WT1-specific Serum Antibodies in Patients with Leukemia


Abstract

WT1 is an oncogenic protein expressed by the Wilms’ tumor gene and overexpressed in the majority of acute myelogenous leukemias (AMLs) and chronic myelogenous leukemias (CMLs). The current study analyzed the sera of patients with AML and CML for the presence of antibodies to full-length and truncated WT1 proteins. Sixteen of 63 patients (25%) with AML had serum antibodies reactive with WT1/full-length protein. Serum antibodies from all 16 were also reactive with WT1/NH2-terminal protein. By marked contrast, only 2 had reactivity to WT1/COOH-terminal protein. Thus, the level of immunological tolerance to the COOH terminus may be higher than to the NH2 terminus. The WT1/COOH-terminal protein contains four zinc finger domains with homology to other self-proteins. By implication, these homologies may be related to the increased immunological tolerance. Results in patients with CML were similar with antibodies reactive to WT1/full-length protein detectable in serum of 15 of 81 patients (19%). Antibodies reactive with WT1/NH2-terminal protein were present in the serum of all 15, whereas antibodies reactive with WT1/COOH-terminal protein were present in only 3. By contrast to results in leukemia patients, antibodies reactive with WT1/full-length protein were detected in only 2 of 96 normal individuals. The greater incidence of antibody in leukemia patients provides strong evidence that immunization to the WT1 protein occurred as a result of patients bearing malignancy that expresses WT1. These data provide further stimulus to test therapeutic vaccines directed against WT1 with increased expectation that the vaccines will be able to elicit and/or boost an immune response to WT1.

Introduction

Overexpressed oncogenic proteins can be considered as potential candidate antigens for cancer vaccines and T-cell therapy (1–3), particularly if the oncogenic proteins are important for maintenance of the malignancy, have restricted tissue distribution, and are immunogenic. WT1 is an oncogenic protein involved in transcriptional regulation, especially during fetal development (4, 5). WT1 was originally identified in Wilms’ tumor (6, 7), but it is also oncogenic and overexpressed in many human leukemias including the majority of AMLs and CMLs (8–15). WT1 overexpression appears to often be an important factor for leukemogenesis and for the viability of leukemic blasts (16–19).

During fetal development, WT1 is normally expressed in a time- and tissue-specific manner and is involved in the regulation of growth and differentiation via interaction with a variety of different target genes (4–6). In the adult, WT1 has a tissue distribution restricted to low levels of expression in the nucleus of some normal tissues, including hematopoietic precursor cells, kidney, and gonad cells (5, 20). The level of expression in hematopoietic precursor cells is substantially less than the level of overexpression in a substantial proportion of leukemias. Thus, WT1 protein is abundant in leukemia cells compared with normal hematopoietic cells (12, 20). WT1 is expressed in the adult kidney, but expression is limited to the kidney podocyte. The extent to which the kidney podocyte is an immunological restricted site is unknown. Of note, in mice immunized to WT1, kidney toxicity has not been observed (3).

There is substantial evidence that WT1 is immunogenic in humans and in mice. Human WT1-specific CTLs can be generated by priming to peptides in vitro and can kill leukemic cells that overexpress WT1 (1, 2). WT1-specific CTLs can have exquisite specificity for leukemic progenitor cells. In one study, CTL-specific for WT1 killed leukemic cells, leaving normal bone marrow progenitor cells intact (2). Murine WT1 is virtually identical to human WT1; thus, many aspects of vaccine studies in mice can be extrapolated to humans. In mice, immunization with peptide fragments of WT1 can elicit Ab, helper T cell, and CTL responses specific for WT1 without apparent toxicity to the small number of normal tissues that express WT1, including the kidney (3). Two murine cancers that “naturally” overexpress WT1 have been identified. Immunization of mice with murine cancer cells that “naturally” overexpress WT1 can induce antibodies specific for WT1, demonstrating that WT1 can be immunogenic in the context of cancer cells (3).

