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Loss of receptors for transforming growth factor β in human T-cell malignancies

(lymphomatoid papulosis/growth inhibitors/tumor suppressor genes/growth factor receptors)

MARSHALL E. KADIN*†, MARC W. CAVAILLE-COLL*, ROBERT GERTZ*, JOAN MASSAGUÉ‡§, SELA CHEIFETZ‡, AND DIANE GEORGE‡

*Department of Pathology, Beth Israel Hospital and Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215; and ‡Cell Biology and Genetics Program and §Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

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ABSTRACT **Ki-1 (CD30)⁺ cutaneous T-cell lymphomas (CTCLs) are slowly progressive lymphomas in which initial spontaneous regression is often observed. To better understand the mechanisms of spontaneous regression and eventual tumor progression in Ki-1⁺ CTCLs, type β transforming growth factor (TGF-β)-mediated growth inhibition of clonally related cell lines derived from two time points, before and after tumor progression, was studied. TGF-β1 inhibited colony-forming efficiency (CFE) of a cell line (Mac-1) derived from clinically indolent Ki-1⁺ CTCLs but failed to inhibit CFE of Mac-2A and -2B cell lines from advanced CTCLs. To determine the basis for TGF-β1 resistance in advanced CTCL cells, we looked for possible defects in the expression of cell surface TGF-β receptors. Mac-1 cells were found to express TGF-β receptors I and II, which mediate growth inhibition, and the TGF-β-binding proteoglycan betaglycan. In contrast, receptors I and II were not detected in CTCL lines Mac-2A and -2B even though these cell lines did express betaglycan. Various treatments that unmask or induce TGF-β receptors in other cells failed to show evidence for these receptors in advanced CTCL cells. Loss of TGF-β receptor expression in these cells correlated with a marked decrease in TGF-β receptor II mRNA levels. Loss of cell surface TGF-β receptors was also found in two of five other patients with T-cell lymphomas including the Sezary syndrome and a noncutaneous T-cell lymphoma, suggesting that loss of TGF-β receptor expression may be a recurrent feature of human T-cell malignancies.**

Ki-1⁺ cutaneous T-cell lymphomas (CTCLs) comprise a spectrum of slowly progressive lymphoproliferative disorders in which skin lesions initially regress spontaneously but eventually persist and metastasize to regional lymph nodes (1). The most benign of these disorders, lymphomatoid papulosis, was first reported as a continuing self-healing eruption that is histologically malignant but clinically benign and able to remain without clinical progression for decades (2). However, 10–20% of these patients develop a systemic malignant lymphoma during an average 13.5-year period (3–5). Patients with regressing atypical histiocytosis whose skin lesions resemble a high grade malignant "histiocytic" neoplasm but who have an initially indolent clinical course (6) may subsequently develop disseminated lymphoma (7), and patients with Ki-1⁺ anaplastic large cell lymphomas who present with initially regressing skin lesions may subsequently have tumor cell infiltration of regional lymph nodes and progressive lymphoma (8, 9). Immunophenotypic and molecular genetic studies have shown that all of these disorders (lymphomatoid papulosis, regressing atypical histiocytosis, and Ki-1⁺ anaplastic large cell lymphoma) are histogenetically related disorders derived from activated T cells, which express activation antigens CD30, CD25, CD71, and HLA-DR (1, 7, 8). Because the clinical behavior of these disorders cannot be predicted from histopathologic criteria—all appear malignant—it is necessary to seek new prognostic criteria based on the molecular characteristics of the atypical cells.

