Constitutive Activation of NF-κB Causes Resistance to Apoptosis in Human Cutaneous T Cell Lymphoma HuT-78 Cells

AUTOCRINE ROLE OF TUMOR NECROSIS FACTOR AND REACTIVE OXYGEN INTERMEDIATES

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How tumor cells develop resistance to apoptosis induced by cytokines and chemotherapeutic agents is incompletely understood. In the present report, we investigated apoptosis induction by tumor necrosis factor (TNF) in two human T cell lines, Jurkat and HuT-78. While TNF inhibited the growth of Jurkat cells and activated caspase-3, it had no effect on HuT-78 cells. It was further found that HuT-78 cells constitutively expressed the nuclear transcription factor NF-κB, TNF activated NF-κB in Jurkat cells but not in HuT-78 cells. HuT-78 cells were also resistant to NF-κB activation induced by phorbol ester, H₂O₂, ceramide, endotoxin, and interleukin-1. Despite the presence of preactivated NF-κB, HuT-78 cells also expressed high levels of IκB-α, the inhibitory subunit of NF-κB and, unlike Jurkat cells, were resistant to TNF-induced degradation of IκB-α. Its half-life in HuT-78 cells was 12 h as opposed to 45 min in Jurkat cells. Antibodies against TNF blocked the constitutive activation of NF-κB and proliferation of HuT-78 cells but had no significant effect on Jurkat cells, suggesting an autocrine role for TNF. The antioxidant pyrrolidine dithiocarbamate also suppressed constitutive NF-κB activation and it reversed the cell’s sensitivity to TNF-induced cytotoxicity and activation of caspase-3. Overall, these results suggest that constitutive activation of NF-κB, TNF, and prooxidant pathway in certain T cell lymphomas causes resistance to apoptosis, and this can be reversed by antioxidants.

Development of resistance to apoptosis induction by cytokines and chemotherapeutic agents is one of the major problems in cancer therapy (1). Overcoming this resistance has been largely unsuccessful, because the mechanism of development of resistance is not understood. Multiple drug resistance (MDR) P-glycoprotein, Bcl-2, inactivation of p53 and related proteins, glutathione S-transferase, protein kinase C, MDRI-related proteins, transglutaminase, and heat shock proteins (e.g. hsp 27) all play a role (2–5). Besides these factors, an activated form of the nuclear transcription factor NF-κB has recently been implicated in development of resistance to tumor necrosis factor (TNF) (6–8).

Under normal conditions, NF-κB is present in the cytoplasm in its inactive state as a heterotrimer consisting of p50, p65, and IκB-α (9). When activated IκB-α undergoes ubiquitination, phosphorylation, and degradation, and the p50-p65 complex is released to be translocated to the nucleus where it causes gene activation. The activation of NF-κB is initiated by a wide variety of stress stimuli, which themselves cause apoptosis. Among these are TNF, IL-1, x-rays, γ-radiation, phorbol ester, ceramide, endotoxin, calcium ionophores, and H₂O₂ (9, 10). Interestingly, several chemotherapeutic drugs such as the anthracyclines doxorubicin and daunorubicin (11, 12), taxol, the vinca alkaloids vinblastine and vincristine (12), camptothecin (13) and etoposide (14) also cause NF-κB activation. Several genes whose proteins are involved in tumor promotion and metastasis, such as ICAM-1, VCAM-1, ELAM-1, cyclooxygenase-2, and matrix metalloproteinase-9, are regulated by NF-κB (15–17).

A progressive activation of constitutive NF-κB has recently been correlated with progression of breast cancer, melanoma, and juvenile myelomonocytic leukemia (18–21). How NF-κB is constitutively activated in some tumor cells and what role it plays in induction of resistance to apoptosis is not clear. In the present study, we show that in contrast to acute T cell leukemic Jurkat cells, cutaneous T cell lymphoma HuT-78 cells were resistant to the apoptotic effects of TNF and constitutively expressed high levels of activated form of NF-κB and of IκB-α simultaneously. The latter was resistant to TNF-induced IκB-α degradation and had a very long half-life. Neutralizing anti-TNF antibodies down-regulated the constitutive NF-κB activation and induced apoptosis in HuT-78 but not in Jurkat cells. Pyrrolidine dithiocarbamate (PDTC), a quencher of reactive oxygen intermediates, also inhibited constitutively activated NF-κB and rendered HuT-78 cells susceptible to TNF-induced killing.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Bacteria-derived human rTNF, purified to homogeneity, was kindly provided by Genentech (San Francisco, CA). PMA, lipopolysaccharide, hydrogen peroxide, PDTC, ceramide, and cycloheximide were purchased from Sigma. Okadaic acid (LC Laborato-}