Studies demonstrating that priming in vitro can generate human WT1-specific CTLs (1, 2) imply that WT1 vaccines can be immunogenic in humans. Previous studies had demonstrated the presence of Ab to the NH2-terminal portion of the WT1

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3 The abbreviations used are: WT1, Wilms’ tumor 1; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; TRX, thioredoxin; Ab, antibody.
protein in the sera of three patients with AML by Western blot analyses (3). The current study was undertaken to confirm and extend those findings. Antibodies reactive to WT1 were shown to be present in the sera of many patients with AML and CML. The demonstration of antibodies specific for WT1 provides direct evidence that WT1 is immunogenic in humans. The presence of antibodies provides substantial evidence for the concurrent presence of WT1-specific helper T cells in the same individuals. The demonstrated presence of existent immunity to WT1 adds great credece to the prospect that vaccination of humans with formulations of WT1 will be able to elicit and boost Ab and T-cell immunity to WT1.

Materials and Methods

Recombinant Proteins. For protein expression, cDNA constructs representing the human WT1/full-length (amino acids 1–449), the NH2-terminal (amino acids 1–249; denoted WT1/NH2-terminal), and COOH-terminal (amino acids 267–449; denoted WT1/COOH-terminal) regions were subcloned into a modified pET28 vector. Because of the insolubility of the recombinant WT1/full-length and WT1/NH2-terminal proteins as well as issues with expression in Escherichia coli, it became necessary to express these two proteins as fusion proteins. Ra12 is the COOH-terminal fragment of a secreted Mycobacterium tuberculosis protein, denoted as MTB32B (21). The WT1 full-length region was subcloned into a modified pET28 vector that has a 5’ histidine-tag, followed by the Ra12-WT1/full-length fusion region, followed by a 3’ histidine-tag. The WT1/NH2-terminal region was subcloned into a modified pET28 vector that has a 5’ histidine-tag, followed by the TRX-WT1/NH2-terminal fusion region, followed by a 3’ histidine-tag. The WT1/COOH-terminal coding region was subcloned into a modified pET28 vector without a fusion partner containing only the 5’ histidine-tag. Table 1 lists the three WT1 proteins included in this study.

Recombinant BL21 pLysS E. coli (Stratagene, La Jolla, CA) containing the expression constructs for the Ra12-WT1/full-length, TRX-WT1/NH2-terminal, or the WT1/COOH-terminal proteins were grown overnight and induced with isopropyl-β-D-thiogalactoside. All of the proteins behaved similarly and were purified following essentially the same protocol. Cells were harvested and lysed by incubation in 10 mM Tris (pH 8.0) with Complete Protease Inhibitor Tablets (Boehringer Mannheim Biochemicals, Indianapolis IN) at 37°C, followed by repeated rounds of sonication. Inclusion bodies obtained were washed twice with 10 mM Tris (pH 8.0)/0.5% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate and solubilized in 8 M urea containing 10 mM Tris at pH 8.0 (buffer A). Proteins were then purified by metal chelate affinity chromatography over nickel-nitrilotriacetic acid resin (Qiagen, Inc., Valencia, CA) as described (22). Proteins were analyzed by SDS-PAGE, and fractions containing the protein of interest were pooled and dialyzed overnight against excess 10 mM Tris (pH 8.0). Dialyzed samples were brought to 8 M urea and loaded onto a Source Q anion exchange resin (Amersham Pharmacia Biotech, Uppsala Sweden) equilibrated in buffer A. Proteins were eluted in buffer A with a gradient from 0 to 1 M NaCl. Fractions containing the proteins of interest were pooled, dialyzed overnight against excess 10 mM Tris (pH 8.0), and stored at −80°C for further use. The identity of the WT1 proteins was confirmed by NH2-terminal sequencing.

