Signal Transduction Abnormalities in T Lymphocytes from Patients with Advanced Renal Carcinoma: Clinical Relevance and Effects of Cytokine Therapy

Ronald M. Bukowski, Patricia Rayman, Robert Uzzo, Tracy Bloom, Kati Sandstrom, David Peereboom, Thomas Olencki, G. Thomas Budd, Denise McLain, Paul Elson, Andrew Novick, and James H. Finke

Experimental Therapeutic Program, Cleveland Clinic Cancer Center [R. M. B., K. S., D. P., T. O., G. T. B., D. M., J. H. F.] and Departments of Immunology [R. M. B., P. R., T. B., J. H. F.], Biostatistics [P. E.], Hematology and Medical Oncology [R. M. B., D. P., T. O., G. T. B.], and Urology [R. M. B., R. U., A. N., J. H. F.], Cleveland Clinic Foundation, Cleveland, Ohio 44195

ABSTRACT

Studies have demonstrated abnormalities of the CD3/T-cell antigen receptor (TCR) and pathways of signal transduction in T lymphocytes from animals and patients with advanced malignancy. Diminished expression of TCRγ and p56^ck^ that are associated with the TCR and reduced nuclear localization of RelA containing nuclear factor κB (NFκB) complexes have been noted. These defects have been described in T cells from patients with malignant melanoma, renal cell carcinoma (RCC), ovarian cancer, and colorectal cancer. Preliminary observations also indicate possible correlation with clinical variables such as stage in selected instances. To further characterize altered expression of TCRγ, p56^ck^, and impaired activation of NFκB, T lymphocytes were obtained from 65 patients with RCC, the majority of whom were receiving combination cytokine therapy [interleukin (IL)-2, IFNα-containing regimens] and 37 control individuals. In 29 of these patients, levels of TCRγ and p56^ck^ were determined by Western blots of T-cell lysates and semiquantitated using densitometry. Relative levels were then correlated with a series of clinical variables including response to therapy, performance status, survival, disease sites, age, and others. In another group of 28 patients, and overall, reduced levels were noted visually in 12 of 29 (41%) and 13 of 29 (45%) individuals, respectively. When levels were semiquantitated using densitometry, significant decreases of TCRγ (P = 0.029) and p56^ck^ (P = 0.029) but not CD3ε (P = 0.131), compared with control levels, were found. In patients treated with IL-2/IFNα-based therapy, relative levels of TCRγ increased significantly (P = 0.002) on day 15 of cycle one compared with the baseline. Correlations of TCRγ or p56^ck^ levels with response or disease variables, except for lower TCRγ levels (P < 0.001) in the presence of bone metastases, were not found. Abnormal NFκB activation after stimulation with phorbol myristate acetate/ionomycin and/or anti-CD3 monoclonal antibody was found in 59% of patients (17 of 28) and was not accounted for by the advanced age of the study cohort. Activation of NFκB in peripheral blood T cells was inducible during cytokine therapy in four of six individuals who displayed impaired NFκB activity prior to therapy. Moreover, impaired activation of NFκB does not appear linked to a reduction of TCRγ expression, because in five patients, normal TCRγ levels were present although κB binding was not inducible. In the majority of patients with advanced RCC, peripheral blood T cells express TCRγ and p56^ck^, and in a subset, reduced levels of these TCRγ-associated molecules are seen that may increase during cytokine-based therapy. Abnormal activation of NFκB is also present in >50% of patients and may also revert to normal during IL-2/IFNα-based treatment. This alteration in NFκB activation occurred in the presence of normal expression of TCRγ-associated signaling elements. The clinical significance of these findings remains unclear.

INTRODUCTION

Abnormalities of the CD3/TCRγ (TCRγ) and signal transduction pathways have been described in splenic and tumor-infiltrating lymphocytes obtained from tumor-bearing animals (1) and patients with advanced cancer (2). These include defects in the expression of the TCRγ chain and protein tyrosine kinases, e.g., p56^ck^, that are associated with the TCRγ. These alterations result in impaired tyrosine phosphorylation after li-
gation of the CD3 complex. Although less frequent, similar findings in peripheral blood T-cells from melanoma (3, 4), RCC (2, 5), ovarian carcinoma (6), Hodgkin’s disease (7), and colorectal cancer (8) patients have also been reported and in the last instance may be correlated with disease stage (9). Recently, we and others have reported that T cells from tumor-bearing animals (10) and RCC patients (11) also have reduced 6B sequence motif-specific binding activity. The major alteration appears to be in the nuclear localization of Rel A containing NFκB complexes after T cell activation.

