that end, we developed an immunoassay for circulating CTLA-4 in human serum, and show in this communication that a soluble form of CTLA-4 is present in patients with ATD.

Materials and Methods

Patients

All patients in this study had a recent diagnosis of Graves’ disease (n = 17) or Hashimoto’s thyroiditis (n = 3) based upon clinical presentation and laboratory findings. The patients ranged in age from 19 to 47 years, and 16 were female and 4 were male. Serum was obtained at the time of diagnosis, and none of the patients studied had remarkable co-morbidity. All patients were studied before any treatment for ATD. Control sera were from normal healthy laboratory volunteers of similar age and sex mix relative to the

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2 Abbreviations used in this paper: ATD, autoimmune thyroid disease; sCTLA-4, soluble CTLA-4; EIA, enzyme immunoassay.
Enzyme immunoassays (EIA)

A sandwich EIA was used for detection of CTLA-4 in human serum. For this purpose, wells of a 96-well microtiter plate were coated with anti-CTLA4 mAb (clone BNI3; PharMingen, San Diego, CA). After blocking, 100 μl of a 1:3 dilution of the test samples were applied to the wells, and the plates were incubated for 60 min at room temperature and then washed to remove unbound material. Next, a biotinylated anti-CTLA4 mAb (clone AS-33, Antibody Solutions, Palo Alto, CA) was added, and the reactions were incubated for 1 h. Reactions were developed using a streptavidin-peroxidase complex (Zymed, South San Francisco, CA) and 3,3′,5,5′-tetramethyl-benzidine substrate. Optical density (OD) was read at 450 nm. A standard curve was generated with the use of a dilution series of a commercially available CTLA4-Ig fusion protein (AnCell, Bayport, MN). Intrassay coefficient of variation (CV) was 3% (n = 16), and the interassay CV was 11% (n = 20). Cross-reactivity of the Abs used in these studies was determined with the use of a CD28-Ig fusion protein prepared in this laboratory and was <0.1%. This fusion protein was chosen for cross-reactivity studies because of the relatively high degree of amino acid sequence similarity (27%) between the V domains of the CTLA-4 and CD28 polypeptides (19). Each sample was run in triplicate and was corrected for background by subtraction of OD obtained when the sample was incubated in wells coated with an isotype-matched mAb of irrelevant specificity (anti-rat CD4). Inhibition experiments were performed by the addition of 200 ng of a B7.1-Ig fusion protein (described below) to 100 μl of test serum for 1 h before immunoassay.

A B7.1-Ig fusion protein was generated by cloning the extracellular domains of the cDNA into the signal-pIG vector (Novagen, Madison, WI). For this purpose, cracks were the As of Raji cell (American Type Culture Collection, Manassas, VA) RNA using the following sense (s) and anti-sense (as) primers: B7.1s = AAACTTGCTTTTCACCTTTGTCA; B7.1as = GGATCCGCATCAGGAAAATGCTCTTGC. CHO cells were transfected with the use of Fugene-6 (Boehringer Mannheim, Indianapolis, IN) and selected with G418. Cell culture supernatants were collected in serum free medium and tested in a sandwich ELISA for human B7.1 and IgG1 epitopes. Positive culture supernatants were pooled and purified by protein A chromatography. The fusion protein was reactive with CTLA4-Ig as determined by an ELISA binding assay (data not shown). SDS-PAGE analysis of the fusion protein showed major products of ~150 kDa under nonreducing conditions and a product of 74 kDa when analyzed under reducing conditions.

Polyclonal Abs

Rabbit anti-human CTLA-4 Abs were generated by standard methods in New Zealand White rabbits immunized with a keyhole limpet hemocyanin-conjugated peptide. Antiserum 8K was raised to the carboxyl-terminal sequence of the predicted sCTLA-4 protein (KPSYNRLGENAPNRARM). This is a novel sequence predicted from the alternate transcript that arises from a frame-shift mutation that occurs during RNA splicing.3 This amino acid sequence shares no significant similarity with proteins cataloged on the available protein databases. The peptide was synthesized and conjugated by Research Genetics (Huntsville, AL).

