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Suppression of NF-κB Activation in Normal T Cells by Supernatant Fluid from Human Renal Cell Carcinomas

T lymphocytes from patients with renal cell carcinoma (RCC) show reduced immune function and impaired activation of the transcription factor, NF-κB. We determined the mechanism of NF-κB suppression in T cells of RCC patient and determined whether supernatant fluid from RCC explants (RCC-S) induced the same phenotype of NF-κB suppression in normal T cells that is observed in patient T cells. The pattern of κB-binding activity in T cells of RCC patient was altered as compared to that seen in T cells obtained from normal volunteers. In some patients, no activation of RelA/NFκB1-binding activity was detectable, while in others κB-binding activity was modestly induced but the duration was reduced. IκBα was degraded normally following stimulation in both normal controls and T cells from RCC patients. RCC-S did not alter the cytoplasmic levels of RelA and NF-κB1 but did suppress their nuclear localization and inhibited the activation of RelA/NF-κB1 binding complexes. These results show that RCC-S can induce in normal T cells the same phenotype of impaired NF-κB activation that is detected in T cells of RCC patient. It also appears that NF-κB suppression by RCC-S may contribute to the immunosuppression of host immunity.

Key Words: *Transcription factors; T-lymphocytes; Kidney neoplasms; Proteins, NF-κB*

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INTRODUCTION

Among renal cell carcinoma (RCC) patients, 30% have metastases at the time of diagnosis. Two-year survival rate of RCC patient is 10-20% (1, 2). Metastatic RCC is ordinarily resistant to both radiation therapy and chemotherapy (3). Although the development of immunotherapy, including interferon-α, interleukin-2 and interleukin-12 has resulted in improved response rates in metastatic RCC, the treatment of metastatic RCC remains difficult with less than 25% of patients responding favorably (4). Several mechanisms contribute to the immune unresponsiveness of cancer patients, including immune suppression directly induced by the cancer through its production of soluble immune suppressive factors (5). T cells represent an important component of the host immune response against human tumor cells. The impairment of T cell function and signaling pathways observed in cancer patients may hinder the development of T cell immunity and reduce the effectiveness of immunotherapy. NF-κB is important to T cell activation and regulates the inducible expression of a large number of genes necessary for the development of T cell immunity. The κB/Rel family of proteins is composed of p105/p50 (NF-κB1), p65 (RelA), p100/p52 (NF-κB2), c-Rel and RelB which bind the κB sequence motif in homo or heterodimeric form (6-10). NF-κB is present in an inactive form in the cytoplasm of T cells through interaction with inhibitory proteins, collectively termed IκBs. IκBα inhibits both DNA binding activity and nuclear localization of RelA and c-Rel proteins (6, 11). The principle steps in the activation of NF-κB involve the dissociation of dimeric forms containing RelA, c-Rel and NF-κB1 from IκBα after phosphorylation and subsequent degradation of IκBα (6, 11, 12). We determined the mechanism of NF-κB suppression in T cells of RCC patient and determined RCC-S induced suppression of NF-κB activation in normal T cells is similar to that observed in T cells of RCC patient.

MATERIALS AND METHODS

Cell culture and isolation of T cells

Peripheral blood was obtained from patients with con-

firmed diagnosis of renal cell carcinoma and healthy volunteers. Tumor tissue was obtained from RCC patients and used to make tumor supernatants. Normal T cells were used as control and compared to patient T cells. Normal T cells from healthy donors were used for culturing with tumor supernatants. Peripheral blood lymphocytes (PBL) were isolated from healthy volunteers and RCC patients. T cells were purified as previously described (13, 14): PBL were subjected to Ficoll-Hypaque (Pharmacia, Piscataway, NJ, U.S.A.) density gradient centrifugation and then depleted of macrophages, B cells and NK cells by negative selection using magnetic separation with microbeads coated with antibodies to CD14, CD16 and CD19 (Miltenyi Biotec Inc., Sunnyvale, CA, U.S.A.). To obtain activated T cells, T cells (5×10^5/mL) were cultured in the absence and presence of stimulus for various lengths of times. The medium used was RPMI1640 supplemented with 5% fetal calf serum. PMA (20 ng/mL) (Sigma, St. Louis, MO, U.S.A.) plus ionomycin (0.75 μg/mL) (Sigma) or cross-linked anti-CD3 (10 μg/mL) (Ortho Biotech, Rariton, NJ, U.S.A.) plus IL-2 (1000 IU) (Hoffman LaRoche, Nutley, NJ, U.S.A.) were used as stimulus.

