Loss of functional cell surface transforming growth factor β (TGF- β) type 1 receptor correlates with insensitivity to TGF- β in chronic lymphocytic leukemia

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ABSTRACT Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in Western countries, and there is significant variability in survival within CLL clinical stages. Earlier studies showed that CLL cells produce and are usually growth inhibited by transforming growth factor β type 1 (TGF- β 1), suggesting a mechanism for the clinically indolent course of most CLL. Here we studied the mechanism by which CLL cells from about one-third of the patients are insensitive to TGF- β 1. Of the 13 patients studied, CLL cells isolated from the peripheral blood of 8 patients were sensitive to growth inhibition by TGF- β 1, as determined by incorporation of tritiated thymidine, whereas those from 5 patients were completely resistant to TGF- β 1. As judged by binding of radiolabeled TGF- β 1 followed by cross-linking and immunoprecipitation with anti-receptor antisera, CLL cells sensitive to TGF- β 1 exhibited normal cell surface expression of both types 1 and 2 TGF- β receptors. In contrast, all CLL cells resistant to TGF- β 1 exhibited no detectable surface type I receptors able to bind TGF- β 1, but normal expression of type II receptors. Both TGF- β 1-sensitive and TGF- β 1-resistant CLL cells contained normal amounts of both type 1 and type 2 receptor mRNAs. Specific loss of type 1 receptor expression represents a new mechanism by which cells acquire resistance to TGF- β 1-mediated growth inhibition in the development and progression of human lymphoproliferative malignancies.

In Western countries, chronic B-cell lymphocytic leukemia (CLL) is the most common adult leukemia. The diagnosis and clinical staging of CLL is usually straightforward, but there is wide variability in the rate of progression and the frequency of disease-related complications. Some CLL patients live for 20 years or longer, while others succumb to the disease within a few months. At present, the clinical stage of CLL probably remains the strongest predictor of patient survival, but there remains significant variability in survival within a given clinical stage. This variability has led to the search for biological mechanisms that may underlie the progression of CLL (1–3).

Endogenous production of transforming growth factor β type 1 (TGF- β 1) has been proposed as one of the factors that contributes to the low proliferative rate of CLL cells. In short term cultures of freshly isolated CLL cells, the majority of patient samples exhibit increased proliferation in the presence of neutralizing antibodies to TGF- β 1 in a specific and dose-dependent manner. Similarly, in the majority of patients, exogenous TGF- β 1 inhibits proliferation of CLL cells induced

by phorbol esters, anti-CD40, anti-IgM, interleukin (IL)-2, IL-10, or anti-TGF- β 1 (4, 5).

The three major cell surface receptors for TGF- β are termed types I, II, and III (T β RI, II, and III, respectively) (6, 7). T β RI and T β RII are the signaling receptors, while T β RIII appears to promote ligand binding to T β RI and T β RII (6–13). The type I and II receptors are serine/threonine kinases with approximately 40% identity in their kinase domains (6–8, 11, 12); like their ligands, they are members of a large superfamily (6). Both endogenous and exogenous TGF- β 1 mediate growth inhibition by interacting with a functional complex of T β RI and T β RII. TGF- β 1 binds to T β RII and recruits T β RI into a functional complex, which leads to transphosphorylation of T β RI by T β RII and generation of an anti-proliferative signal (13–16).

Loss of growth inhibition by TGF- β is thought to contribute to the development and progression of a variety of tumors. In retinoblastoma, colon and gastric cancer, hepatoma, and some T cell malignancies, loss of TGF- β responsiveness correlates with loss of cell surface T β RII expression due to mutations or deletions in the T β RII gene (17–23). A recent report implicated genetic changes in T β RI with insensitivity to TGF- β 1 in the human prostate cancer cell line, LNCaP, but there are no reports of a specific T β RI defect associated with resistance to TGF- β 1 in human lymphoproliferative malignancies or primary tumor samples (24).