Immunoprecipitation

Immunoprecipitation was used to detect sCTLA-4 in serum. For this purpose, 2–3 ml of serum was incubated overnight with 3 μg of a cocktail of anti-CTLA4 mAbs in an equal volume of PBS. The cocktail consisted of 1 μg each clone BNI3, AS33P, and AS32P (both from Antibody Solutions, Palo Alto, CA). Precipitates were collected by the addition of 50 μl of goat anti-mouse magnetic beads (Dynal, Great Neck, NY), and were separated by 10–20% gradient PAGE. The separated components were electrophoblotted onto nitrocellulose membranes. The blots were reacted with the 8K anti-CTLA-4 Ab for 1 h at room temperature, washed, and then reacted with reporter Ab (HRP-conjugated anti-rabbit IgG). The blots were then developed with the use of a commercially available chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturers instructions.

Results

To determine whether a circulating form of CTLA-4 was present in human serum, we devised a sensitive EIA using available mAbs to CTLA-4. The assay was optimized using commercially available CTLA4-Ig as a standard and then applied to testing of human serum samples. Fig. 1 shows combined data from the EIA on patients with ATD and normal apparently healthy volunteers. Circulating CTLA-4 was virtually undetectable in healthy volunteers as defined by the limit of sensitivity of 4 ng/ml for this assay. A single serum sample among 30 from healthy controls had detectable CTLA-4. By contrast, 11 of 20 patients with ATD had detectable circulating CTLA-4 levels in the range of 28–78 ng/ml. Of the 11 patients with detectable sCTLA-4, 8 had Graves’ disease (8 of 17 studied) and the remaining 3 had Hashimoto’s thyroiditis (3 of 3 studied). There was no obvious relationship between the sex and age of the patient and levels of sCTLA-4. sCTLA-4 was detectable using several commercially available mAbs to CTLA-4 as capture Abs (data not shown), arguing against the possibility that autoantibodies or anti-idiotypic Abs were responsible for these effects.

To determine whether CTLA-4 immunoreactivity in serum consisted of an intact and functional molecule as opposed to degraded or shed CTLA-4 polypeptides, we attempted to block the CTLA-4 immunoassay by preincubating positive sera with B7-Ig fusion proteins. Data from a representative experiment is shown in Fig. 1. In this case, a sample containing 28 ng/ml sCTLA-4 was completely neutralized in the presence of 200 ng B7.1-Ig fusion protein. Neutralization of immunoreactivity was obtained on three independent serum samples and ranged from 33 to 95% inhibition. Negligible inhibition (<5%) was observed when a control fusion protein (Muc18-Ig) was tested in these experiments. These data show that the CTLA-4 immunoreactive material present in human serum represents an intact functional receptor for the B7.1 ligands.

The primary limitation of the EIA for circulating CTLA-4 described here and that used by others (18) is that they are based on the use of Abs reactive with epitopes within the B7-binding region of the molecule. As a result, they cannot distinguish native soluble

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CTL-A4 receptors derived from the transcript lacking the transmembrane domain from those that might be present in serum due to proteolytic digestion or shedding of the CTL-A4 integral membrane protein. To determine whether the immunoreactive material in serum of ATD patients was derived from the gene product of the alternate transcript, we designed immunoprecipitation experiments using a pool of commercially available mAbs as a precipitin and a polyclonal Ab raised against a novel epitope within the carboxyl terminus of sCTLA-4 that is generated by a frame shift mutation that occurs during RNA splicing. Fig. 2 shows a representative experiment. This combination of Abs predominantly identifies a polypeptide species ~23 kDa, which is consistent with the predicted size based on the amino acid sequence and predicted N-linked glycosylation pattern of sCTLA-4. Western blotting of serum proteins from several patients with ATD also showed a predominant species of about 23 kDa when tested directly against the 8K antiserum (data not shown). This species was not evident when probed with a pre-immune serum from the same animal used for immunization, even when run 50-fold concentrated than immune sera.