ELISA. Sera from adult patients with de novo AML or CML were tested for the presence of WT1-specific Ab. Fifty μl/well of a 5 μg/ml recombinant protein in coating buffer (15 mM Na2CO3, 30 mM NaHCO3, pH 9.6) was adsorbed to TC microwell plates 12 × 8 (Nunc, Roskilde, Denmark) overnight at 4°C. Plates were washed with PBS/0.5% Tween 20 and blocked for 2 h at room temperature with 200 μl/well of 1% BSA/PBS/0.1% Tween 20. After washing, 50 μl/well of serum dilutions, ranging from 1:50 to 1:20,000, in 1% PBS/BSA/0.1% Tween 20 were added and incubated overnight at 4°C. Plates were washed, and 100 μl/well diluted secondary Ab/1% PBS/BSA/0.1% Tween 20 were added (donkey antihuman IgG-horsersradish peroxidase; Jackson-Immunoresearch, West Grove, PA) and incubated for 2 h at room temperature. Plates were washed, incubated with 50 μl/well of substrate solution (TMB peroxidase/substrate; Kirkegaard and Perry Laboratories, MA) for 10 min at room temperature, stopped with 50 μl/well of 1 N H2SO4, and immediately read (Cyto-Fluor 2350; Millipore, Bedford, MA).

For the serological survey, human sera were tested by ELISA over a range of serial dilutions from 1:50 to 1:20,000. A positive reaction is defined as an absorbance of a 1:500 diluted serum that exceeds the mean absorbance of sera from normal donors (n = 96) by three (WT1/full-length and WT1/COOH-terminal proteins) SDs. Because of a higher background in normal donors to the WT1/NH2-terminal protein, a positive reaction to the WT1/NH2-terminal region is defined as an absorbance of 1:500 diluted serum that exceeds the mean absorbance of sera from normal donors by 4 SDs. To verify that the patient Ab response was directed against WT1 and not to the Ral2 or TRX fusion part of the protein or possible E. coli contaminant proteins, controls included the Ra12 and TRX protein alone purified in a similar manner. Samples that showed reactivity against the Ra12 and/or TRX proteins were excluded from the analysis.

Results

WT1-specific Abs in Sera of Normal Individuals. To evaluate for the presence of immunity to WT1, we analyzed for the prevalence of Ab to recombinant full-length and truncated
WT1 proteins in the sera of normal individuals and patients with leukemia. Ab reactivity was determined by ELISA reactivity to WT1/full-length protein, WT1/NH2-terminal protein, and WT1/COOH-terminal protein. The proteins used are defined in Table 1. A positive reaction was defined as an absorbance of a 1:500 diluted serum that exceeds the mean absorbance of all sera from normal donors (n = 96) by either 3 SDs (WT1/full-length protein and WT1/COOH-terminal protein) or 4 SDs (WT1/NH2-terminal protein). The ELISA for WT1/NH2-terminal protein had a higher background in normal individuals and leukemia patients. Thus, the cutoff was set at a higher level.

Summary results (Table 2) show that 2 of 96 normal donors have serum antibodies reactive with WT1/full-length protein. One of these individuals had Ab to WT1/NH2-terminal protein, and one had Ab to WT1/COOH-terminal protein.

**WT1-specific Abs in Sera of Patients with AML.** The presence of immunity to WT1 in patients with AML was similarly analyzed in the same experiments. Summary results (Table 2) show that 16 of 63 patients (25%) with AML had serum antibodies reactive with WT1/full-length protein. Serum from all of the 16 patients with reactivity to WT1/full-length protein also reacted with the WT1/NH2-terminal protein. By marked contrast, only 2 of 63 patients (3%) had reactivity to the WT1/COOH-terminal protein.

Fig. 1 depicts serum reactivity to WT1/full-length protein for each of the 63 individual patients. The WT1/full-length protein was expressed as a fusion protein with Ra12, a foreign protein. The WT1/NH2-terminal protein was expressed in E. coli as a fusion protein with TRX, another foreign protein. To verify that the patient Ab response was directed against WT1 and not to the Ra12 or the TRX fusion portion of the protein or possible E. coli contaminant proteins, controls included the Ra12 and TRX protein alone (Fig. 2). Reactivity against the Ra12, TRX fusion, or possible E. coli contaminant proteins was minimal, if at all present in all of the positive patients tested, including the two representative patients depicted.

**WT1-specific Abs in Sera of Patients with CML.** Studies of immunity to WT1 in patients with CML provided similar results (Table 2). In total, 15 of 81 patients (19%) with CML had serum antibodies reactive with WT1/full-length protein, and all 15 had serum antibodies reactive with the WT1/NH2 terminus. Only 3 of 81 patients (4%) had reactivity to the WT1/COOH-terminal protein.