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HuT-78, a cutaneous T cell lymphoma derived from peripheral blood of a Caucasian patient with Sezary syndrome with the properties of a mature T cell line of inductor/helper phenotype, was a generous gift from National Institutes of Health AIDS Research and Reference Reagent Program of National Institutes of Health. These cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM glutamine, and antibiotics at 37 °C in an atmosphere of 5% CO\textsubscript{2} in air. GST-IkB-\alpha was prepared as described previously (31).

Antibodies—The polyclonal antibodies used were as follows: anti-p65, against the epitope corresponding to amino acids mapping within the amino-terminal domain of human NF-\kappa B p65; anti-p50, against a peptide 15 amino acids long mapping at the NLS region of NF-\kappa B p50; anti-IkB-\alpha, against amino acids 297–317 mapping at the carboxyl terminus of IkB-\alpha/MAD-3 and anti-IkB-\beta (amino acid 339–358) and anticyclin D1 against amino acids 1–295, which represents full-length cyclin D1 of human origin. All these antibodies were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA.). Anti-PARP monoclonal antibody that recognizes PARP and its degradation product was purchased from PharMingen (San Diego, CA.). Anti-TNF antibodies were raised in rabbits using recombinant TNF as described (22).

Electrophoretic Mobility Shift Assays (EMSA)—The details of the preparation of nuclear extracts and the assay procedure have been described elsewhere (23, 24). Nuclear extracts were either used immediately or stored at −70 °C. Typically, 4–6 μg of protein was used per assay. The protein content of the extract was measured by the method of Bradford (25). EMSAs were performed by incubating nuclear extract with \textsuperscript{32}P-end-labeled 45-mer double-stranded NF-\kappa B oligonucleotide from the human immunodeficiency virus terminal repeat, 5'TTGTTCACAAGGGACATTTTCGTGGGAGGAGCCGTGG-3'. A double-stranded mutated oligonucleotide, 5'TTGTTCACAACCTCATTTCAGGGGCGGTGG-3', was used to examine the specificity of binding of NF-\kappa B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, antibodies against p50 or p65 subunits of NF-\kappa B were used as described (26). Visualization and quantitation of radioactive bands were carried out using a PhosphorImager (Molecular Dynamics) using Image Quant software (National Institutes of Health, Bethesda, MD).

Determination of IkB by Western Blot—IkB-\alpha and -\beta Western blot assays were carried out with 25–30 μg of cytoplasmic extracts. Following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline with 0.5% Tween 20 (PBST) containing 5% fat-free milk and then exposed to IkB-\alpha antibodies at 1–3000 dilution. The membranes were washed with PBST and treated with secondary antibody conjugated to horseradish peroxidase. The antigen-antibody reaction was visualized by an enhanced chemiluminescence (ECL) assay using Amersham ECL reagents and exposure to film.

Transient Transfection and CAT Assay—HuT-78 or Jurkat cells were transiently transfected with −243RMICAT (wild-type) or −243RMI-CAT (mutant) and cultured for 24 h before the assay as described under “Experimental procedures.” B, analysis of PARP cleavage by TNF in HuT-78 and Jurkat cells. Cytoplasmic extracts (50 μg) were prepared from 2 × 10\textsuperscript{6} cells treated with 1 nM TNF or 10 μg/ml cycloheximide (CHX) or both for 2 h at 37 °C, resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed by anti-PARP antibody.
TNF and NF-κB Expression in Resistance to Apoptosis

Fig. 3. A, kinetics of TNF-induced degradation of IκB-α. 30 μg of cytoplasmic extract from 100 μl TNF-treated cells were resolved by SDS-PAGE using 8.5% gel, and the separated proteins were transferred to nitrocellulose membrane and probed with anti-IκB-α antibodies. Antigen-antibody reaction was visualized using ECL assay. B, rate of disappearance of IκB-α in the presence of cycloheximide. 2 × 10^6 cells were treated with 20 μg/ml cycloheximide for the indicated times. Cytoplasmic extracts (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, probed with anti-IκB-α antibodies, and visualized by ECL assay.