Altered expression of various signal transduction molecules may be associated with functional defects noted in T cells isolated from tumor-bearing hosts (4) and may be a contributing factor responsible for the inability to develop an effective anti-tumor immune response. Defects in proliferation (12, 13), lytic capacity (12), and cytokine secretion (14) have been described for tumor-infiltrating lymphocytes derived from patients with various types of solid tumors. In mice with transplantable neoplasms (RENCa and MCA-38), tumor progression is associated with a reduction in expression of signaling elements and a decrease in production of the TH1 cytokines IL-2 and IFNγ by splenic T cells (15). Likewise, in patients with metastatic melanoma, whose T cells displayed reduced TCRγ expression, diminished production of IL-2 and IFNγ (4) has been observed. Moreover, studies in animals suggest that cytokine-based Immunotherapy may result in the reversal of some signaling defects (15). RCC represents a tumor in which cytokine therapy is used frequently; however, the clinical significance of the observed signaling abnormalities and the effects of cytokine therapy on these findings are unclear. The present study was therefore initiated to further characterize the frequency of TCRγ and p56κκ abnormalities in peripheral blood T cells from patients with RCC, correlate these abnormalities with clinical characteristics, and investigate alterations occurring during cytokine therapy. Additionally in view of recent findings cited previously, the frequency of abnormal NFκB activation in T cells from RCC patients was studied. The results demonstrate reduced expression of TCRγ and p56κκ in T cells from a subset of patients with advanced RCC and possible increases in TCRγ expression during therapy with rIL-2- and IFNa-containing regimens. Abnormal activation of NFκB was also found in 59% of patients, which reverted to a normal pattern in some individuals during cytokine therapy. Moreover, the alterations in signal transduction that are associated with defective NFκB activation are distinct and downstream from early CD3/TCR-linked signaling events.

MATERIALS AND METHODS

Patient Selection. Patients with metastatic and/or recurrent renal cell carcinoma, the majority of whom were treated in a series of Phase I or II clinical trials, were studied. A total of 65 patients were investigated. The treatment regimens administered to the various groups are outlined in Table I. Peripheral blood was sampled pretherapy and at various time points during and after treatment (see Table I). Informed consent was obtained in accordance with institutional and National Cancer Institute guidelines.

Control Subjects. Peripheral blood T cells were obtained from 37 healthy donors to serve as controls. In studies using Western blotting, whole-cell lysate from the same healthy donor was included in each experiment when TCRγ, CD3ε, and p56κκ from patient T cells were analyzed. To evaluate the variation in signaling element expression among individuals, cell lysates from five healthy donors were run in the same experiment. To assess variations in the levels of TCRγ, p56κκ, and CD3ε over time, these signaling elements were measured in three additional normal donors at three separate time points. T-cell nuclear lysates from 20 different healthy individuals were used as controls when NFκB activation was evaluated in patient T cells. These T cells were obtained from redundant leukopheresis samples (Cleveland Clinic Blood Bank). Finally, the level of stimulus-dependent 6B binding activity from RCC patient T cells was compared with an age-matched control group (n = 8).

Isolation of PBL-T. PBLs were isolated, and PBL-Ts were purified as described previously (2, 10). Briefly, PBLs were subjected to Ficoll-Hypaque (LKD Biotech, Piscataway, NJ) density gradient centrifugation and depleted of B cells, natural killer cells, and macrophages by negative selection using magnetic separation with microbeads coated with antibodies to