Preparation of tumor supernatants

To generate tumor supernatant fluid, tumor tissues from primary renal cell carcinoma were obtained from patients undergoing radical nephrectomy. 3×3 mm explants were made from tumors using a No. 10 Bard-Parker scalpel and incubated overnight in RPMI1640 medium. Thereafter, 1 g of 3×3 mm explants were placed into a T-75 flask with 15 mL of Dulbecco's Modified Eagles Medium without additional supplements. After three days of culture at 37℃ with 95% air and 5% CO_2 the supernatant fluid was harvested, filtered and stored at -70℃ until use. To determine the effect of tumor-derived soluble products on normal T cells, T cells were cultured in complete RPMI1640 with and without RCC-S. The supernatant fluid was added to T cell cultures, using 20-50% dilution.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared according to Schreiber et al. (15) with minor modifications. T cells (10×10^6/mL) were harvested and washed with cold PBS, and then sedimented by centrifugation. 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 100 μg/mL chymostatin) by gentle pipetting. The cells were incubated on ice for 15 min and then 10 μL of 10% Nonidet P-40 solution (Sigma) was added and centrifuged. The supernatant fluid containing the cytoplasmic extracts was aliquoted. Pelleted nuclei were resuspended in 50 μL of buffer C (25% glycerol, 20 mM HEPES, pH 7.9, 0.4 M 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℃ for 20 min and centrifuge, supernatants containing the nuclear proteins were taken and stored at -70℃. Protein concentration was measured with a commercial kit (Pierce BCA kit, Rockford, IL, U.S.A.).

Western blot analysis

Protein samples (5-10 μg) were mixed with equal volume of 2X Laemmli sample buffer, boiled and resolved by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred from the gel to nitrocellulose membranes. The membranes were blocked by incubating in 5% nonfat dry milk in TBST overnight. The membranes were sequentially incubated with specific antibody (two hr for anti-IκBα and anti-c-Rel, one hr for anti-RelA and anti-NF-κB1) and then with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Chicago, IL, U.S.A.; 1:2000 dilution in TBST). The membranes were washed with TBST and specific immune complexes were detected by enhanced chemiluminescence (Dupont, Boston, MA, U.S.A.).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using nuclear extracts obtained at various times after stimulation with either PMA plus ionomycin or cross-linked anti-CD3 plus IL-2. Nuclear extracts (5-10 μg) were incubated in a 25 μL total reaction volume containing 20 mM HEPES, pH 7.9, 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 8% glycerol and 2 μL of poly (dI-dC) (Pharmacia, Piscataway, NJ, U.S.A.) for 15 min at 4℃. The reaction mixture was then incubated with radiolabeled oligonucleotide (2×10^5 cpm) for 20 min at room temperature. The samples were analyzed by electrophoresis in 6% non-denaturing polyacrylamide gel with 0.25X TBE buffer (22.3 mM Tris, 22.2 mM boric acid, 0.5 mM EDTA). The gels were dried and analyzed by autoradiography. An oligonucleotide corresponding to κB element from the IL-2Rα gene was prepared by using an Applied Biosystems oligonucleotide synthesizer (model 381A). The sequence of the oligonucleotide was: 5'-CAACGGCAGGGGAATCTCCCTCTC-CTT-3'. The underlined sequence represents the κB motif.

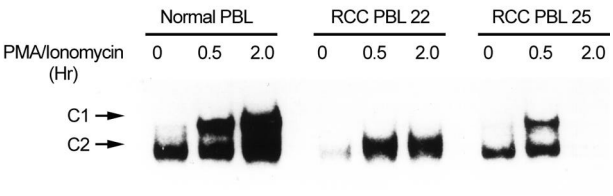

Fig. 1. Analysis of stimulus-induced κB binding activity in normal and patient T cells by EMSA. Normal PBL, normal T cells; RCC PBL, T cells of RCC patient.