Cytotoxicity was represented by detection of both cleaved (86 kDa) and active forms of PARP. Antigen-antibody reaction was visualized using ECL assay. The absorbance was read at 570 nm and the percent cell viability was calculated.

RESULTS

HuT-78 Cells Are Resistant to Apoptosis by TNF—The aim of the present study is to understand the molecular basis for induction of sensitivity or resistance of tumor cells to the apoptotic effects of TNF. Two human T cell lines, Jurkat and HuT-78, were examined. The proliferation of Jurkat cells was completely inhibited by TNF, whereas it had no significant effect on the proliferation of HuT-78 cells (Fig. 1A). Activation of caspase-9 leading to cleavage of PARP is one of the major hallmarks for apoptosis. When examined for the ability of TNF to induce PARP cleavage, it was found that PARP was cleaved by TNF in Jurkat cells but not in HuT-78 cells (Fig. 1B). Cycloheximide arrested the TNF-induced PARP cleavage only in Jurkat cells, not in HuT-78 cells. These results indicate that HuT-78 cells are resistant to growth inhibition and apoptosis induction by TNF.

HuT-78 Cells Constitutively Express Activated NF-κB—As NF-κB activation has been implicated in induction of resistance, we examined the constitutive and inducible NF-κB activation in the two cell types. Gel shift assay showed that under normal conditions, Jurkat cells did not express activated NF-κB, but on treatment with TNF, activated NF-κB was noted within 5 min, reached maximum at 30 min, and declined thereafter (Fig. 2A). In contrast, HuT-78 cells constitutively expressed high levels of activated NF-κB and could not be further activated by TNF (Fig. 2A). Supershift assays using antibodies to either the p50 or p65 subunits of NF-κB retarded the mobility of NF-κB proteins in both Jurkat and HuT-78 cells (Fig. 2B). This shift was specific, as the irrelevant antibodies anti-cyclin D1 and preimmune serum had no effect. Thus it is possible that HuT-78 cells were resistant to TNF-induced apoptosis, because they constitutively expressed activated NF-κB.

HuT-78 Cells Constitutively Express High Levels of IκB-α—We used Western blot analysis to further investigate why HuT-78 cells constitutively express activated NF-κB. NF-κB is present in its inactive state in the cytoplasm where it is bound to IκB-α. The degradation of IκB-α is critical for NF-κB activation and translocation to the nucleus. Western blot analysis of the levels of IκB-α in the cytoplasm before and after TNF treatment showed that in Jurkat cells, TNF caused the degradation of IκB-α within 15 min; it was resynthesized by 60 min (Fig. 3A). In contrast, HuT-78 expressed large amounts of IκB-α, and these were not affected by TNF treatment. Thus HuT-78 cells constitutively express high levels of IκB-α along with activated NF-κB.

IκB-α Turnover Is Slow in HuT-78 Cells—Why IκB-α does not inhibit NF-κB activation in HuT-78 cells was investigated. The synthesis of IκB-α was blocked by treatment of cells with cycloheximide, which allowed us to determine the constitutive rate of degradation. The half-life of IκB-α was found to be less than 60 min in Jurkat cells but greater than 12 h in HuT-78 cells (Fig. 3B). These results suggest that IκB-α from HuT-78 cells is either resistant to proteolysis or its protease is not as active in HuT-78 cells.

HuT-78 Cells Express Various Rel Proteins and IκB-α and -β—To determine why NF-κB is constitutively activated in HuT-78, but not in Jurkat cells, we examined the levels of p50, p65, p52, c-Rel, IκB-α, and IκB-β in the cytoplasm and nucleus of these cell types by Western blot analysis. As shown in Fig. 4A, all these NF-κB proteins are expressed in the cytoplasm of both cell types, but levels may be somewhat higher in HuT-78 cells. In addition, HuT-78 cells expressed two different isoforms of p52, whereas Jurkat cells expressed only one form. Besides
Heat-denatured GST-I from either cell type in a concentration dependent manner. The possibility, the p50-p65 complex prepared from TNF activated cells was incubated etantly in Jurkat cells than in HuT-78 cells. To investigate this possibility, the p50/p65 complex prepared from TNF activated cells was incubated in vitro with recombinant GST-IκB-α and then examined for binding to DNA by EMSA. As shown in Fig. 4B, IκB-α interacted with DNA binding activity of p50/p65 through pathways consisting of both overlapping and nonoverlapping steps. FMA, lipopolysaccharide, H2O2, ceramide, okadaic acid, and IL-1 activated NF-κB in Jurkat cells, but none had any effect on NF-κB in HuT-78 cells (Fig. 6). It is possible that NF-κB is constitutively maximally activated in HuT-78 cells.

