Tumor necrosis factor-α (TNF-α) stimulates proliferation of Mo7e, CMK, HU-3, and M-MOK human leukemic cell lines. We report here the signal transduction pathway involved in TNF-α-induced Mo7e cell proliferation. Mo7e cells spontaneously die in the absence of growth factors, but treating the cells with interleukin (IL)-3, IL-6, thrombopoietin, granulocyte/macrophage colony-stimulating factor, or TNF-α promotes their survival and proliferation. Although most of these factors activate MAP kinase and Jun NH₂-terminal kinase/signal transducer and activators of transcription signaling pathways, TNF-α fails to activate either pathway. When Mo7e cells were treated with TNF-α, nuclear factor κB (NF-κB) was activated transiently. The activated NF-κB consisted of heterodimers of p65 and p50 subunits. The degradation of IκBα coincided with activation of NF-κB in TNF-α-treated cells. To investigate the role of activated NF-κB in TNF-α-induced Mo7e proliferation, a cell-permeable peptide (SN50) carrying the nuclear localization sequence of p50 NF-κB was used to block nuclear translocation of activated NF-κB. Pretreating Mo7e cells with SN50 blocked TNF-α-induced nuclear translocation of NF-κB and inhibited TNF-α-induced Mo7e cell survival and proliferation. A mutant SN50 peptide did not affect TNF-α-induced Mo7e cell growth. SN50 had no effects on IL-3- or granulocyte/macrophage colony-stimulating factor-induced Mo7e cell proliferation. The results indicate that activation of NF-κB is involved in TNF-α-induced Mo7e cell survival and proliferation.

Tumor necrosis factors (TNFs)† are produced by neutrophils, activated lymphocytes, macrophages, natural killer cells, endothelial cells, and smooth muscle cells, and were reported initially to induce tumor necrosis (1, 2). Subsequent studies, however, indicated that TNFs also promote the proliferation and survival of some tumor cell lines (3–5). In patients with leukemias or lymphomas, elevated levels of serum TNF-α, which are associated with a poor prognosis, have been reported (6–13). The reasons for the TNF-α-related poor prognosis of leukemia patients are not well understood. We and others reported that TNF-α significantly stimulates the growth of several human leukemic cell lines, including Mo7e (14, 15), CMK (3, 5), HU-3, and M-MOK (4). TNF-α also stimulates the proliferation of primary leukemia cells isolated from patients (16–18). In an ultrastructural study of primary leukemia cells from patients, TNF-α did not induce direct cytotoxicity or apoptosis, but instead stimulated leukemia cells (19). Furthermore, we and others have found that low doses of TNF-α act synergistically with other cytokines, such as IL-4, to stimulate Mo7e leukemic cell growth (14, 15). However, the molecular mechanisms of the signaling pathway(s) in TNF-α-induced leukemic cell proliferation remain unclear.

Extensive studies show that TNF-α is an extremely pleiotropic factor, which can induce activation of phagocytic and endothelial cells, induction of prostaglandins, alterations in lipid metabolism, and regulated expression of major histocompatibility complex antigens, oncogenes, and transcription factors (1, 2). The capacity for TNF-α to induce such a wide variety of effects is attributable, in part, to its ability to activate multiple signal transduction pathways including mitogen-activated protein kinases (MAPKs), Jun NH₂-terminal kinase (JNK), nuclear factor κB (NF-κB), and/or STATs in numerous cell lines (20–24). Although primarily involved in cell proliferation (25, 26), the activation of MAPK may have also been implicated in apoptosis in some circumstances (27–29). TNF-α-induced recruitment of the signal transducer FADD to type I TNF receptors may mediate TNF-α-induced apoptosis, but TNF-α-induced activation of NF-κB protects against TNF-α-induced apoptosis in some cell lines (30). Conventional NF-κB is a heterodimer that consists of p65 and p50 subunits. Both subunits of NF-κB are members of the NF-κB/Rel family of transcription factors, which also includes c-Rel, RelB, and p52 (31). The activity of NF-κB is strictly regulated by an inhibitor, IκBα, that forms a complex with NF-κB and keeps NF-κB in the cytoplasm (23). When cells receive signals that activate NF-κB, IκBα is phosphorylated and degraded through a ubiquitin/proteasome pathway. The degradation of IκBα triggers the translocation of NF-κB from the cytoplasm to the nucleus. Although increasing evidence shows that the activation of NF-κB is involved in cell activation and proliferation, several lines of evidence also indicate that activation of NF-κB may result in apoptosis (32–34).