To this end, we studied the growth regulation of clonally related tumor cell lines derived from a clinically indolent stage and an advanced tumor-forming stage of disease in a patient whose disease progressed from lymphomatoid papulosis to a Ki-1⁺ anaplastic CTCL. Our studies indicate that the growth of a tumor cell line from a clinically indolent stage of disease could be suppressed by low concentrations of type β transforming growth factor (TGF-β), a multifunctional growth factor that can inhibit proliferation of cells from various lineages (for reviews, see refs. 10 and 11), limit the clonal expansion of activated lymphocytes (12), and induce extracellular matrix production in cultured cells and after subcutaneous injection in test animals (13–15). The biological activity of TGF-β could explain both the regression of T-cell clones and the subsequent healing of skin lesions with fibrosis in this patient's Ki-1⁺ CTCLs. In contrast, two tumor cell lines derived from an advanced stage of disease in this patient were resistant to the growth inhibitory effect TGF-β. This resistance in advanced disease was associated with a specific loss of TGF-β receptor expression, a defect also observed in two other T-cell lymphoma lines. Thus, diminished TGF-β receptor expression may be involved in the pathogenesis and progression of T-cell malignancies.

MATERIALS AND METHODS

Case History. Details of the patient's clinical history, pathology, and cell lines have been described (16). Briefly, a 43-year-old white male presented in 1983 with a history of lymphomatoid papulosis diagnosed in 1971 and mixed cellularity Hodgkin disease involving inguinal lymph nodes, stage IIA, treated to remission with radiation to inverted Y, splenic pedicle, and mantle fields in 1975. He continued to experience spontaneously regressing skin lesions with the morphology and immunophenotype of lymphomatoid papulosis, type A (17). In 1985, he developed a new erythroderma with circulating Sezary-like cells, while skin nodules continued to appear and regress spontaneously. Biopsies of several skin nodules again revealed lymphomatoid papulosis, type A, while biopsies of the erythroderma revealed an epidermotropic CTCL. A cell line (Mac-1) was developed from Sezary-like cells in the blood. In 1987, clinical progression of disease

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Abbreviations: TGF-β, type β transforming growth factor β; CTCL, cutaneous T-cell lymphoma; CFE, colony-forming efficiency; TβR-I and -II, TGF-β receptors I and II.

†To whom reprint requests should be addressed.

was evident from persistent enlarging skin tumor nodules with increased numbers of large lymphoid cells, interpreted as large cell lymphoma. Cell lines Mac-2A and -2B were derived from separate skin tumors. The patient's clinical status continued to deteriorate with development of a malignant pleural effusion and multiple infections to which he succumbed in November 1988. Autopsy showed involvement of retroperitoneal lymph nodes by a Ki-1$^+$ anaplastic large cell lymphoma (18).

Tissue Culture. Clonal tumor cell lines were developed from early regressing (Mac-1) and advanced tumor-forming (Mac-2A, -2B) stages of this patient's disease. The Mac-1 cell line contains a majority of small lymphoid cells and few large Reed–Sternberg-like cells. Mac-1 cells have the phenotype of activated helper T cells (CD2$^+$, CD4$^+$, CD25$^+$, CD30$^+$, HLA-DR$^+$). Cell lines Mac-2A and -2B contain a higher proportion of large Reed–Sternberg-like cells and have a less well differentiated phenotype than Mac-1 cells, having lost antigens CD2 and CD4, and expressing HD-associated antigen CD15. Moreover, while most Mac-1 cells express the low-affinity interleukin 2 (IL-2) receptor (CD25), very few cells in lines 2A and 2B do. Cell lines Mac-2A and -2B did not require IL-2 for their initiation. All three cell lines are clonally related as revealed by a common balanced translocation t(8;9)(p22;p24) and the same T-cell receptor α-chain gene rearrangement (16).

Five additional tumor cell lines from patients with cutaneous and noncutaneous T-cell lymphomas were studied. Cell lines Se-Ax and HUT 78 were derived from peripheral blood of patients with the Sezary syndrome, an erythrodermic form of CTCL (19, 20). OCILy 13.2 was derived from bone marrow tumor cells at relapse of a patient with a noncutaneous diffuse large T-cell lymphoma (21). JB6 and SR786 were derived from tumor cells in the blood (JB6) and brains (SR786) of children with noncutaneous Ki-1$^+$ anaplastic large T-cell lymphomas (22). All cell lines are maintained in complete RPMI 1640 medium (GIBCO) with 15% heat-inactivated fetal bovine serum.