### Table I

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Dose (s)</th>
<th>Schedule</th>
<th>No. of patients</th>
<th>Type of trial</th>
<th>Day of PBL study</th>
</tr>
</thead>
<tbody>
<tr>
<td>A rIL-2/rHuIFNa2b</td>
<td>37.5 MIU/m2/day</td>
<td>CIV d1-5, 8-12, 15-19, 22-26</td>
<td>4</td>
<td>Phase I/II</td>
<td>8 to 12 weeks postnephrectomy or surgery for metastatic disease</td>
</tr>
<tr>
<td>rHuIFNa2b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B rIL-2/rHuIFNa2a ± 5-FU</td>
<td>5.0 MIU/m2/day</td>
<td>SQ d1-5, 8-12, 15-19, 22-26</td>
<td>12</td>
<td>NA</td>
<td>Prior to surgery or therapy for metastatic disease</td>
</tr>
<tr>
<td>rHuIFNa2a</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SFU</td>
<td></td>
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</tr>
<tr>
<td>C None</td>
<td>NA</td>
<td>NA</td>
<td>11</td>
<td>Pilot study, adjuvant rIL-2</td>
<td></td>
</tr>
<tr>
<td>D None</td>
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<td>NA</td>
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Fig. 1  Western blots demonstrating levels of TCRζ, p56^ck, and CD3ε in four patients compared with a control individual (+). Corresponding densitometry data are included illustrating the differences between patients and controls (□, patient; ■, control). Representative data illustrate various patterns in which T cells from one patient (S. F.; Pt. SF) display normal levels of signaling elements, those from two patients (D. I. and W. A.; Pt. Di and Pt. WA, respectively) display reduced levels of TCRζ, and those from one patient (D. G.; Pt. DG), which display no detectable levels of TCRζ and p56^ck.

CD19, CD16, and CD14 (Miltenyi Biotec, Inc., Sunnyvale, CA). The cell populations isolated (PBL-T) contained >90% cells positive for CD3 as determined by immunostaining.

**Western Blot.** PBL-Ts were lysed by incubation in lysis buffer (PBS (calcium and magnesium free), 10 mM sodium phosphate, 20 mM HEPES, 1% Triton X-100, 100 mM NaCl, 1 mM ZnCl₂, 1 mg/ml NaF, 100 µg/ml PMSF, 200 µg/ml chymotrypsin, 100 µg/ml Pefabloc, and 200 µg/ml trypsin/chymotrypsin inhibitor, 1 mM sodium orthovanadate, 2 µg/ml aprotinin, and 2 µg/ml leupeptin) for 15 min at 4°C. The lysate was centrifuged at 12,000 rpm for 10 min at 4°C, after which the supernatant was carefully harvested to not disturb the pellet. Cellular lysates (10–20 µg of equivalent loading in each one) were electrophoresed in 2% (for TCR) or 7.5% (for p56^ck) SDS page under reducing conditions and then transferred to NitroBind (Nitrocellulose) transfer membranes (MSI, Westboro, MA). The membranes were incubated overnight at 4°C with a 5% solution of nonfat dried milk. Then the membranes were blotted for 1 h at room temperature with anti-TCRζ antibody (Coulter Corp., Hialeah, FL) at 5 µg/ml, anti-p56^ck (Upstate Biotechnology, Inc., Lake Placid, NY) at 1 µg/ml, and anti-CD3ε (DAKO Co., Carpinteria, CA) at 5 µg/ml. The membranes were washed with 1 M Tris base, 5 M NaCl, and 0.1% Tween 20 (pH 7.5) and incubated with anti-rabbit or anti-mouse immunoglobulin horseradish peroxidase diluted 1:2000 (Amersham). After washing with 1 M Tris base, 5 M NaCl, and 0.1% Tween 20 (pH 7.5), the membranes were developed with the chemiluminescence kit ECL (Amersham) for 1 min. X-Omat AR film (Kodak Co., Rochester NY) was used to detect the chemiluminescence. Patient samples and the same normal control were included on each Western blot. All samples for a given patient were run in the same Western blots so that comparisons between pretreatment and treatment samples could be made.

**Densitometry Scanning.** The developed X-omat AR Film was placed on a white light box (Vari Quest 100) by Fotodyne, and its images were captured by a High Resolution CCD Camera (Sierra Scientific). Image 1.44 was the program used to analyze the density of each band by graphically plotting the images and calculating the area under each peak.

**NFκB Activation/T-Lymphocyte Culture.** PBL-Ts (1 × 10⁶/ml) were cultured in the presence and absence of stimulus. The medium was RPMI 1640 supplemented with 2% FCS. T Cells were stimulated with 10 ng/ml of PMA (Sigma Chemical Co., St. Louis, MO) plus 0.75 µg/ml ionomycin (Sigma) for 0.25, 0.5, 2, and 4 h. In some experiments, T cells were stimulated with cross-linked anti-CD3 monoclonal antibody (Ortho Biotech, Raritan, NJ).