PDTC Inhibits NF-κB Activation and Renders HuT-78 Cells Susceptible to TNF-induced Apoptosis—The mechanism of NF-κB activation is not fully understood. Activation by most stimuli, however, requires the generation of reactive oxygen species (ROS). PDTC is known to inhibit the formation of ROS and NF-κB activation by certain stimuli in a cell type-specific manner (28). Whether PDTC can also inhibit constitutive NF-κB activation is not known. We showed that PDTC suppressed the TNF-induced NF-κB in Jurkat cells (Fig. 7A). PDTC also suppressed NF-κB activation in HuT-78 cells (Fig. 7B). Thus, like inducible activation of NF-κB in Jurkat cells, ROS was involved in constitutive activation of NF-κB in HuT-78.
To determine if constitutive NF-κB activation in HuT-78 cells is linked to its resistance to apoptosis by TNF, cells were treated with the cytokine in the presence of PDTC. PDTC sensitized HuT-78 cells but not Jurkat cells to TNF-induced cytotoxicity (Fig. 8A). When examined for caspase-3-dependent PARP cleavage, in agreement with the cytotoxicity data, PDTC sensitized HuT-78 cells to TNF-induced PARP cleavage (Fig. 8B). Thus constitutive NF-κB activation was responsible for resistance to TNF-induced apoptosis in HuT-78 cells. As shown in Fig. 1, Jurkat cells, when treated with TNF, were growth inhibited and underwent PARP cleavage. The treatment of these cells with PDTC seemed to partially protect them from TNF-induced cytotoxicity (Fig. 8A) and PARP cleavage (Fig. 8B). These results suggest a dual function for ROS, i.e. apoptotic and antiapoptotic. In Jurkat cells ROS is needed for apoptosis, whereas in HuT-78 cells ROS prevents apoptosis.

Anti-TNF Antibodies Inhibit NF-κB Activation and Proliferation of HuT-78—TNF is known to induce ROS (28). In HuT-78 cells constitutive synthesis of TNF may be responsible for production of ROS and thus for constitutive expression of activated NF-κB. To determine this, we treated HuT-78 cells for 24, 48, and 72 h with anti-TNF antibodies and then assayed them for NF-κB. Anti-TNF treatment down-regulated the nuclear levels of activated NF-κB in a time-dependent manner (Fig. 9A). This decrease correlated with a decrease in the constitutive levels of IκB-α in the cytoplasmic pool (Fig. 9B). Anti-TNF antibodies also decreased HuT-78 cell proliferation, but not Jurkat cell
proliferation in a dose-dependent manner (Fig. 9C). Consistent with these results, anti-TNF antibodies induced PARP cleavage in HuT-78 cells but not in Jurkat cells (Fig. 9D). In addition, when measured for TNF production, HuT-78 cells were found to express TNF but not Jurkat cells (data not shown). These results indicate that HuT-78 cells constitutively express TNF, which causes NF-κB activation through ROS and that in turn induces resistance to apoptosis, also consistent with the effect of PDTC, indicating that NF-κB activation leads to resistance to apoptosis.

DISCUSSION

In the present study we investigated the mechanism by which tumor cells develop resistance to apoptosis induced by TNF. Two human T cell lines, Jurkat and HuT-78, were examined. While TNF inhibited the growth of Jurkat cells and activated caspase-3, it had no effect on HuT-78 cells; constitutive activation of NF-κB was demonstrated in HuT-78 cells and may account for this difference in growth and apoptosis induction. TNF and various other stimuli activated NF-κB in Jurkat cells but not in HuT-78 cells. Despite preactivated NF-κB, HuT-78 cells also expressed high levels of IκB-α, which unlike Jurkat cells, were resistant to TNF-induced degradation. The half-life of the IκB-α in HuT-78 cells is much longer than Jurkat cells. Antibodies against TNF down-regulated the constitutive activation of NF-κB and proliferation of HuT-78 cells, suggesting an autocrine role for TNF. The antioxidant PDTC also suppressed constitutive NF-κB activation and reversed sensitivity to TNF for cytotoxicity and caspase-3 activation. These results suggest that constitutive NF-κB activation causes resistance to apoptosis through generation of ROS and TNF.