Colony-Forming Assays in Methylcellulose. Mac-1, -2A, and -2B lymphoma cells at a viability of >95% were adjusted to a final concentration of 500, 1000, or 2000 cells per 100 μl in 0.8% methylcellulose containing 20% fetal bovine serum and Iscove's modified Dulbecco's medium with penicillin, streptomycin, and Fungizone. Aliquots (100 μl) of the methylcellulose cell mixture were plated into individual microtiter plate wells and placed in a humidified incubator maintained at 37°C containing 5% CO_2/95% air. Human TGF-β1 derived from platelets (R & D Systems) was added just before plating. A murine monoclonal antibody (1D11.16), which neutralizes the bioactivity of human TGF-β1 and TGF-β2 (generously provided by Larry Ellingsworth, Collagen Corp.), was added to cultures in some experiments. The 1D11.16 antibody neutralizes the growth inhibitory effect of TGF-β1 on mink lung epithelial cells and thymocytes (23). Culture plates were inspected for colony formation beginning at 4 days and every 2 days thereafter. Colonies containing >20 cells were counted by using a phase-contrast microscope. Assays were done in quadruplicate.

TGF-β Receptor Assays. Porcine TGF-β1 and -β2 were from R & D Systems. ^{125}I-labeled TGF-β1 (^{125}I-TGF-β1) was prepared as described (24). Before incubation with ^{125}I-TGF-β1, cells were washed for 30 min at 37°C in binding buffer (Krebs–Ringer salts buffered with 25 mM Hepes, pH 7.5) containing 0.5% bovine serum albumin (BSA). For some studies, washed cells were pretreated for 2 min at 4°C with pH 3 buffer according to the procedure of Birchenall-Roberts *et al.* (25) in order to unmask occupied receptors.

For affinity labeling, cells were resuspended in binding buffer (24) with BSA and incubated 3 h at 4°C with ^{125}I-TGF-β1 in the presence or absence of native TGF-β1 or -β2. Unbound ligand was removed by repeated centrifugation from binding buffer or by centrifugation (15 min × 3000 rpm) through a 1-ml solution of 7% sucrose in protein-free binding buffer in silicon-treated tubes. Bound ^{125}I-TGF-β1 was cross-linked to the cells with disuccinimidyl suberate (24). Triton extracts from labeled cells were analyzed by SDS/PAGE and autoradiography.

For TGF-β receptor II (TβR-II) mRNA assays, total RNA was prepared by guanidine hydrochloride extraction using the RNazol B isolation method (Biotecx Laboratories, Houston). The integrity and quantitation of the RNA preparations were confirmed by agarose gel electrophoresis of aliquots and staining with ethidium bromide. cDNA was prepared from 5 μg of total RNA using random hexamers as reverse transcriptase primers. The cDNA was amplified by PCR using two sets of primer oligonucleotides designed according to the human TβR-II sequence (26). These include a set of oligonucleotides (204, CCGGGATCCCGCTTTGCTGAGGTC-TATAAGGC; 104, CCGGAATTCCAGGGTCCCGCACCT-TGGAACC) that amplify a 685-bp region within the cytoplasmic kinase domain of the receptor cDNA and a set of oligonucleotides (208, CCGGGATCCCCGCACGTTCA-GAAGTCG; 108, CCGGAATTCCAGGATTGCTGGTGT-TATA) that amplify a 365-bp region within the extracellular domain of the receptor cDNA (26). Amplification was performed on a Perkin–Elmer thermal cycler using the GeneAmp PCR kit. The cycling parameters were 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 35 cycles, followed by a 72°C incubation for 10 min at the end.

Northern blots were prepared by blotting gels onto a nylon membrane (Biotrans, ICN) and probing with a ^{32}P-labeled (Megaprime DNA labeling system, Amersham) cDNA probe encoding the full-length TβR-II (26, 27).

Immunohistochemical Detection of TGF-β1. Tissues were stained with a rabbit polyclonal IgG antibody, anti-CC(1-30) (kindly provided by L. Ellingsworth, Collagen Corp.), which reacts with the secreted form of TGF-β1 (28). Details of the immunoperoxidase method have been described (29).