In approximately one-third of patients with CLL, cultures of freshly isolated CLL cells are resistant to TGF- β 1-mediated growth inhibition (5). The mechanism of TGF- β 1 resistance in these patients is unknown and TGF- β receptor expression in CLL has not been analyzed (4, 5). To this end, we compared surface expression of T β RI and T β RII in both TGF- β 1-sensitive and -resistant CLL cells obtained from patient peripheral blood samples. Resistance to TGF- β 1 was associated with loss-of-functional surface T β RI; surface T β RII was normal. Thus, loss-of-functional T β RI on the cell surface may represent a new mechanism of TGF- β 1 resistance involved in the pathogenesis and progression of human CLL.

MATERIALS AND METHODS

Patients. Thirteen unselected patients with a diagnosis of CLL were studied; determination of the clinical stage (Table 1) was according to the Rai classification (1, 2). All patients had a sustained lymphocytosis in excess of 20×10^9 /liter;

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Abbreviations: CLL, chronic lymphocytic leukemia; TGF- β 1, transforming growth factor β type 1; IL, interleukin.

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Table 1. Summary of responsiveness to TGF- β 1 and cell surface T β RI and T β RII expression in CLL cells

Patient	CS	Growth in presence of TGF- β 1 and			Growth in presence of anti-TGF- β 1 and			Cell surface expression of	
		PMA	IL-2	IL-10	PMA	IL-2	IL-10	T β RI	T β RII
1	3	0.58	0.68	0.73	1.36	1.61	1.67	+	+
2	3	0.95	1.05	0.97	1.02	1.00	1.11	—	+
3	2	1.04	1.01	0.98	0.87	0.94	0.98	—	+
4	1	0.99	1.03	1.04	1.12	0.98	1.06	—	+
5	2	0.21	0.14	0.29	2.77	2.25	1.14	+	+
6	4	0.46	0.72	0.49	1.59	2.32	1.47	+	+
7	3	1.00	1.03	0.98	1.06	0.91	0.95	—	+
8	3	0.91	0.98	1.04	0.96	1.02	0.98	—	+
9	2	0.22	0.74	0.35	1.51	1.63	1.85	+	+
10	0	0.67	0.71	0.51	1.38	1.58	1.35	+	+
11	3	0.49	0.72	0.68	1.11	1.27	1.59	ND	ND
12	2	0.30	0.55	0.69	1.37	1.19	3.04	ND	ND
13	2	0.56	0.43	0.57	1.77	1.29	1.89	ND	ND

CLL cells were stimulated to proliferate in the presence of anti-IgM and combinations of PMA, IL-2, or IL-10. Responsiveness to TGF- β 1 was determined by comparing proliferation in the presence or absence of added TGF- β 1 and the presence or absence of anti-TGF- β 1. Growth in the presence of TGF- β 1 is expressed as the ratio of proliferation in the presence of TGF- β 1 to proliferation in the absence of TGF- β 1 for each of the stimulation conditions. Growth in the presence of anti-TGF- β 1 is expressed as the ratio of proliferation in the presence of anti-TGF- β 1 to proliferation in the presence of a control antibody for each of the stimulation conditions. CS, clinical stage of disease; PMA, phorbol 12-myristate 13-acetate; ND, not determined.

values ranged from 37–119 $\times 10^9$ /liter. Morphologic analysis of peripheral blood films showed small, mature appearing lymphocytes having condensed chromatin, indistinct nucleoli, and scant cytoplasm; there were fewer than 10% prolymphocytes. The morphologic diagnosis was substantiated by immunophenotypic analysis using flow cytometry. Peripheral blood mononuclear cells were purified by Ficoll density gradient separation and analyzed for the expression of the markers CD45, HLA-DR, CD19, CD20, CD22, CD2, CD3, CD4, CD5, CD7, CD8, CD23, CD10, CD11c, CD25, κ and λ light chains, IgM, IgD, and IgG. In all patients, the lymphocytes had an immunophenotype characteristic of B-CLL that included expression of HLA-DR, the pan B cell antigens CD19 and CD20, CD23, the T cell marker CD5, monotypic immunoglobulin light chains, and dim expression of surface immunoglobulin (25). In all patients, CLL cells accounted for more than 95% of the total cell population.