Fig. 3 depicts serum reactivity to WT1/full-length protein for each of the 81 individual patients. Fig. 4 shows a lack of reactivity to TRX and Ra12 proteins in two representative individual sera to confirm that the patient Ab response was directed against WT1.

**Discussion**

The data presented demonstrate that Ab responses to WT1 are detectable in some patients with AML and CML. Sera from 144 leukemia patients and 96 normal volunteers were examined and demonstrated the presence of Ab to WT1 in 25% of AML and 19% of CML patients but only 2% of normal individuals. The greater incidence of Ab in leukemia patients provides strong evidence that immunization to the WT1 protein occurred.

### Table 2

<table>
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<tr>
<th></th>
<th>WT1/full-length</th>
<th>WT1/NH2 terminus</th>
<th>WT1/COOH terminus</th>
</tr>
</thead>
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<tr>
<td>Normal individuals (n = 96)</td>
<td>2/96 (2%)</td>
<td>1/96 (1%)</td>
<td>1/96 (1%)</td>
</tr>
<tr>
<td>AML patients (n = 63)</td>
<td>16/63 (25%)</td>
<td>16/63 (25%)</td>
<td>2/63 (3%)</td>
</tr>
<tr>
<td>CML patients (n = 81)</td>
<td>15/81 (19%)</td>
<td>15/81 (19%)</td>
<td>3/81 (4%)</td>
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**Fig. 1** Serum Ab reactivity to WT1 in 63 patients with AML. Reactivity of serum Ab to WT1/full-length protein was evaluated by ELISA in patients with AML. The first and second columns represent the positive and negative controls, respectively. Commercially obtained WT1-specific Ab WT180 was used for the positive control (column 1). Serum obtained from a normal individual was used for the negative control (column 2). The next 63 lanes represent results using sera from each individual patient. The absorbance values depicted were from ELISA using a 1:500 serum dilution. The figure includes cumulative data from three separate experiments.

**Fig. 2** Serum Ab reactivity to WT1 proteins and control proteins in two patients with AML. Reactivity of serum Ab to WT1/full-length, WT1/NH2-terminal, TRX, and Ra12 proteins was evaluated by ELISA in two patients with AML. The absorbance values depicted were from ELISA using a 1:500 serum dilution. Positive control (WT180) was WT1-specific Ab WT180. Negative control (Normal) was serum from a normal individual. AML-1 and AML-2 denote serum from two of the individual patients in Fig. 1 with demonstrated Ab reactivity to WT1/full-length. The WT1/full-length protein was expressed as a fusion protein with Ra12. The WT1/NH2-terminal protein was expressed as a fusion protein with TRX. The control Ra12 and TRX proteins were purified in a similar manner. The results confirm that the serum Ab reactivity against the WT1 fusion proteins is directed against the WT1 portions of the protein.

**Fig. 3** Serum Ab reactivity to WT1 proteins and control proteins in two patients with AML. Reactivity of serum Ab to WT1/full-length, WT1/NH2-terminal, TRX, and Ra12 proteins was evaluated by ELISA in two patients with AML. The absorbance values depicted were from ELISA using a 1:500 serum dilution. Positive control (WT180) was WT1-specific Ab WT180. Negative control (Normal) was serum from a normal individual. AML-1 and AML-2 denote serum from two of the individual patients in Fig. 1 with demonstrated Ab reactivity to WT1/full-length. The WT1/full-length protein was expressed as a fusion protein with Ra12. The WT1/NH2-terminal protein was expressed as a fusion protein with TRX. The control Ra12 and TRX proteins were purified in a similar manner. The results confirm that the serum Ab reactivity against the WT1 fusion proteins is directed against the WT1 portions of the protein.
Antibodies to WT1