Table 1. κB/Rel protein expression and NF-κB activity in N-PBL, P-PBL and N-PBL with RCC-S

Expression & activity	N-PBL (n=5)	P-PBL (n=10)	N-PBL+RCC-S (n=10)
+	5		
−		6	6
+/−		4	4

N-PBL, normal T cells; P-PBL, T cells of RCC patient; RCC-S, supernatant from RCC explants

RESULTS

κB-binding activity in T cells from RCC patients

T cells from normal donors and RCC patients were stimulated with PMA/ionomycin and κB-binding activity of those cells were analyzed by EMSA. In normal T cells, stimulation with PMA/ionomycin resulted in the appearance of two distinct κB motif-specific DNA-binding activities (designated C1 and C2 in Fig. 1). Both complexes were observed within 30 min of stimulation and remained elevated for at least two hr. In six of patient-derived T cells (n=10), the C1 complex was not detectable. In T cells obtained from the other four patients, the C1 activity was observed after 30 min of stimulation but returned to basal levels in two hr (Fig. 1) (Table 1). Nuclear extracts from patient T cells showed constitutive expression of the C2 complex and the abundance of this activity was increased by stimulation. Similar patterns of response were observed when normal and patient T cells were stimulated with anti-CD3/IL-2 (data not shown).

Expression of NF-κB and IκBα in T cells from RCC patients

The nuclear localization of RelA, c-Rel and NF-κB1 in the different cell populations was examined by Western analysis of cytoplasmic and nuclear extracts at various times following stimulation. In normal T cells, expression of c-Rel, RelA and NF-κB1 was increased in the nucleus after 30 min and two hr of stimulation. There were decreased expression of RelA and c-Rel in the nuclei of patient T cells suggesting impaired translocation (Fig. 2). Alterations in levels of nuclear RelA were the most dramatic. In four of 10 RCC patient T cells modest but significant levels of RelA were detected within the nucleus after 30 min of stimulation but faded into the background in two hr (Fig. 2A). In six other samples nuclear RelA was undetectable at any time (Fig. 2B) (Table 1). Levels of NF-κB1 and c-Rel in the nucleus were increased modestly following stimulation. Interestingly, no C1 κB-binding activity was seen under these conditions. T cells from normal donors and RCC patients were stimulated for various times with PMA/ionomycin to determine the change in IκBα, an inhibitor of NF-κB activity, by Western analysis. A characteristic decrease in IκBα expression was observed within 30 min in normal T cells (Fig. 3) which coincides with the nuclear appearance of RelA (Fig. 2). In patient-derived T cells (n=10), the magnitude and kinetics of IκBα degradation were similar to that of normal T cells (Table 2). These observations demonstrate that early events in NF-κB acti-

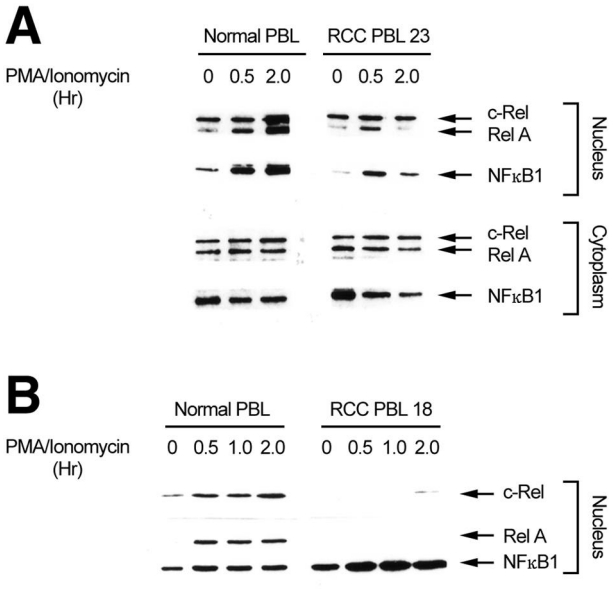

Fig. 2. Altered nuclear translocation of RelA and c-Rel showed in patient T cells.

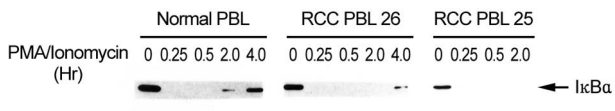

Fig. 3. Normal degradation of IκBα following stimulation is observed in normal T cells and patient T cells.