Discussion

We3 and others (18) have recently described an alternate transcript of the CTL-A4 gene that encodes a protein that lacks a transmembrane region and likely represents a native soluble form of CTL-A4. The work presented in this report documents the finding of CTL-A4 immunoreactive material in serum of patients with ATD including Graves’ disease and Hashimoto’s thyroiditis. Because the immunoreactive material contains two epitopes, one of which is unique to the putative sCTLA-4 protein, we propose that sCTLA-4 is a novel polypeptide product of the CTL-A4 gene. The fact that CTL-A4 immunoreactivity can be blocked by one of its known ligands (namely, B7.1) supports the notion that sCTLA-4 encodes a functional receptor. Like other soluble receptors (20–25), sCTLA-4 may have important immunoregulatory functions. The effect of sCTLA-4 binding to B7 molecules might depend on the activation status of the cells involved. For example, on resting cells, sCTLA-4 may block B7-CD28 interactions, thereby interfering with T cell costimulation. On the other hand, inhibition of B7-CTL-A4 interactions on activated T cells (conditions under which the transmembrane form of the molecule is selectively expressed) may prevent down-regulation of T cell responses. The functional activity of sCTLA-4 adds an additional level of complexity to the current description of the role of CTL-A4 in immunoregulation. For example, what are the relative roles of sCTLA-4 versus the transmembrane form of the molecule in the immune dysfunction observed in CTL-A4 knockout mice? CTL-A4 knockouts develop a profound polyclonal lymphoproliferative disorder that has commonly been attributed to inhibition of the attenuating effect of CTL-A4 on activated cells; however, because these animals contain deletions within the exon 2 which includes the B7-binding domains, they would lack both the soluble as well as the transmembrane forms of CTL-A4. Our data differs from that reported by Magistrelli et al. (18) in that they report detection of circulating CTL-A4 in 14 of 64 healthy subjects (18). By contrast, we observed a value of <4 ng/ml in only one sample of 30 healthy individuals. It is possible that these differences are due to technical variables such as the different mAbs used for detection. Alternatively, they may be attributed to differences in the ethnic background of the populations tested or to the relatively undefined immunological status of the two control groups, for example, recent infection, allergy, etc.

We believe our findings to be provocative because they may also provide a link between the genetic susceptibility to ATD and differences in the expression of the various forms of the CTL-A4 molecule. Population genetics data clearly suggest a role for the CTL-A4 gene region in the susceptibility to ATD; however, a specific change in CTL-A4 structure or function has not been described. Two polymorphisms within the CTL-A4 gene have been studied with respect to population genetic associations with endocrine autoimmune disease. One of them represents a single nucleotide polymorphism that results in an amino acid substitution (Thr/Ala) within the signal sequence of the CTL-A4 polypeptide (19). The Ala allele has been reported to have statistically significant higher frequency among patients with Graves’ disease (17) as well as insulin dependent diabetes mellitus (10–12). No relationship between this dimorphism and CTL-A4 structure, function, or expression has been described. A small number of patients from this study (n = 5) who were positive for sCTLA-4 were typed for the Thr/Ala polymorphism, but this analysis revealed no exclusive association with a specific genotype (data not shown). A larger population needs to be studied to fully examine the relationship, if any, between CTL-A4 polymorphisms and circulating levels of sCTLA-4.

A second polymorphism with population genetic associations with autoimmune endocrine disease is a dinucleotide repeat (AT)n within exon 3 of the human CTL-A4 gene (9, 26). The dinucleotide repeat is within a noncoding region of the gene, but is potentially important because it might effect mRNA stability. Long runs of A and T are found in the 3’ untranslated regions of a variety of transcripts that are transiently expressed including mRNAs for cytokines, lymphokines, and protooncogenes (27). It is possible that polymorphisms within this repeat unit effect stability or splicing of one or more of the alternate CTL-A4 transcripts, resulting in changes in expression observed in this study. This study does not, of course, provide support for such a concept, and it is certainly possible that the polymorphisms described to date merely serve as markers for variation within the CTL-A4 gene, the CD28 gene (which is closely linked to CTL-A4 (19)), or unknown genes in linkage disequilibrium with CTL-A4. Nevertheless, our findings of increased levels of sCTLA-4 in patients with ATD may reveal important information regarding CTL-A4 function as well as the pathogenesis of autoimmune disease. An interesting question that our data raises is whether elevated levels of sCTLA-4 represent a constitutive effect of a CTL-A4 susceptibility gene per se or rather are due to a physiologic response to the activation status of T cells.
with reactivity to thyroid autoantigens. To that end, we are currently examining the relationship between sCTLA-4 levels and disease onset in ATD.

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