Non-Malignant Cell Lung Cancer-derived Soluble Mediators Enhance Apoptosis in Activated T Lymphocytes through an IκB Kinase-dependent Mechanism

Raj K. Batra, Ying Lin, Sherven Sharma, Mariam Dohadwala, Jie Luo, Mehis Pold, and Steven M. Dubinett

ABSTRACT

T lymphocyte survival is critical for the development and maintenance of an effective host antitumor immune response; however, the tumor environment can negatively impact T-cell survival. Lymphocytes exposed to tumor supernatants (TSNs) were evaluated for apoptosis after mitogen stimulation. TSN was observed to significantly enhance phorbol 12-myristate 13-acetate/ionomycin- and anti-CD3-stimulated lymphocyte apoptosis. Enhanced lymphocyte apoptosis was associated with an impairment of nuclear factor-κB (NF-κB) nuclear translocation and diminished IkBα degradation. In lymphocytes stimulated after exposure to TSNs, cytoplasmic IkBα persisted as a result of alterations in IκB kinase (IKK) activity. Accordingly, although there were no apparent differences in IKK component concentrations, lymphocytes preexposed to TSNs exhibited markedly reduced IKK activity. We conclude that non-small cell lung cancer-derived soluble factors promote apoptosis in activated lymphocytes by an IKK-dependent pathway.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the United States in both men and women. The tumors’ capacity to avert host antitumor responses may be an important determinant contributing to the morbidity and mortality incurred by this disease. A number of mechanisms whereby tumors evade the immune system have been described (1–6), including those that directly abrogate effector cell function by limiting lymphocyte survival (7–10). Our preliminary studies suggested that normal donor lymphocytes exposed to NSCLC tumor cell supernatant exhibited increased apoptosis, and we proceeded to model and investigate the mechanisms responsible for this effect. Because tumor-induced alterations in T-cell NF-κB activation resulted in the induction of T-lymphocyte apoptosis by renal cell carcinoma (8–10), and because NF-κB has been implicated as an important inhibitor of apoptosis (11, 12), we hypothesized that a similar process may be operative in NSCLC.

Transcription factors of the NF-κB family mediate signal transduction into the nucleus after exposure to a variety of endogenous and exogenous stimuli including tumor necrosis factor α, interleukin 1, lipopolysaccharide, ionizing/UV irradiation, viral infections, and CD3 ligation (13–15). In the nucleus, these proteins selectively bind oligomeric DNA consensus sites in the regulatory regions of a variety of immune/inflammatory response genes to induce the transcription of an array of cytokines, growth factors, and adhesion molecules. In most cell types, NF-κB nuclear translocation requires a release from inhibitor (IκB) proteins that sequester NF-κB in the cytoplasm (16, 17). IkBα, which interacts with the RelA/p65 subunit, is a major regulator of NF-κB activation (14, 17, 18). For NF-κB release, IkBα is inducibly phosphorylated at specific serine sites, which prompts its ubiquination and proteosomal degradation (16, 19). Thus, inducible phosphorylation is a key regulatory step, and is-mediated by a large (M, ~900,000) protein kinase complex termed IKK (20–22). Whereas IKK activation is rapid in response to diverse stimuli (22), prompt deactivation is probably just as important to limit kinase activation after removal of an activating stimulus. This deactivation of IKK is mediated, at least in part, by an autoregulatory mechanism that serves to autophosphorylate the IκKβ subunit of IKK (23).

In this study, we investigated the fate of T cells after activation in the tumor environment. The effects of A549 TSNs on Jurkat T-cell apoptosis were investigated. We found that TSNs strongly inhibited T-cell NF-κB activation and, as a result, markedly enhanced T-cell apoptosis after activation. The decreases in NF-κB activation and IkBα degradation in the Jurkat T cells could be attributed to TSN-mediated inhibition of IKK activity. Thus, this report is the first documentation of tumor-induced lymphocyte apoptosis through a mechanism that involves impaired IKK activity.

MATERIALS AND METHODS

Reagents. Purified mouse anti-human CD3 (PharMingen, San Diego, CA), the phorbol esters PMA and phorbolecin acetate (Life Technologies, Inc., Grand Island, NY), and ionomycin (Sigma, St. Louis, MO) were used for lymphocyte activation. 7-AAD (Sigma) was used to detect lymphocyte apoptosis using flow cytometry.