**EMSA.** Cytoplasmic and nuclear extracts were prepared according to Schreiber et al. (16) with minor modifications. Briefly, PBL-T (10⁷) were harvested and washed with cold PBS and then sedimented by centrifugation at 1500 rpm for 5 min. The cell pellet was resuspended in 150 µl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1
mm DTT, 100 µg/ml PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 100 µg/ml Pefabloc, and 0.1 µg/ml chymostatin) by gentle pipetting. The cells were incubated on ice for 15 min and then 10 µl of 10% NP40 solution (Sigma) were added, and cells were vigorously mixed for 10 s and then centrifuged. The supernatant containing cytoplasmic proteins was transferred to another tube and aliquoted. Nuclei were resuspended in 50 µl of buffer C [25% glycerol, 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 µg/ml PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 100 µg/ml Pefabloc, and 100 µg/ml chymostatin]. After mixing at 4°C for 20 min, the nuclei were centrifuged for 10 min at 13,200 rpm, and supernatants containing the nuclear proteins were stored at ~70°C. Protein concentration was measured with a commercial kit (Pierce BCA kit, Rockford, IL) as specified by the manufacturer.

As described previously (10, 17), nuclear extracts (5 µg of protein) were preincubated in a 25-µl total reaction volume containing 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 8% glycerol, and 2 µl of poly(dI-dC) (Pharmacia, Piscataway, NJ) for 15 min at 4°C. The reaction mixture was then incubated with the radiolabeled oligonucleotide (2 x 10⁵ cpm) for 20 min at room temperature. The samples were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel with 0.25× TBE buffer (22.3 mM Tris, 22.2 mM boric acid, and 0.5 mM EDTA). The gels were dried and analyzed by autoradiography.

For the preparation of the probe, radiolabeled double-stranded oligonucleotides were prepared by annealing coding strand template to a complementary 10-base primer and filling in the overhang with the Klenow fragment of DNA polymerase I in the presence of [α-32P]dCTP. Oligonucleotide corresponding to 6b element from IL-2NRα gene was prepared by using an Applied Biosystems oligonucleotide synthesizer (model 381 A). The sequence of the oligonucleotide was: 5'-CAACGGCAGGGGAATCTCCCTCTCCITT-3'.

Supershift experiments were performed using standard EMSA procedure described previously, with the exception that 25 µl of reaction mixture were preincubated with 2 µl/2 µg of the appropriate antibody at 4°C for 15 min before the addition of labeled oligonucleotide. Thereafter, the samples were electrophoresed in a 6% nondenaturing polyacrylamide gel. The antibodies used included, anti-NFκB1, anti-RelA, and anti-c-Rel (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Normal rabbit serum 2 µl (2 µg; Jackson Immuno Research Laboratory, West Grove, PA) was used as a control.

Statistical Analysis. Relative levels of TCRζ, p56kk, and CD3ε were determined by densitometry scanning. Because of the variability between Western blots performed at different times, direct comparisons could not be made. Therefore, the ratios of control and pretreatment values were used. These values were used to compute summary statistics and P values for various patient groups. The Wilcoxon signed rank test (two-sided; Ref. 18) was used to test for a difference between the control and pretreatment values after computing the following:

\[ \frac{\text{Patient value}_{\text{pre}}}{\text{Control value}} \]

To determine changes associated with treatment, the following value was calculated using control and patient signaling molecule levels on either day 8 or 15:

\[ \frac{\text{Patient value}_{\text{pre}}}{\text{Control value}} - \frac{\text{Patient value}_{\text{day 8 or 15}}}{\text{Patient value}_{\text{pre}}} \]

The Wilcoxon rank sum (two-sided) test (18) was used to test for a difference between responders and nonresponders using the ratios of pretreatment to control values.

To assess the relationships between patient clinical characteristics and levels of signaling molecules, a multiple linear regression model (19) was fit to the logarithm of the ratio of the pretreatment value over the control value for p56kk, TCRζ, and CD3ε. The common logarithm was computed in an attempt to stabilize variances; one was added to the pretreatment values to avoid taking the logarithm of zero. Factors were eliminated in a backward fashion and consisted of age, prior therapy, gender, performance status, primary tumor in place, individual metastatic sites of lung, lymph node, liver, adrenal, soft tissue, and bone.

ANOVA (20) was used to test for a significant variability within and between control subjects run on the same Western blot (three subjects and three time points). The F statistic (21) was used to test for a difference in variances between different samples and within the same sample run on this same Western blot. The Mantel-Haenszel log-rank test (22) was used to test for a survival difference by response.