Table 2. Degradation of IκBα in N-PBL, P-PBL and N-PBL with RCC-S

Degradation	N-PBL (n=5)	P-PBL (n=10)	N-PBL+RCC-S (n=10)
+	5	10	8
−			2

Abbreviations are as noted in Table 1

vation are normal in patient T cells.

Effect of RCC-S in NF-κB activation

Normal T cells were cultured in RCC-S overnight and then stimulated with PMA/ionomycin to determine whether RCC-S could alter activation of NF-κB in normal T cells. NF-κB proteins remained in the cytoplasm but failed to undergo nuclear translocation after stimulation (Fig. 4A). κB-binding activity was also suppressed by RCC-S as determined by EMSA (Fig. 4B) (Table 1). In most cases (n=8), RCC-S did not alter the normal degradation of IκBα. In a few experiments (n=2), the degradation of IκBα was reduced by RCC-S (Table 2). This frequency is similar to that observed in T cells from RCC patient (n=10).

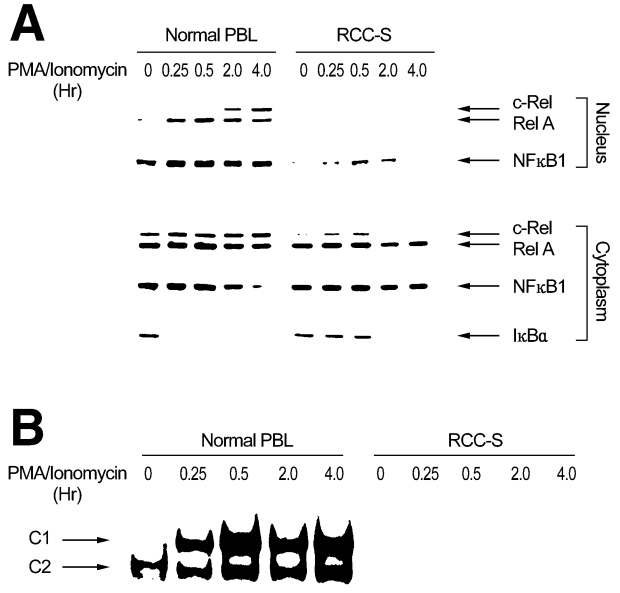

Fig. 4. RCC-S suppresses the activation of NFκB in normal T cells. (A) Western blot: RCC-S inhibits the nuclear translocation of RelA, c-Rel and NFκB1. The degradation of IκBα occurs normally in normal T cells exposed to RCC-S in some experiments. (B) EMSA: RCC-S suppressed κB DNA binding activity.

DISCUSSION

The development of cellular immunity is dependent on the activation of T cells. Activation-dependent stimulation of new gene transcription is now well documented, involving transacting factors such as NF-κB which regulates the expression of many genes important to T cell function and growth (6, 16, 17).

T cells from cancer patients or tumor-bearing animals are impaired in their response to extracellular stimuli including the activation of κB-specific DNA-binding activity (14, 18). Our results show that there are alterations in both the expression and DNA-binding activity of NF-κB family members of RCC patient T cells. The major inducible κB-specific binding complex (C1) either did not appear in the nucleus or was modestly activated and exhibited a markedly reduced time course. The failure of c-Rel and RelA to accumulate normally in the nuclei of patient T cells was due to an event occurring after phosphorylation and degradation of the inhibitor IκBα since this latter process occurred normally in RCC patient T cells.

Although the biological nature of the soluble products in tumor supernatants is not known, the culture supernatant of tumor cells may produce a variety of immunosuppressive molecules such as IL-10, TGF-β1 and prostaglandin E2 (5). They may contribute to immune unresponsiveness of cancer patients and inhibit the activation of NF-κB. Our results demonstrate that RCC-S can induce in normal T cells the same phenotype of impaired NF-κB activation that is detected in T cells of RCC patient. These alterations in NF-κB activity may be due to a product(s) of renal tumor cells. Stimulus-dependent degradation of IκBα in the cytoplasm was not impaired by RCC-S in most experiments. However, the degradation of IκBα was reduced by RCC-S in two out of 10 cases. Thus, there appears to be at least two different mechanisms by which RCC-S can suppress NF-κB activation. First, NF-κB proteins do not translocate to the nucleus at all. Second, NF-κB proteins degrade in the nucleus after translocation.