Cell Cultures. The human lung adenocarcinoma cell line A549 and human Jurkat T cells were obtained from American Type Culture Collection (Manassas, VA). Human Jurkat T cells are human CD4+ T cells that constitutively proliferate and can be induced (by activation with concanavalin A, phytohemagglutinin, or PMA plus a calcium ionophore) to produce interleukin 2. These cells were maintained at 37°C in a 5% CO2 atmosphere in air, in tissue culture flasks containing RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 2 mM glutamine (R-10 complete medium; JRH Biosciences, Lenexa, KS). TSNs were generated by culturing 1 × 106 A549 NSCLC cells for 24 h in 1 ml of R-10 complete medium. Jurkat T-cell cultures (6 × 105 cells) were exposed to 1 ml of TSN or control R-10 complete medium for 18 h in parallel, before activation with anti-CD3 or PMA/ionomycin. Similarly, normal donor PBLs were cultured in RPMI 1640 and 10% fetal bovine serum or TSN for 18 h, activated with PMA/ionomycin for 24 h, and evaluated for apoptotic fractions by flow cytometry.

Measurement of Apoptosis. 7-AAD staining, which can be used to identify the characteristic pattern of DNA damage that accompanies apoptosis, was used to distinguish apoptotic from live cells (24). Twenty-four h after activation, aliquots of 106 Jurkat cells were washed with PBS and incubated for 20 min at room temperature with 10 μg/ml 7-AAD (Sigma). At least 106 cells were evaluated by FACScan flow cytometer (Becton Dickinson, San Jose, CA) in the University of California Los Angeles Jonsson Cancer Center Flow Cytometry Core Facility. Between 5,000 and 15,000 gated events were col-
lected and analyzed using Cell Quest software (Becton Dickinson), and live cells were distinguished from apoptotic cells as described previously (25).

Western Blot Analysis. Jurkat cells, stimulated with PMA (40 nM) and ionomycin (1 μg/ml) in culture medium or TSNs, were sedimented at predetermined time points, the culture medium was aspirated, and the cells were washed with PBS. The cells were then lysed in 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF (cell lysis buffer), and 20 μg of protein from the lysates were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked using 5% nonfat milk in PBS and probed with 0.2 μg/ml rabbit anti-IκB antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing, the blot was labeled with a 1:5000 dilution of horseradish peroxidase-coupled anti-rabbit antibody (Santa Cruz Biotechnology) and developed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Preparation of Nuclear Extracts. After culture and activation as described, Jurkat T-cells (1 × 10$^6$) were collected and washed twice with cold PBS, and the cell pellet was suspended in 40 μl of cell lysis buffer for 10 min on ice. Nuclei were extracted by sedimentation (microcentrifugation at 6,500 rpm) for 10 min at 4°C. The nuclear pellet was then suspended in 15 μl of extraction buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.4 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF] and incubated for 10 min at 4°C with brief intermittent mixing. The mixture was microcentrifuged (14,000 rpm for 10 min at 4°C), and the nuclear protein was resuspended in 60 μl of extraction buffer D [20 mM HEPES (pH 7.9), 25% glycerol, 50 mM NaCl, 1.5 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF].

Electrophoretic Mobility Shift Assay. Ten μg of nuclear extract were preincubated in 20 μl (total reaction volume) of 1× binding buffer containing 20 mM HEPES (pH 7.9) 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 8% glycerol, and 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech) for 15 min at 4°C. The reaction mixture was then incubated with 1 × 10$^5$ cpm of [γ-32P]ATP (3000 Ci/mM)-labeled double-stranded NF-κB consensus binding oligonucleotide (AGTTGAGGGGACCTTCCCCAGGC; Santa Cruz Biotechnology) for 20 min at room temperature. The samples were then resolved by nondenaturing 6% PAGE in 0.5× Tris-borate EDTA buffer, dried, and analyzed by autoradiography.