RESULTS

Loss of TGF-β Responsiveness Associated with Tumor Progression. Colony-forming efficiency (CFE) without growth factors or antibodies against growth factors added was 29% (range, 24–37%) for Mac-1, 29% (range, 23–35%) for Mac-2A, and 39% (range, 32–48%) for Mac-2B cell lines. CFE of cell line Mac-1 from clinically indolent disease was inhibited by >70% in the presence of 1.0–10.0 ng of TGF-β1 (Fig. 1).

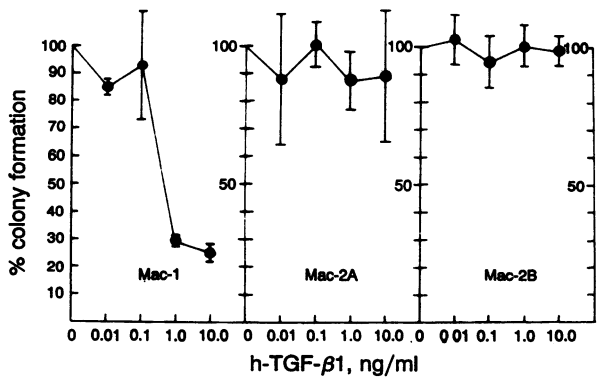

FIG. 1. Differential inhibition of Mac-1, -2A, and -2B colony formation by human (h) TGF-β1. Colonies of 20 or more lymphoma cells were counted on day 8 after plating 1000 cells per well in 0.8% methylcellulose. Bars show SD of four experiments. Percentage colony formation compared to control (100%) is shown.

In contrast, CFE of cell lines Mac-2A and -2B from advanced stage disease was not inhibited by these concentrations of TGF-β1. TGF-β1 appeared to be an autocrine inhibitory growth factor for Mac-1 cells since CFE of these cells was increased 2-fold in the presence of monoclonal antibody 1D11.16, which neutralizes the inhibitory effect of TGF-β1 (data not shown).

Presence of TGF-β in Lymphoma Tissues. A skin tumor nodule adjacent to the tumors from which cell lines 2A and 2B were derived was stained with antibody CC(1-30), which detects secreted TGF-β (28, 29). TGF-β staining was abundant in the extracellular matrix and on the surface of some tumor cells in this nodule (Fig. 2), suggesting that failure of skin nodules to regress was not due to a lack of local secretion of TGF-β.

Loss of TGF-β Receptors. Affinity labeling of Mac-1 cells with ^{125}I-TGF-β1 revealed that these cells express the TGF-β binding proteoglycan, betaglycan, and TβR-I and -II (Fig. 3). Ligand competition assays indicated that TGF-β1 bound to all three proteins with high affinity and specificity, which is typical of these receptors in other cell lines (ref. 11; see also below). The TGF-β-resistant cell lines Mac-2A and -2B expressed betaglycan at levels higher than those in Mac-1 cells but did not show detectable TβR-I or -II (Fig. 3).

Cell line Mac-2A was examined in more detail to determine whether a low level of TβR-I and -II might exist in these cells that escaped detection in the previous experiments. Experiments were done using higher concentrations of iodinated ligand in order to optimize the visualization of any TGF-β receptors present in Mac-2A. When Mac-1 cells and Mac-2A cells (Fig. 4) were labeled with 100 pM ^{125}I-TGF-β1 in the presence of increasing concentrations of native TGF-β1 or -β2, the labeling of betaglycan was competed for by TGF-β1 and somewhat more potently by TGF-β2. This competition pattern is typical of endogenous betaglycan in some other cell lines (11, 24, 30–32) and molecularly cloned betaglycan expressed in transfectant cell hosts (33). TβR-I and -II in Mac-1 cells showed a higher affinity for TGF-β1 than for TGF-β2 (Fig. 4), as is typical of these receptors in all other cell lines described (11, 24, 31). Various low molecular weight bands labeled in Mac-2A cells resembled betaglycan rather than receptor I or II in their labeling competition pattern; TGF-β1 and -β2 were equally effective at competing for the labeling of all these bands (Fig. 4). These minor bands might derive from limited cleavage of the labeled betaglycan present in the Mac-2A samples.