RESULTS

Patient Characteristics. A total of 54 patients with metastatic/unresectable RCC and 11 patients after resection of all disease were studied from October 1993 to August 1997 (Table 1). Forty-two individuals were participants in two different clinical trials using slightly different regimens of rIL-2 and IFNα with/without 5-FU (23, 24). Seven patients were treated with rIL-2 and IFNα, and the remaining 35 received this same combination plus 5-FU. In this group, all patients had an Eastern Cooperative Oncology Group performance status of ≤1 (0–17 patients and 1–25 patients). The majority (n = 32) of these patients and 1–25 patients). The majority (n = 32) of these
Fig. 3 Western blots and corresponding densitometry data in three patients receiving therapy with rIL-2, IFNα, and 5-FU. Levels of TCRζ (●) and CD3ε (□) in T cells obtained on days 0, 8, and 15 of cycle 1, with an accompanying control, are illustrated. Increasing levels of TCRζ during cytokine therapy are demonstrated in each patient. The levels of CD3ε showed minimal variation. Pt. WA, patient W. A.; Pt. DO, patient D. O.; Pt. RS, patient R. S. D8, day 8; D15, day 15.

Fig. 4 Alterations in TCRζ in patients treated with IL-2/IFNα ± 5-FU (means; bars, SE). Changes in relative levels of TCRζ (determined by densitometry) on days 8 and 15 of cytokine therapy in 29 patients. Alterations in TCRζ levels were calculated as follows: TCRζD8 or D15 = TCRζD8 or D15/Control. Relative levels of TCRζ were then compared with pretreatment values for all patient for day 8 (D8; P = 0.187) and day 15 (D15; P = 0.002).

individuals were previously untreated. Twelve patients with metastatic and locally advanced RCC prior to surgery or systemic therapy were also studied. Finally, 11 patients with locally advanced or metastatic disease who underwent resection of all visible disease were studied prior to treatment in a pilot study of adjuvant rIL-2.

Frequency and Extent of Signaling Element Defects in Peripheral Blood-derived T Cells from RCC Patients. Previously, in a small number of RCC patients, we reported that the levels of TCRζ, p56ik, and p59syn were decreased in PBL-Ts from some individuals. This report extends these findings and determined the levels as well as the frequency of altered expression of TCRζ, CD3ε, and p56ik in PBL-Ts from RCC patients. Twenty-five patients receiving rIL-2/IFNα-based therapy and four additional patients with locally advanced and/or metastatic disease were studied. Data on these 29 patients was pooled and examined for differences between patients and normal controls. Correlations of pretreatment protein levels with survival, clinical response, number or location of metastatic sites, performance status, prior therapy, age, gender, and nephrectomy status were carried out.

Fig. 1 demonstrates Western blots illustrating levels of TCRζ and p56ik in PBL-Ts from selected RCC patients compared with controls. In the 29 patients examined, normal levels of TCRζ and p56ik were displayed in 59 and 55% of individuals, respectively. The majority of remaining individuals expressed detectable but reduced levels of these two signaling elements. In 2 of 29 patients, TCRζ and p56ik were not detected in PBL-Ts. CD3ε levels (determined on 23 patients) remain relatively constant in most instances; however, some variability in these levels was also noted. Visual inspection of the Western blots clearly demonstrated some decrease in the various signaling molecules compared with controls as follows: TCRζ, 12 of 29 (41%); p56ik, 13 of 29 (45%); and CD3ε, 2 of 23 (8%). Because visual inspection of Western blots is at best qualitative,
Fig. 5 EMSAs are illustrated that were performed on T cells from four normal individuals and four RCC patients (indicated by initials) plus four age-matched controls. The studies demonstrate induction of 6B-specific binding complexes after stimulation with PMA (P, 10 μg/ml) and ionomycin (I, 0.75 μg/ml) or cross-linked anti-CD3 monoclonal antibody (CD3). Various incubation times including 0.25, 0.5, 2.0 and 4.0 h are illustrated. T cells from the four age-matched control individuals demonstrate normal induction of C1 (RelA and NFKB 1 homodimer). Additionally, T cells from four different normal and four RCC patients demonstrate normal NFKB activation in the former group compared with no induction of the C1 and C2 nuclear complexes after stimulation in the patients.

densitometry scanning was used to provide relative estimates of signaling molecule levels. Fig. 1 illustrates Western blots and relative levels of these proteins based on the densitometry data in selected patients (prior to therapy) and normal individuals. For the entire group of patients, CD3ε levels were not significantly different compared with control individuals (P = 0.131); however, TCRζ (P = 0.029) and p56ck (P = 0.029) were lower.