Immune Complex Protein Kinase Assays. Eighty μg of cytoplasmic protein from Jurkat T cells were incubated with 1 μg of rabbit anti-IκB antibody (Santa Cruz Biotechnology) for 1 h at 4°C. Immune complexes were precipitated using protein A/G plus agarose (20 μl, incubation overnight at 4°C under gentle agitation). The immunoprecipitates were collected by sedimenting the agarose (2,500 rpm, 5 min, 4°C). The pellets were washed four times with 1.0 ml of PBS and resuspended in 20 μl of protein kinase assay buffer (50 mM HEPES, 0.1 mM EDTA, 0.01% Brij 35, 0.1 mg/ml BSA, 0.1% β-mercaptoethanol, and 0.15 mM NaCl). One hundred ng of IκBα protein (Santa Cruz Biotechnology) and 10 μl of ATP mix [930 μl of protein kinase buffer, 6 μl of 50 mM ATP, 20 μl of 20 mM MgCl$_2$, and 44 μl of [γ-32P]ATP (10 mCi/ml)] were added per sample. After a 20-min incubation at room temperature, the kinase reaction was terminated by adding 2× SDS-PAGE sample buffer and then boiling samples for 3 min. The agarose beads were then sedimented (14,000 rpm for 30 s), the supernatant was separated in SDS-PAGE, and the results were analyzed by autoradiography.

RESULTS

TSN (A549 NSCLC) Enhances Apoptosis of CD3-activated Jurkat T Cells. Normal donor lymphocytes exposed to TSNs exhibited greater apoptosis after PMA and ionomycin stimulation than PBLs stimulated under control conditions (Fig. 1A). These results...
prompted us to model and investigate the mechanisms responsible for this effect. To determine whether factors released from NSCLC cells influence T cell survival, Jurkat T cells were exposed to TSNs and stimulated with anti-CD3 for 24 h, and the fraction undergoing apoptosis was measured by 7-AAD staining and flow cytometry. After exposure to TSNs, Jurkat T cells exhibited a marked increase (38% compared with 2%) in the apoptotic fraction after anti-CD3 stimulation (Fig. 1B), as compared with cells exposed to medium alone. In contrast, the fraction of unstimulated Jurkat T cells undergoing apoptosis did not vary between the control and TSN-exposed groups (Fig. 1B). Thus, soluble factors within A549 TSNs are contributing to apoptosis in Jurkat T cells upon anti-CD3 stimulation. Similar results were obtained after PMA and ionomycin stimulation of Jurkat cells (data not shown).

**Nuclear Extracts from Jurkat T Cells Exposed to TSNs Display Altered NF-κB Binding.** To determine the role of NF-κB-dependent apoptosis in lymphocytes cultured and activated in TSNs, Jurkat T cells were activated using PMA plus ionomycin after culture in either medium or A549 TSNs, and nuclear extracts were evaluated for NF-κB translocation. Nuclear extracts (10 μg) from these cells were admixed with the labeled NF-κB consensus sequence (5'-AGTTGAGGGACTTTCCCAGGC-3'), and the mixture was resolved by 6% nondenaturing PAGE for determination of NF-κB translocation. NF-κB activation (p65/p50) was inhibited in the presence of NSCLC TSNs.

**Jurkat T Cells Exposed to A549 TSNs Display Altered IKK Activity.** Because IκB phosphorylation (by IKK) precedes its degradation, we sought to determine whether the intracellular IKK concentrations or IKK activity was impacted in the activated Jurkat T cells after growth in TSNs. As described recently, IKK is a multicomponent complex comprised of related yet distinct subunit kinases termed IKKα and IKKβ and a regulatory subunit, IKKγ (27). The reported kinetics of IKK activity suggests rapid activation with prompt deactivation after stimulus transduction (20, 21, 28). For these studies, activated Jurkat T cells were grown in medium or TSNs, stimulated with PMA plus ionomycin, and lysed. First, cell lysates were examined for IKKα and IKKβ by Western blot analysis after SDS-PAGE separation. The results, as depicted in Fig. 4, suggest that the cytoplasmic concentrations of IKK complexes (as gauged by the presence of IKKα and IKKβ components) were not altered after exposure of Jurkat T cells to TSNs. These data suggest that A549 TSNs do not impact IKK component expression and led us to consider whether tumor factors interfered with the formation of the IKK complex and/or its activity.