We also investigated whether the loss of detectable TβR-I and -II in Mac-2A cells might be due to masking of these

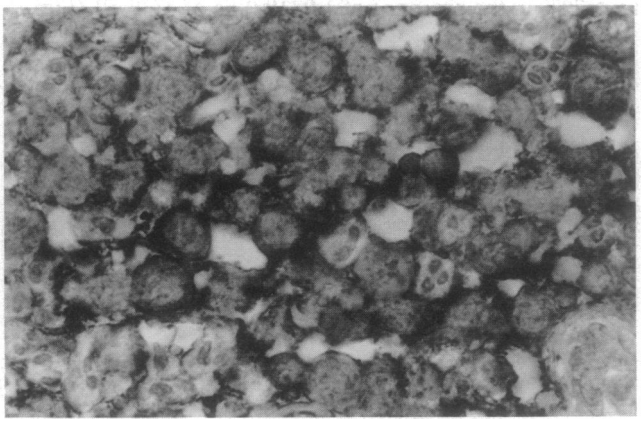

FIG. 2. Immunoperoxidase stain of human TGF-β1 as dark precipitate on the surface of tumor cells and extracellular matrix in skin adjacent to tumors from which Mac-2A and -2B cell lines were derived. Paraffin-embedded tissue sections were stained with polyclonal antibody CC(1-30). (×460.)

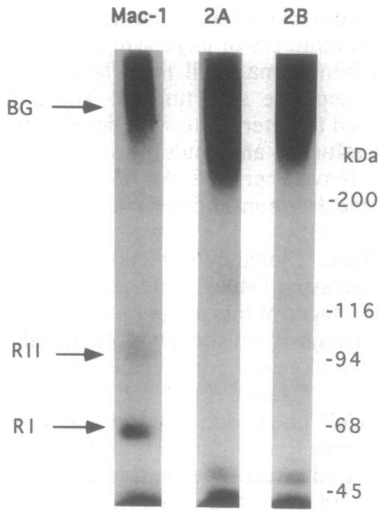

FIG. 3. TGF-β receptor phenotype in Mac-1, -2A, and -2B cells. Cells were affinity-labeled in suspension with 25 pM ^{125}I-TGF-β1, and detergent extracts were subjected to gel electrophoresis and autoradiography. TβR-I (RI), TβR-II (RII), and betaglycan (BG) are indicated. Positions of protein markers are indicated with their molecular mass (kDa).

receptors by endogenous TGF-β. Cells were pretreated with acidic buffer conditions, which unmask TGF-β receptors occupied by endogenous ligand (25). Acidification of Mac-2A cells following this procedure did not reveal receptor I or II (data not shown). Furthermore, treatment of Mac-2A cells with phytohemagglutinin, which has been reported to upregulate TGF-β receptors on primary cultures of thymocytes (34), had no effect on the TGF-β receptor levels of these cells (data not shown).

Loss of TβR-I and -II was also found for Sezary line Se-Ax and noncutaneous T-cell lymphoma line OCILy 13.2 (Table 1). In contrast, no receptor loss was detected in Sezary cell line HUT 78 or Ki-1$^+$ lines JB6 and SR786 from noncutaneous anaplastic large cell lymphomas.

TGF-β receptor loss was correlated with a marked decrease in TβR-II mRNA levels in T-lymphoma cells, as determined by Northern blot assay. TβR-II mRNA was detectable in Mac-1 cells but not in Mac-2A and Mac-2B (Fig. 5). In the Se-Ax and OCILy 13.2 cell lines, which showed no TβR-I and -II by affinity labeling, TβR-II mRNA was de-