The correlations of pretreatment TCRζ and p56ck levels with various clinical characteristics and objective responses (3 of 25 partial responses) in patients receiving cytokine therapy were also investigated. Lower levels of TCRζ were associated with the presence of bone metastases (P < 0.001). No significant decreases were associated with the other variables such as number of metastatic sites or nephrectomy status. Additionally, higher levels of p56ck and TCRζ were not associated with clinical response (P = 0.068 and 0.223, respectively).

Because variability of protein levels from experiment to experiment is an issue, the level of expression was examined in PBL-T cells isolated from healthy volunteers. Some variability in levels of CD3ε, p56ck, and TCRζ were observed in T cells isolated from five different normal individuals (data not shown). The stability of signal transduction molecule levels over time in three control individuals (Fig. 2) without cancer was also studied longitudinally at three different time points (days 0, 8, and 15). These findings illustrate that levels of TCRζ, p56ck, and CD3ε varied minimally within a single individual over a time
Identification of the NFκB family members that are present in the 6B-specific complexes of patient and normal T cells. Supershift experiments were performed on nuclear extracts from normal and patient PBL-T cells after 4 h of stimulation with anti-CD3. Standard EMSA procedure was followed with the exception that 25 μl of reaction mixture were preincubated with 2 μl (2 μg) of the appropriate antibody at 4°C for 15 min. The labeled probe (IL2Rx sequence) was then added before electrophoresis. The antibodies used included anti-NFκB1, anti-RelA, and anti-c-Rel. Normal rabbit serum (2 μg; Jackson Immuno Research Laboratory) was used as a control. Nuclear extract from normal activated T cells that was not incubated with any antibody was included in every experiment to show the migration of C1 and C2 complexes. Identical results to those shown in this figure were obtained in a second experiment.

A: Normal PBL

<table>
<thead>
<tr>
<th>No Antibody</th>
<th>Rabbit IgG</th>
<th>Anti-NFκB1</th>
<th>Anti-RelA</th>
<th>Anti-c-Rel</th>
<th>Anti-NFκB1 + Anti-RelA</th>
<th>Anti-NFκB1 + Anti-c-Rel</th>
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</table>

B: RCC PBL

<table>
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<tr>
<th>No Antibody</th>
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<th>Anti-NFκB1</th>
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<th>Anti-c-Rel</th>
<th>Anti-NFκB1 + Anti-RelA</th>
<th>Anti-NFκB1 + Anti-c-Rel</th>
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</table>

period of 2 weeks. When the densitometry data in Fig. 2 were analyzed, there was significant variability between individual control subjects (TCR, P = 0.018; p56k, P = 0.46; and CD3ε, P = 0.031), this was not seen within the same subjects tested on separate days (TCR, P = 0.38; p56k, P = 0.26; CD3ε, P = 0.94). These data suggest that signaling molecule levels remain relatively constant within a normal individual over time.

Treatment with rIL-2/IFNα-based Regimens Produces an Increase in the Expression of TCRζ in Patient-derived T Cells. The effects of cytokine administration on PBL-T levels of TCRζ and p56k were then investigated in 21 patients in whom serial studies were available. Fig. 3 illustrates selected patients who were receiving rIL-2/IFNα regimens. Increases of TCRζ are evident on inspection of the Western blots and are also demonstrated by the corresponding densitometry readings. In the 12 patients with reduced levels of TCRζ before therapy, increases were noted in nine after initiation of treatment. In contrast, levels of CD3ε remained relatively constant. Changes in protein levels during cytokine administration (rIL-2/IFNα regimens) were then examined by comparing relative levels on days 8 and 15 to baseline. Fig. 4 illustrates changes in the relative levels of TCRζ levels determined by densitometry and demonstrates that by day 15 of treatment, these increased significantly compared with baseline (P = 0.002). No significant changes in p56k levels during treatment were found.