To determine whether TSNs impacted IKK activity, the IKKβ component was immunoprecipitated from cell lysates, and the specific IKK activity was measured by assessing recombinant IκBα phosphorylation. As shown in Fig. 5, Jurkat T cells in control conditions possess normal IKK activity, exhibiting prompt cyclical activation and deactivation after phosphol ester stimulation. In contrast, however, IKK activity is not evident in Jurkat cells that have been exposed to TSNs. These data indicate that A549 TSNs contain factors that interfere with the formation of the IKK complex and/or its activity. This interference prohibits signal-induced IκB degradation, resulting in the inhibition of NF-κB nuclear translocation, thus predisposing activated T cells in the tumor milieu to apoptosis.
DISCUSSION

Complex interactions between the tumor and the host immune system contribute to the development of immunosuppressive networks within the tumor milieu. Immune disarray occurs (a) by the induction of local and systemic lymphocyte apoptosis or immune non-responsiveness in effector lymphocytes (5, 8–10, 29–34), (b) by decreased numbers and function of antigen-presenting cells in the tumor milieu (35–38), and (c) by tumor secretion of suppressive cytokines/prostanoids or other soluble factors, as well as by tumor induction of immunosuppressive cytokine secretion by surrounding stroma (37, 39–41). Although these immunosuppressive mechanisms are categorized discretely, the clinically observed deficits that arise are characteristically interrelated. For example, induction of selective cell apoptosis or signaling defects may eventuate in abnormalities in paracrine cytokine secretion. The importance of these cumulative defects is reflected by a predictable poor prognosis in cancer patients who harbor such defects (42).

The molecular and cellular defects underlying clinical tumor-mediated immunosuppression are being gradually unveiled. Tumor-derived factors can subvert effective antitumor cell-mediated immunity (37, 39–41) and induce cytotoxic T-cell mediators of cell death (34) through induced apoptosis or signaling defects. A novel mechanism of tumor-derived immunosuppression, one that impairs T-cell survival in the tumor milieu by inhibiting effector cell NF-κB activation, has recently been described (8–10). The renal cell cancer-induced alterations in T-cell NF-κB activation also appear to be mediated by soluble factors and provided a rationale to investigate whether similar alterations were present in lung cancer. The role of NF-κB as a survival factor has been extensively described (11, 12), and this ubiquitous factor has been implicated in the transcriptional regulation of many inhibitors of apoptosis (43, 44).

Preliminary studies suggested that A549 TSNs could induce apoptosis in activated normal donor lymphocytes. To investigate the basis for this observation, we stimulated Jurkat T cells in the presence of tumor cell supernatant as a model for lung cancer-induced alterations in lymphocyte apoptosis. Our data indicate that Jurkat T cells are indeed predisposed to apoptosis after phorbol ester stimulation in the presence of A549 TSNs. Whereas Jurkat T cells under control conditions exhibit predictable kinetics in terms of NF-κB activation, IkB degradation, and IKK activity, NF-κB activation is impaired in the presence of TSNs. Thus, increased T-cell apoptosis after mitogen stimulation is a result of inhibition of NF-κB activation in the tumor milieu. These data are consistent with those of Uzzo et al. (9, 10), as well as with a recent report that inactivates NF-κB activation as a necessary survival factor for the mitogenic effects of PMA on Jurkat T cells (45). Furthermore, our studies indicate that the impairment in NF-κB nuclear translocation is a downstream result of impaired IKK activity, suggesting that factors secreted by NSCLC cells either (a) disable IKK complex formation or (b) directly or indirectly (through signal-mediated events) inhibit IKK activity. The mechanism of IKK inactivation has not yet been elucidated, and it may involve regulatory autophosphorylation or dephosphorylation by activated phosphatases, as suggested by the ability of okadaic acid to activate IKK in mamalian cells (20).

In summary, these results suggest that tumor-derived soluble factors interfere with IKK activation or regulation in lymphocytes, and this may contribute to enhanced activation-induced T-cell apoptosis in the tumor environment. As such, this is the first report of tumor-mediated enhancement of lymphocyte apoptosis implicating defects in IKK activity, and additional studies are necessary to isolate and define the specific tumor-derived products responsible.

REFERENCES


Non-Small Cell Lung Cancer-derived Soluble Mediators Enhance Apoptosis in Activated T Lymphocytes through an IκB Kinase-dependent Mechanism

Raj K. Batra, Ying Lin, Sherven Sharma, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/3/642

Cited articles
This article cites 44 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/3/642.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/3/642.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.