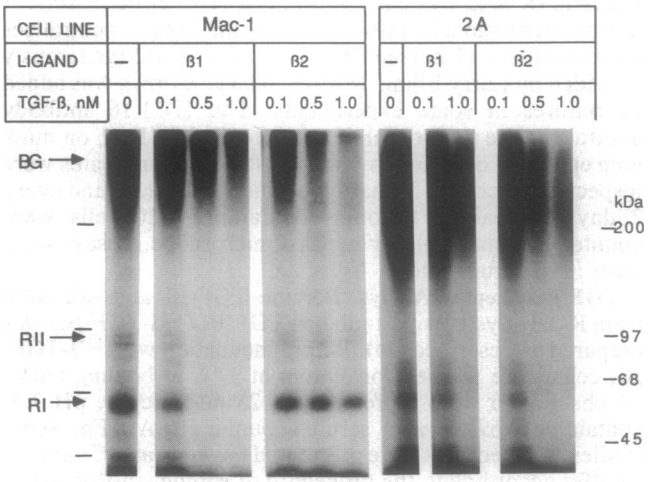

FIG. 4. TGF-β receptor competition profiles. Mac-1 and -2A cells were affinity-labeled with 100 pM ^{125}I-TGF-β1 alone or in the presence of the indicated concentrations of unlabeled TGF-β1 or -β2. RI, TβR-I; RII, TβR-II; BG, betaglycan.

Table 1. Affinity labeling, Northern blotting, and PCR data in T-cell lymphoma lines tested

Cell line	Binding data	Northern blot	PCR 208–108	PCR 204–104
A549	+	+	+	+
Mac-1	+	+	+	+
Mac-2A	−	−	+	+
Mac-2B	−	−	+	+
OCILy 13.2	−	+	+	+
Se-Ax	−	+	+	+
HUT 78	+	+	+	+
JB6	+	+	+	+
SR786	+	+	+	+

PCR 208–108 amplifies a 365-bp region within the extracellular domain of TβR-II. PCR 204–104 amplifies a 685-bp region within the cytoplasmic kinase domain of TβR-II.

tected by Northern blot assay (Table 1). The HUT 78, JB6, and SR786 cell lines, which showed TGF-β receptors by affinity labeling, also showed TβR-II mRNA by Northern blot assay (Table 1).

To increase the sensitivity of the receptor mRNA assays, reverse transcription PCRs were carried out with primers that amplify fragments of 685 and 365 bp corresponding to independent regions in the human type II receptor kinase domain and extracellular domain, respectively. Using equivalent amounts of RNA from each T-lymphoma cell line and from the A549 human lung adenocarcinoma cell line that expresses TGF-β receptors (35) as a positive control, fragments of the appropriate size could be amplified from all the cell lines tested, including Mac-2A and -2B cells, which gave negative results in the Northern blot assays (Table 1). These results show that the type II receptor is expressed in these cells at very reduced levels (<10% of that expressed in Mac-1 cells), which are detectable by the more sensitive reverse transcriptase PCR assay but not by Northern blotting.

DISCUSSION

The present results show that tumor progression in a human Ki-1$^+$ CTCL was associated with loss of TGF-β binding to receptors I and II and loss of TGF-β growth inhibitory responses. Tumor progression apparently was not correlated with the absence of TGF-β, which could be demonstrated immunohistochemically in tumor tissue. The patient from whom these cell lines were derived had a long history of spontaneously regressing skin lesions derived from activated T cells. An abrupt change in the clinical course of this patient was marked by persistence and enlargement of skin lesions with dissemination to distant lymph nodes. The development of clonally related tumor cell lines at two time points, before and after tumor progression, allowed us to study molecular events that might explain this change in clinical behavior.

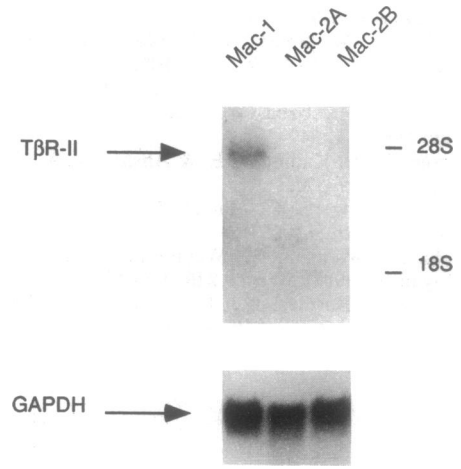

FIG. 5. Northern blot showing lack of detectable TβR-II mRNA in cell lines Mac-2A and -2B. Amount and integrity of RNA in each lane were confirmed by ethidium bromide staining. Filter was stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a control for mRNA loading.