Cell Preparation. Ten to 20 ml of heparinized whole blood was obtained from each of the patients following informed consent. The blood was diluted 1:2 with Hanks' balanced salt solution (HBSS; GIBCO), overlaid on Ficoll-Hypaque (Pharmacia), and centrifuged at 500 g for 30 minutes at room temperature. Cells at the interface were harvested and washed twice in HBSS, suspended in aliquots of HBSS containing 10% DMSO and 40% FCS, and cryopreserved in liquid nitrogen until needed. The viability of cryopreserved cells was assessed by trypan blue dye exclusion immediately prior to use in all assays and typically exceeded 95%.

Proliferation Assays. Responsiveness to TGF- β 1 was measured using a tritiated thymidine incorporation assay (4, 5, 26). CLL cells (500,000) were suspended in 200 μ l of RPMI medium 1640 containing 10% fetal calf serum, and placed in wells of a 96-well microtiter plate in the presence or absence of recombinant human TGF- β 1 (R & D Systems; 10 ng/ml), or in the presence of a neutralizing antibody to human TGF- β 1 (R & D Systems; 10 μ g/ml), or in the presence of a control antibody. Cells were stimulated to proliferate by adding goat-

anti-human IgM F(ab')₂ antibodies (Cappel, NC; 10 μ g/ml) in combination with phorbol 12-myristate 13-acetate (Sigma; 1 ng/ml), recombinant human IL-2 (R & D Systems; 100 units/ml), or recombinant human IL-10 (R & D Systems; 10 ng/ml). All assay conditions were performed in triplicate. Cells were incubated with [³H]thymidine (1 μ Ci per well; 1 Ci = 37 GBq) during the final 18 hr of a 5-day culture period and collected using an automated harvester. Radioactivity incorporated by the cells was measured by liquid scintillation counting. Growth inhibition by TGF- β 1 was calculated as the ratio of the mean value of radioactivity incorporated in the presence of TGF- β 1 to that in its absence. Growth stimulation by anti-TGF- β 1 was calculated as the ratio of the mean value of radioactivity incorporated in the presence of a neutralizing antibody to TGF- β 1 to that in the presence of a control antibody.

Measurement of Cell Surface TGF- β Receptors. Radioiodination of TGF- β 1 was performed according to ref. 27. Cryopreserved CLL cells were thawed and washed twice in PBS. Ten million cells were suspended in KRH binding buffer [Krebs-Ringer salts buffered with 25 mM HEPES, pH 7.5/5 mM MgSO₄/0.5% bovine serum albumin (BSA)] and incubated for 2–3 hr at 4°C with 250 pM [¹²⁵I]-TGF- β 1. Unbound ligand was removed by repeated centrifugation with binding buffer in silicon-treated tubes. Bound [¹²⁵I]-TGF- β 1 was then cross-linked to cells in 5 ml of KRH buffer containing 50 μ l of a 10 mg/ml stock solution of disuccinimidyl suberate for 15 min at 4°C and the cells were lysed in 1 ml of lysis buffer (10 mM Tris, pH 7.5/1 mM EDTA, pH 7.5/1% Triton X-100). Cell lysates were incubated with 10 μ l of specific antisera (8, 10) against the C terminus of T β RII or the juxtamembrane region of T β RI for 4 hr at 4°C. Protein A Sepharose (50 μ l) was then added for 90 min and the precipitate was collected by centrifugation and washed twice with lysis buffer. TGF- β receptors were analyzed by SDS/PAGE followed by exposure to x-ray films or to a phosphorimager screen for detection in a Fuji Phosphorimager.