Impaired activation of NFκB in PBL-T Cells from RCC Patients. In view of our previous report (10) noting abnormal activation of NFκB/Rel proteins in T cells, 28 patients with RCC were then investigated to determine the induction of 6B binding activity. Twenty patients prior to therapy with rIL-2/IFNα/5-FU and 8 additional untreated patients with metastatic RCC were studied. In the 20 patients receiving cytokine therapy, three were simultaneously investigated for alterations in TCRζ and p56k. PBL-Ts were isolated and stimulated with PMA/ionomycin prior to performing EMSA (25 patients). Normal PBL-T cells show two 6B-specific binding complexes after stimulation (Fig. 5). Antibody-mediated supershift experiments showed that the slower moving complex (C1) was composed of RelA (p65) and NFκB1 (p50). Anti-NFκB1 and anti-RelA shifted the C1 complex effectively, whereas anti-c-Rel had very little effect (Fig. 6). The faster migrating complex (C2) was a homodimer of NFκB1 (Fig. 6) because antibody to NFκB1
Fig. 7 Five selected patients in whom studies of TCR, p56\textsuperscript{ick}, and CD3\textsuperscript{e} levels and Nf\kappa B activation performed concomitantly. PBL-T cells were isolated; and Western blots to detect levels of TCR, p56\textsuperscript{ick}, and CD3\textsuperscript{e} were performed. Additionally, T cells were stimulated for 4 h with PMA (10 \( \mu \)g/ml), ionomycin (0.75 \( \mu \)g/ml), or cross-linked monoclonal anti-CD3 antibody prior to isolating cytoplasmic and nuclear extracts. The nuclear extracts were used for EMSA to detect nuclear expression of 6B-specific binding activity. This is indicated as positive (+) or negative (−) and illustrates abnormal Nf\kappa B activation in the presence of normal TCR and p56\textsuperscript{ick} levels. Parentheses, patients’ initials.

The presence of immune dysfunction in animals and patients with advanced malignancies has been recognized (1, 2), but the responsible mechanisms are unclear. A variety of cell-mediated functions such as cytotoxicity (12) and cytokine production (14) are deficient, and the possibility these abnormalities may have a negative impact on therapy and/or patient outcome does exist.

Abnormalities of signal transduction in T-lymphocytes from spleens of mice with progressive tumors (1, 25) have been demonstrated, including decreased levels of the protein tyrosine kinases p56\textsuperscript{ick} and p59\textsuperscript{shc}, as well as the \( \zeta \) chain of the TCR/CD3 complex. In humans, similar abnormalities in tumor-infiltrating lymphocytes and PBLs from individuals with RCC (2, 5), colon cancer (8, 9), ovarian cancer (6), melanoma (3, 4), and Hodgkin’s disease (26) have been described. Reduced TCR/CD3-associated tyrosine phosphorylation, p56\textsuperscript{ick} kinase activity, and cytokine production have also been reported in tumor-infiltrating lymphocytes from non-Hodgkin’s lymphoma patients, although they express normal levels of TCR\textsubscript{z}–associated kinases and TCR\textsubscript{z} (27).

The presence of signaling defects may provide an explanation for the functional abnormalities reported previously, but correlations between these findings remain preliminary. Several reports have noted an association of these defects with decreased cytokine production (4, 5, 26) or cell proliferation (27). It is also possible that the reduced expression of TCR\textsubscript{z} in tumor-associated T cells may occur secondary to the release of proteases from granulocytes and monocytes during protein extraction (28).

The results in the present report suggest that levels of TCR\textsubscript{z} and p56\textsuperscript{ick} are reduced in peripheral blood T cells in selected patients with advanced renal cell carcinoma, although virtually all patients express these signaling elements. When the entire population was compared with normal individuals, the relative levels of TCR\textsubscript{z} and p56\textsuperscript{ick} but not CD3\textsuperscript{e} were significantly lower. A reduction of TCR\textsubscript{z} in T cells was noted in \( \sim \)41% of patients, similar to results reported previously for RCC patients using flow cytometry (29).