Our results show that HuT-78 cells express activated NF-κB and IκB-α simultaneously. This is not too surprising as the synthesis of IκB-α requires NF-κB activation (29), but why IκB-α fails to inhibit constitutively activated NF-κB, however, is not clear. Our results show that IκB-α in HuT-78 is degraded very slowly compared with Jurkat cells. In WEHI-3 cells, which were also shown to express constitutively activated NF-κB, the rate of degradation of IκB-α is faster than normal (30). Previously, we have shown that IκB-α phosphorylated at Tyr-42 is refractory to TNF-induced degradation (31). Thus, it is possible that IκB-α from HuT-78 cells is phosphorylated at Tyr-42.
Phosphotyrosine blots, however, did not reveal any tyrosine phosphorylation (data not shown). A lack of retarded mobility of IxB-α from HuT-78 cells on SDS-PAGE gels also indicated a lack of phosphorylation. It is possible that the p50-p65 heterocomplex is mutated so that it can no longer bind to endogenous IxB-α. However, it is unlikely because we found that the p50-p65 heterocomplex can bind both exogenously added IxB-α, can bind to the NF-κB binding site in the DNA, and is supershifted by the antibodies.

NF-κB activation in HuT-78 cells was not only refractive to TNF, the constitutive DNA binding activity in these cells remained unchanged by a host of other NF-κB activators, including lipopolysaccharide, H₂O₂, ceramide, IL-1, and phorbol 12-myristate 13-acetate. Western blot analysis indicated that besides a nuclear pool there is a cytoplasmic pool of p50-p65 in HuT-78 cells. Coprecipitation followed by Western blot analysis results indicated that the cytoplasmic pool of p50-p65 subunits in HuT-78 cells exists in complex with IxB-α. The absence of further activation by various stimuli suggest the lack of nuclear translocation of p50-p65 from the cytoplasm, perhaps because of decreased proteolytic activity of the enzyme required to degrade IxB-α, as noted earlier.

Our results indicate that NF-κB activation and apoptosis are linked, but how constitutive activation of NF-κB prevents apoptosis of HuT-78 cells is not clear. Several genes that are known to down-regulate apoptosis are regulated by NF-κB activation, including the zinc finger protein A20 (32), manganese superoxide dismutase (33), and cIAP2 (cellular inhibitor for apoptosis) (34). It is possible that these genes are constitutively expressed in HuT-78 cells, thus leading to initiation of apoptosis. Our results are consistent with reports that mice lacking the NF-κB p65 gene die early in embryonic development from massive cellular death of hepatic parenchyma (35). The antiapoptotic role of NF-κB was also demonstrated from the observation that embryonic fibroblast from IxB knockout mice are resistant to TNF (6). Similarly, transfection of a dominant negative form of IxB-α cDNA prevented the TNF-induced apoptosis of cells (7, 8).

Our results show that pretreatment of HuT-78 cells with PDTC inhibited constitutive NF-κB activation and sensitized the cells to TNF-induced apoptosis, thus suggesting a critical role of ROS. PDTC is a potent inhibitor of inducible NF-κB activation (28, 36) in most cells. It displays antioxidant property both by metal chelating and by acting as a radical scavenger (37, 38). Generation of ROS has been proposed as an important mechanism to mediate the apoptotic and gene regulatory effects of TNF (39, 40). Our results, however, indicate ROS is also involved in protection of cells from apoptosis by activating NF-κB.

Besides PDTC, treatment of cells with anti-TNF antibodies also down-regulated NF-κB and inhibited cell growth. These results, which are consistent with previous report (41), suggest an autocrine role for TNF in induction of resistance. In addition, studies employing a TNF expression vector and an anti-sense TNF mRNA expression vector transfected into TNF-sensitive and -insensitive cells clearly demonstrated that endogenously made TNF is protective against TNF-induced cytotoxicity (42). These observations further confirm the inhibitory role of NF-κB in apoptosis. Additionally, our study demonstrates the autocline growth-promoting role of TNF through the generation of reactive oxygen intermediates. Inhibitors of both nuclear factor-κB and activator protein-1 activation have been shown to block the neoplastic transformation response (43). In summary, our study demonstrates the role of constitutively expressed NF-κB in the induction of resistance in HuT-78 cells through generation of TNF and ROS.

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