Northern Blot Analysis. Total cellular RNA was isolated by the RNeasy B method (Cinna/Biotec Laboratories, Friendswood, TX), and poly(A)⁺ RNA was purified using a Poly(A)⁺ Tract Isolation System kit (Promega) according to the manufacturers' instructions. Thereafter, 4 μ g of poly(A)⁺ RNA was resolved on a 1% agarose/2.2 M formaldehyde gel before being blotted onto a nylon membrane (Hybond N, Amersham). The filter was hybridized overnight in 5 $\times$ SSC buffer containing 50% formamide at 45°C. Blots were washed at 50°C in 0.2 $\times$ SSC containing 0.1% SDS and exposed to x-ray film. A 642-bp PCR-amplified cDNA product between nt 870 and 1512 of T β RI (8) and a 902-bp PCR-amplified cDNA product between nt 802 and 1704 of T β RII (11) were labeled by the random priming method and used as probes.

RESULTS

Tritiated thymidine incorporation assays allowed us to determine whether the malignant B cells from individual CLL patients were sensitive or resistant to growth inhibition by TGF- β 1. As summarized in Table 1, 8 of the 13 CLL patients examined displayed sensitivity to TGF- β 1. This was evidenced both by a decrease in proliferation when grown in the presence of TGF- β 1 and by an increase in proliferation in the presence of a neutralizing antibody to TGF- β 1. This effect was consistently seen with all combinations of agents used to stimulate the proliferation of CLL cells. In five of the patients (2, 3, 4, 7, and 8), the malignant B cells showed complete resistance to TGF- β 1-mediated growth inhibition, again regardless of the proliferative stimulus applied to the cells.

To determine the basis of the resistance to TGF- β 1, we examined CLL cells from the five TGF- β 1-resistant patients and five of the TGF- β 1-sensitive patients for surface expres-

sion of TGF- β receptors. We employed binding of radiolabeled TGF- β and cross-linking, followed by immunoprecipitation with antisera specific for the cytoplasmic domains of T β RI and T β RII. As exemplified by Patient 1 in Fig. 1, cells sensitive to growth inhibition by TGF- β showed, as expected, normal surface expression of both T β RI and T β RII. In contrast, as exemplified by Patients 2 and 3 in Fig. 1, all CLL patients resistant to growth inhibition by TGF- β showed a complete lack of expression of cell surface T β RI able to bind and become cross-linked to radioiodinated TGF- β . This was evidenced by the amount of 125 I-TGF- β bound to surface T β RI and immunoprecipitated with anti-T β RI serum. In all patients, T β RII surface expression was normal, as demonstrated by the amount of 125 I-TGF- β bound to surface T β RII and immunoprecipitated by anti-T β RII. In the case of Patient 3, no 125 I-TGF- β bound to T β RII could be immunoprecipitated with the anti-T β RI antiserum, suggesting that either there is no T β RI on the cell surface or that surface T β RI cannot be incorporated into a complex with T β RII. In contrast, with cells from Patient 2, 125 I-TGF- β bound to surface T β RII can be immunoprecipitated with the T β RI-specific antiserum. Thus, with Patient 2, T β RI is expressed on the cell surface and can form a complex with T β RII, but cannot bind or be cross-linked to the 125 I-TGF- β ligand. Presumably Patient 2 has a mutation(s) in the extracellular domain of T β RI that affects binding to TGF- β .

To determine whether a loss of surface T β RI expression in CLL patients was due to an absence of T β RI mRNA, we used Northern blot analysis to evaluate mRNA expression. As shown in Fig. 2, we detected normal levels of both T β RI and T β RII mRNA in all freshly isolated CLL cells, including those that did not exhibit T β RI surface expression. This finding suggests that a defect in the level of transcription of the T β RI or T β RII genes was not responsible for the absence of functional surface T β RI in the five patients resistant to TGF- β -mediated growth inhibition.