In the present study, we attempted to semiquantitate levels of TCR\textsubscript{z} and p56\textsuperscript{ick} and correlate them with clinical findings and response. Significant associations were not found, except for decreased levels of TCR\textsubscript{z} in the presence of bone metastases. No association with response was found, but the numbers of patients responding represent \( \leq 15\% \) of the population. The patient population in our study was composed of individuals with unresectable and advanced RCC; therefore, differences between early and advanced disease stages as reported by Matsuda et al. (9) for colorectal cancer patients were not detectable. Finally, the use of Western blots to assess signaling molecule...
Fig. 8 EMSAs are illustrated that were performed on T cells from six control individuals and six RCC patients (indicated by initials). Patient T cells were obtained prior to cytokine therapy (day 0) and on day 15 of cycle 1. T cells were stimulated with either PMA (P, 10 μg/ml) and ionomycin (I, 0.75 μg/ml) or cross-linked anti-CD3 monoclonal antibody (CD3). Various incubation times including 0.25, 0.5, 2.0, and 4.0 h are illustrated. The six RCC patients demonstrate abnormal induction of the C1 complex prior to therapy. During therapy, activation of NFκB was noted in four patients (MM, VA, RH, and EW) on day 15 of treatment.
Signal Transduction Abnormalities contribute to the findings in the present report. In T cells cycle one (versus different cytokine regimen used, the timing of our studies (day therapy, normalization was reported. Farace et al. reported no effect of rIL-2 therapy on TCR levels in melanoma patients; however, in responding patients with low levels before therapy, normalization was reported. Farace et al. (29) reported that rIL-2 therapy did not produce a significant change in TCR expression. The findings in our study may be related to the different cytokine regimen used, the timing of our studies (day 15), or the methodology used.

In view of previous reports (10, 11), the NFκB family members were also examined. NFκB is a transcription factor that regulates multiple genes important for T-cell function and growth (30). The NFκB complexes translocate to the nucleus, where they regulate transcription of κB-dependent genes. Examination of NFκB activation demonstrated abnormalities in >50% of patients. The most prominent alteration was in the nuclear localization of p50 homodimers (C1 complex) as defined by EMSA. Western blot experiments demonstrated that after stimulation, RelA failed to translocate to the nucleus, although it was present in the cytoplasm (data not shown). In addition to the effects of impaired NFκB activation, the presence of the C2 complex (p50 homodimers) may produce suppression of gene expression (31). Age-matched normal donors were included in this study because previous reports have shown that in older individuals (≥72 years), there are alterations in immune function and reduced activation of AP-1 (32) and NFκB (33). Here we show that in healthy donors (mean age, 52 years) there is nuclear localization of the C1 complex (NFκB/RelA) after stimulation with either PMA/ionomycin or anti-CD3 antibody in seven of eight individuals studied. These findings suggest minor alterations in NFκB activation in healthy individuals in their fifth and sixth decades is infrequent and does not account for the defect observed in T cells from RCC patients.

The alterations in NFκB activation appear to be downstream of alterations in TCR-associated signaling events. It is unlikely that the reduced expression of TCRζ and p56κκ contribute to the impaired activation of NFκB by decreasing phosphorylation of 16B proteins and thereby preventing dissociation of NFκB from its inhibitor (34). In the present study, 14 patients were simultaneously investigated for NFκB activation and TCRζ/p56κκ expression. In nine cases, all studies were normal, and in the other five patients, NFκB activation via the TCRζ or by PMA/ionomycin was impaired despite normal levels of TCRζ and p56κκ. The fact that impaired NFκB activation was observed with PMA/ionomycin further suggests that the defect in NFκB is downstream of signaling linked to TCRζ because these events are bypassed by PMA/ionomycin activation. A recent report by Correa et al. (35) noted a similar dissociation of NFκB activation and TCRζ reduction in T cells from tumor-bearing mice. Attempts to correlate these findings with T-cell cytokine production patterns are under way.

This study and others (2–8) clearly demonstrate abnormal levels of various signaling molecules in T cells from patients with advanced malignancies. It is possible that different patterns will be found in various neoplasms, and until the mechanisms responsible for these changes are clarified, the significance of these findings remains unclear. Tumor-derived soluble factors that impair T-lymphocyte functions have been demonstrated (36, 37), and recent reports show that hydrogen peroxide produced by tumor-associated macrophages can inhibit the expression of TCRζ (38, 39). In addition, cell-cell contact between T cells and a human tumor cell was shown to suppress the expression of TCRζ and cytolytic activity (40). Studies from our laboratory suggest that the impairment in NFκB activation noted in T cells from RCC patients can be reproduced in normal T cells by supernatants from cultured renal tumor explants (41). Additional studies are required to further determine the extent of these defects, determine their relevance to stage and outcome with therapy, and the responsible mechanism(s) involved.

REFERENCES


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