The Mac-1 cell line derived from clinically indolent disease is growth-inhibited by low concentrations of TGF-β1. Cell lines Mac-2A and -2B derived from advanced disease are resistant to inhibition by TGF-β1. These two cell lines are clonally derived from the more benign Mac-1 cell line, as demonstrated by the presence of the same T-cell receptor α-chain gene rearrangement and a common balanced translocation, t(8;9)(p22;p24) (16). Development of TGF-β resistance in this lymphoma is associated with loss of TGF-β binding to receptors I and II. In these cell lines, this loss was associated with decreased expression of TβR-II mRNA. The concomitant loss of TGF-β binding to receptor I is probably due to the dependence of this receptor on receptor II for ligand binding (27). This observation suggests that an escape from the growth suppressive function of TGF-β in these cells may have contributed to the acquisition of their overtly malignant phenotype. The initial ability of this lymphoma's lesions to spontaneously regress might be induced by autocrine or paracrine TGF-β from the surrounding stroma. Without TGF-β receptors, the ability to regress in response to TGF-β would be lost.

Our results indicate that the Mac-2A and -2B cells, which have lost their ability to respond to TGF-β, have lower levels of TβR-II message than a TGF-β-responsive cell line. Like other lymphoid cells, Mac-1 cells have relatively low numbers of TGF-β signaling receptors (36–38). Therefore, a 90% reduction in the level of TβR-II mRNA expression in these cells would result in a level of message that is undetectable by Northern blot assay and a number of cell surface receptors that is too low to be detected by affinity labeling and likely insufficient to mount a response to TGF-β.

Loss of TGF-β receptors was found as well in Ki-1$^-$ malignant T-cell lines from patients with Sezary syndrome and a noncutaneous T-cell lymphoma. In these cell lines, TβR-II mRNA was detected by Northern blot assay, indicating that the defect in TβR-II expression occurs at the level of the protein. A previous study demonstrated low levels of TGF-β receptors in cell line HUT 78 and in human T-lymphotropic virus type I-infected malignant T-cell lines HUT 102 and MT-2 (37). We found that the HUT 78 cells demonstrated TβR-II mRNA by Northern blotting as well as TGF-β receptors by affinity labeling, in contrast to Mac-2A and -2B, OCILy 13.2, and Se-Ax cells, which did not demonstrate receptors by affinity labeling.

These results show that loss of TGF-β cell surface receptor expression can be found in both cutaneous and noncutaneous T-cell malignancies. In some cell lines, this loss occurs via a marked decrease in receptor mRNA expression; in other cell lines, it occurs via a defect in the levels or activity of receptor protein. Further studies are needed to determine the complete clinical and pathologic spectrum of T-cell lymphomas in which there is a loss of TGF-β receptor expression and the mechanism by which this loss occurs.

Loss of TGF-β receptor expression is not confined to T-cell lymphomas and was previously described in cell lines from B-cell lymphomas (37), Hodgkin disease (38), and Epstein–Barr virus-transformed B-cell lines (39). Other examples of transformed cells that have lost TGF-β receptor expression have been described (35, 40, 41), but a specific association of this loss with tumor progression was not documented.

TGF-β receptor expression in some B-cell lymphoma lines is low but can be induced by cell treatment with phorbol 12-myristate 13-acetate (37). Receptor components I and II of T-cell lymphoma lines Mac-2A and -2B remained undetectable after attempts to upregulate or unmask occupied receptors. Because TGF-β inhibits the growth of nonmalignant T and B cells (12), diminished expression of TGF-β receptors is likely to contribute to the unregulated growth of lymphocytes in malignant lymphomas.

These studies suggest that loss of TβR-I and -II expression may be a prognostic marker occurring during the progression of some human lymphomas derived from activated T lymphocytes. This marker has been available only in cell lines in which receptor binding can be measured directly. The development of reagents to analyze TGF-β receptor genes and their expression directly in tissues may provide another means of determining prognosis in human lymphomas.

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