DISCUSSION

Our results demonstrate a correlation between sensitivity to growth inhibition by TGF- β and expression of functional T β RI in primary tumor samples obtained from CLL patients. All TGF- β -sensitive CLL cells express both T β RI and T β RII, while CLL cells that are insensitive to TGF- β lack functional

FIG. 1. Binding and cross-linking of 125 I-TGF- β 1 to freshly isolated B-CLL cells followed by immunoprecipitation with anti-T β RI and anti-T β RII antisera. Shown are the results from three representative patients. Lanes 1 and 2 correspond to Patient 1 of Table 1, lanes 3 and 4 correspond to Patient 2, and lanes 5 and 6 correspond to Patient 3. CLL cells from Patient 1 exhibit surface expression of T β RII and T β RI, whereas cells from Patients 2 and 3 exhibit normal surface expression of T β RII but no T β RI is able to bind TGF- β .

FIG. 2. Northern blot analysis of T β RII and T β RI expression in freshly isolated B-CLL cells. Shown are the results from three representative CLL patients. Lanes 1, 2, and 3 correspond to Patients 1, 2, and 3, respectively. The same blot was hybridized to a 32 P-labeled probe specific for T β RI, stripped and reprobed using a 32 P-labeled probe specific for T β RII, and stripped and reprobed with a 32 P-labeled probe to β -actin to verify equal loading of mRNA.

cell surface T β RI able to bind and be cross-linked to the TGF- β 1 ligand.

A heterodimeric complex of T β RI and T β RII is required for TGF- β 1 signaling, and loss of expression of either of these receptors may render cultured cells or tumor cells insensitive to TGF- β -mediated growth inhibition (13, 15, 18, 22–24). Acquired resistance to TGF- β has been observed in many human malignancies, and in all patients studied in detail these tumor cells have lost expression of cell surface T β RII. Because binding of TGF- β to cell surface T β RI requires the presence of T β RII (6–8, 13, 15, 16), these tumors might exhibit normal cell surface T β RI, which cannot be detected by standard 125 I-TGF- β binding and cross-linking assays. Importantly, loss of T β RII can account for the observed resistance to TGF- β . The mechanisms responsible for T β RII loss are varied and include deletions and rearrangements of the gene leading to loss of mRNA expression, and abnormal cytoplasmic sequestration of the protein (17, 19, 20, 28). Missense mutations within highly conserved regions of the serine/threonine kinase domain of T β RII have been implicated in TGF- β resistance in two human head and neck squamous carcinoma cell lines. In one of these cell lines, SqCC/Y1, the missense mutation resulted in defective T β RII autophosphorylation and transphosphorylation of T β RI. In the other cell line, A253, both T β RII autophosphorylation and transphosphorylation of T β RI were dramatically increased, suggesting constitutive activation of the mutant T β RII kinase. Resistance to TGF- β 1 in A253 cells might be explained by desensitization of T β RI receptors due to high levels of T β RI phosphorylation independent of TGF- β 1 treatment (29).

Recently we reported the identification of a dominant inhibitory mutation in the cytoplasmic domain of T β RII isolated from a TGF- β -resistant human cutaneous T cell

lymphoma cell line (23). The mutant receptor was expressed, together with its wild-type counterpart, only in an advanced malignant tumor; a clonally related TGF- β -sensitive cell line isolated from the same patient at an earlier, clinically indolent disease stage showed expression only of the functional wild-type T β RII. Unlike dominant inhibitory mutants of growth factor receptors, which inhibit activation of the cell surface ligand-induced oligomer (30–33), the dominant inhibitory T β RII inhibited cell surface expression of the wild-type T β RII. This was the first description of a dominant inhibitory T β RII isolated from a TGF- β -resistant tumor cell line.