A central problem with targeting oncogenic proteins with cancer vaccines is that the oncogenic proteins are most often "self" proteins. Attempting to elicit immune responses directed against oncogenic proteins raises the reciprocal problems of immunological tolerance and autoimmune toxicity (23). Immunological tolerance to self-proteins can inhibit or dampen the level of immune response. Reciprocally, if an effective immune response is elicited, the result might be autoimmune toxicity against those tissues that normally express the target protein. The observed Ab responses to WT1 most probably result from patients becoming immune to WT1 on their own leukemia cells and provide direct evidence that WT1 can be immunogenic, despite being a self-protein. In prior murine experiments elicitation of an immune response to murine WT1 did not result in autoimmune toxicity, in particular to bone marrow or kidney, i.e., the normal tissues that express some level of WT1 protein (3). The current study was not designed to examine WT1 Ab-positive patients for autoimmune manifestations. However, autoimmune toxicity directed against WT1-positive tissues, such as kidney, is not a commonly recognized syndrome associated with leukemia. Thus, it is unlikely that the observed WT1-specific antibodies are substantially toxic.

The presence of Ab to WT1 strongly implies that concurrent helper T cell responses are also present in the same patients. WT1 is an internal protein. Thus, CTL responses are likely to be the most effective in terms of leukemia therapy and the most toxic arm of immunity. The presence of antibodies to WT1 does not speak directly to the likelihood of existent CTL responses. However, helper T cells, if present, can potentially provide the necessary help for vaccine induction of CTL responses. Thus, these data provide further stimulus to test therapeutic vaccines directed against WT1 with the expectation that the vaccines will be able to elicit an immune response to WT1. Whether that immune response can therapeutically impact on the outcome of leukemia will be the essential question. The extent to which helper T cell and CTL responses can be elicited in humans should be evaluated.

The WT1 protein is a transcription factor that is composed of two functional domains: a proline-glutamine-rich domain at the NH2 terminus, and a zinc finger domain composed of four zinc fingers at the COOH terminus with homology to the EGR1/Sp1 family of transcription factors (24–26). The majority of the Ab detected was reactive with epitopes within the NH2 terminus. Only a small subgroup of patients showed a weak Ab response to the COOH terminus. This is consistent with observations in the animal model, where immunization with peptides derived from the NH2 terminus elicited Ab, helper T cell, and CTL responses, whereas none of the peptides tested from the COOH terminus elicited Ab or T-cell responses (3). WT1 is a self-protein. One possible implication is that the NH2 terminus is unique and thus less immunogenic. By contrast, the COOH terminus is more highly homologous to other self-proteins and is thus less immunogenic, i.e., the subject of a greater degree of immunological tolerance. Of note, the four zinc-finger domains within the COOH terminus have high homology to EGR family members (25). Data presented in this study indicate that tolerance might vary between different portions of a protein, possibly dependent upon sequence homologies and functional domains.

The current studies did not address important issues concerning possible correlations of Ab responses with levels of WT1 gene and protein expression and clinical characteristics such as French-American-British subcategory and response to

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**Fig. 3** Serum Ab reactivity to WT1 in 81 patients with CML. Reactivity of serum Ab to WT1/full-length protein was evaluated by ELISA in patients with AML. The first and second columns represent the positive and negative controls, respectively. Commercially obtained WT1-specific Ab WT180 was used for the positive control (column 1). Serum from a normal individual was used for the negative control (column 2). The next 81 lanes represent results using sera from each individual patient. The absorbance values depicted were from ELISA using a 1:500 serum dilution. The figure includes cumulative data from three separate experiments.

**Fig. 4** Serum Ab reactivity to WT1 proteins and control proteins in two patients with CML. Reactivity of serum Ab to WT1/full-length, WT1/NH2-terminal, TRX and Ra12 proteins was evaluated by ELISA in two patients with CML. The absorbance values depicted were from ELISA using a 1:500 serum dilution. Positive control (WT180) was WT1-specific Ab WT180. Negative control (Normal) was serum from a normal individual. CML-1 and CML-2 denote serum from two of the individual patients in Fig. 3 with demonstrated Ab reactivity to WT1/full-length. The WT1/full-length protein was expressed as a fusion protein with Ra12. The WT1/NH2-terminal protein was expressed as a fusion protein with TRX. The control Ra12 and TRX proteins were purified in a similar manner. The results confirm that the serum Ab reactivity against the WT1 fusion proteins is directed against the WT1 portions of the protein.
therapy in terms of disease-free or overall survival. The demonstration of a high incidence of immunity should allow such issues to be readily evaluated in future studies.

Acknowledgments

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