In conclusion, two patterns of altered NF-κB activation and alterations in NF-κB activity induced by RCC-S may contribute to the lack of antitumor immunity in RCC patients.

REFERENCES

1. Choudhury M, Efros M, Mittelman A. *Interferons and interleukins in metastatic renal cell carcinoma. Urology 1993; 41 (suppl 1): 67-72.*
2. Thrasher JB, Paulson DF. *Prognostic factors in renal cancer.*

Urol Clin N Am 1993; 20: 247-62.

3. Goepel M, Rubben H. *Adjuvant therapy in renal cancer. World J Urol 1991; 9: 232-6.*
4. Sokoloff MH, Figlin RA, de Kernion JB, Belldegrun AS. *Systemic immunotherapy for genitourinary neoplasms. In: Raghavan D, Scher HI, Leibel SA, Lange P, eds. Principles and practice of genitourinary oncology. Philadelphia: Lippincott-Raven, 1997: 869-83.*
5. Ochoa AC, Longo DL. *Alteration of signal transduction in T cells from cancer patients. In: DeVita VT, Hellman S, Rosenberg SA, eds. Important advances in oncology. Philadelphia: Lippincott-Raven, 1995: 43-54.*
6. Nolan GP, Baltimore D. *The inhibitory ankyrin and activator Rel proteins. Current Opinion in Genetics and Development 1992; 2: 211-20.*
7. Ghosh S, Gifford AB, Riviere LR, Tempst P, Nolan GP, Baltimore D. *Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell 1990; 62: 1019-29.*
8. Ruben SM, Dillon PJ, Schreek R, Henkel T, Cohen CH, Maher M, Baeuerle PA, Rosen CA. *Isolation of a rel-related cDNA that potentially encodes the 65-KD subunit of NF-κB. Science 1991; 251: 1490-3.*
9. Neri A, Chang CC, Lombartdi L, Salina M, Corradini P, Maiolo AT, Chaganti RS, Dalla-Favera R. *B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell 1991; 67: 1075-87.*
10. Ryseck RP, Bull P, Takamiya M, Bours V, Siebelist U, Dobrzanski P, Bravo R. *RelB, a new Rel family transcription activator that can interact with DNA. Mol Cell Biol 1992; 12: 674-84.*
11. Gilmore TD, Morin PJ. *The IκB proteins: members of multifunctional family. Trends in Genetics 1993; 9: 427-33.*
12. Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neiah Y, Baeuerle PA. *Rapid proteolysis of IκBα is necessary for activation of transcription factor NFκB. Nature 1993; 365: 182-5.*
13. Finke JH, Zea AH, Stanley J, Longo DL, Mizoguchi H, Tubbs RR, Wiltrout JJ, O'Shea JJ, Kudoh S, Klein E, Bukowski RM, Ochoa AC. *Loss of T-cell receptor ζ chain and p56^{lck} in T-cell infiltrating human renal cell carcinoma. Cancer Res 1993; 53: 5613-6.*
14. Li X, Liu J, Park JK, Hamilton TA, Rayman P, Klein E, Edinger M, Tubbs R, Bukowski R, Finke J. *T cells from renal cell carcinoma patients exhibit an abnormal pattern of κB-specific DNA-binding activity: a preliminary report. Cancer Res 1994; 54: 5424-9.*
15. Schreiber E, Matthias E, Muller MM, Schaffner W. *Rapid detection of octamer binding proteins with "Mini-extracts" prepared from a small number of cells. Nucleic Acids Res 1989; 7: 6419-23.*
16. Ullman KS, Northrop JP, Verweij CL, Crabtree GR. *Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. Ann Rev Immunol 1990; 8: 421-52.*
17. Sen R, Baltimore D. *Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986; 46: 705-16.*
18. Ghosh P, Sica A, Young HA, Ye J, Franco JL, Wiltrout RH, Longo DL, Rice NR, Komschlies KL. *Alterations in NFκB/Rel family proteins in splenic T-cells from tumor-bearing mice and reversal following therapy. Cancer Res 1994; 54: 2969-72.*