In contrast, there is little evidence implicating loss of or defects in T β RI expression as an important mechanism for acquired TGF- β 1 resistance in cancer. In this regard, there is only a single report that correlates loss of T β RI expression with insensitivity to TGF- β 1; in the human prostate cancer cell line LNCaP there is a defect in T β RI protein expression due to a structural alteration in the T β RI gene that led to a loss of mRNA expression (24). Several types of mutations in the T β RI gene were generated when mink lung epithelial cells were treated with the mutagen ethylmethane sulfonate and selected for growth in the presence of TGF- β . All could be complemented by expression of wild-type T β RI. One class (R) lacked cell surface T β RI (15, 34). Three TGF- β -resistant cell lines (S mutants) expressed surface T β RI able to bind TGF- β . One T β RI mutant, (G217E) within the ATP binding site, resulted in a kinase-dead polypeptide, whereas two (G261E and G322D) resulted in nontransphosphorylatable T β RI polypeptides. Coexpression of G217E with G322D was found to complement and restore TGF- β -mediated gene induction and growth arrest (35). These results are consistent with the notion that T β RI exists as a homodimer, and that the protein kinase domain of one T β RI polypeptide interacts with the membrane-proximal GS domain of another, enabling them to be phosphorylated and activated by T β RII polypeptides.

Our study of CLL, which used primary tumor samples and not cell lines, correlates insensitivity to TGF- β 1 with defects in functional T β RI surface expression, but not loss of either T β RI or T β RII mRNA. In at least one patient (Patient 2), some T β RI is expressed on the cell surface and can form a complex with T β RII, but cannot bind or be cross-linked to TGF- β 1. Our results indicate that loss of surface T β RI receptor proteins able to bind TGF- β and form a signaling complex with T β RII may be another common mechanism of acquired TGF- β 1 resistance in human cancer. It is likely that defects in functional cell surface T β RI are responsible for the resistance to TGF- β 1 observed in some cases of CLL (5, 8, 13, 14, 24). In support of this, sensitivity to TGF- β -mediated growth inhibition was restored in the LNCaP cell line by transfection with T β RI cDNA (24).

Because our work involves nonimmortal primary tumor samples, we are unable to transfect these cells with wild-type T β RI cDNA and select those cells that express the wild-type protein. However, cultured tumor cell lines may have acquired secondary mutations that are not relevant to growth control of the primary tumor cells. Unlike LNCaP cells (24), CLL cells lacking functional surface T β RI express normal amounts of T β RI mRNA. The absence of functional T β RI protein on the surface of CLL cells, despite normal mRNA expression, might be explained by point mutations in T β RI that lead to premature stop codons and truncated proteins, or point mutations that generate mutant T β RI proteins that are unstable or incapable of appearing on the plasma membrane or that cannot bind ligand or interact with T β RII. Since we have not yet cloned and sequenced the T β RI mRNAs from the TGF- β -resistant CLL cells, we do not know whether these cells contain wild-type as well as mutant T β RI mRNA. If both wild-type and mutant mRNAs are present, it would imply that a mutant T β RI protein can dominantly inhibit the function of

the wild-type protein in CLL, analogous to the dominant inhibitory T β RII protein we described in the malignant progression of a cutaneous T cell lymphoma (23). The significance of a dominant inhibitory mutant TGF- β receptor in the development of human cancer is that only one of the two alleles encoding the receptor must have been mutated. With the exception of the cutaneous T cell lymphoma we studied (23), when T β RII expression is lost in a tumor both T β RII alleles have undergone loss or mutation.

Our study, which included 13 patients representing all stages of CLL, did not show any obvious correlation between loss of T β RI surface-protein expression and stage of disease. It will be of interest to study other patients with CLL to determine if loss of T β RI surface expression is a predictor of outcome in this most common adult leukemia, or if it contributes to the histologic transformation to an aggressive large cell lymphoma observed in some patients (1, 2).

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