The fact that TNF-α stimulates the proliferation of human leukemic cell lines as well as primary leukemia cells led us to investigate the TNF-α-induced activation of signaling path-
ways in Mo7e and other cell lines. Specifically, we addressed the questions of what signal transduction pathways are activated in leukemic cells treated with TNF-α and whether the activated signal transduction pathway(s) is/are involved in TNF-α-induced Mo7e cell proliferation. We found that TNF-α transiently induced activation of NF-κB, but not the MAPK or STAT signaling pathways, which are activated by IL-3, GM-CSF, and thymopoietin (TPO) in Mo7e and other cell lines. Pretreatment of Mo7e cells with a synthesized membrane-permeable peptide, SN50, blocked the TNF-α-induced translocation of NF-κB to the nuclei in a dose-dependent manner. Pretreating Mo7e cells with SN50 specifically inhibited TNF-α-induced but not IL-3- or GM-CSF-induced Mo7e cell proliferation. Thus, the data presented here provide direct evidence that the activation and function of NF-κB is essential to TNF-α-induced proliferation of Mo7e cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human TNF-α, recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF), and anti-TNF-α antibody were obtained from R&D Systems (Minneapolis, MN). IL-3, IL-6, and TPO were purchased from PeproTech (Rocky Hill, NJ). [methyl-3H]Thymidine (specific activity 70–86 Ci/mmol), [32P]dATP (specific activity >3000 Ci/mmol), and [32P]dCTP (specific activity >3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Anti-γ-interferon antibody labeled with fluorescein isothiocyanate (FITC) was purchased from Zymed Laboratories Inc. Antibodies against human p50, p52, p65, and B-Rel NF-κB subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antihuman IκBα and anti-phosphorylated IκBα antibodies were purchased from New England Biolabs (Beverly, MA).

**Cell Lines**—The human Mo7e megakaryoblastic leukemia cell line, originally described by Avanzi et al. (35), was maintained in Isoeve’s modified Dulbecco’s medium (IMDM; Life Technologies, Inc.) containing 10% fetal bovine serum, 1% glutamine, and 5 ng/ml rhGM-CSF. The human Meg-01 megakaryoblastic leukemia cell line, originally described by Ogura et al. (36), was maintained in RPMI 1640 medium (Life Technologies, Inc.) with 10% fetal bovine serum. The human leukemic HEL cell line, which has both erythroid and megakaryocytic characteristics (14, 37, 38), was maintained in IMDM with 10% horse serum. Both Meg-01 and HEL cell lines were purchased from ATCC, and the Mo7e cell line was obtained from Genetix Institute (Boston, MA). In experiments to detect effects of cytokines, Mo7e cells were prepared by washing three times with serum-free medium and were starved for 18 h in medium without cytokine (14). Meg-01 and HEL cells were cultured in serum-free medium with 1× Nutridoma Hu (Roche Molecular Biochemicals) for 18 h before cytokine treatments.

**Synthesis of the Recombinant Membrane-permeable Peptides—**SN50, a peptide that has been reported to have the capacity to block the nuclear translocation of activated NF-κB (34, 39), contains membrane-permeable signal sequences of Kaposi’s fibroblast growth factor and the nuclear translocation motif (VQRKRQKLMP) of human NF-κB p50.

SN50mt, a mutant SN50, contains membrane-permeable signal sequences of Kaposi’s fibroblast growth factor and the mutant nuclear translocation motif of human NF-κB p50 (39). Both SN50 and SN50mt phosphorylated commercially synthesized peptides (South San Francisco, CA). The peptides were purified by reverse phase high-performance liquid chromatography, and the molecular weight of the purified peptides was verified by mass spectrometry analysis. To track the intracellular localization of the membrane-permeable peptides in Mo7e cells, SN50 was labeled with FITC. The synthesized peptides were dissolved in Me2SO to a final concentration of 100 μg/ml and mixed directly with culture medium (1:500–1000).

**Effects of TNF-α on Cell Growth**—To determine the effects of TNF-α on cell proliferation, DNA synthesis was measured by [3H]thymidine incorporation in freshly prepared cells. The assays were performed in triplicate, using a total of 4 × 10⁶ Mo7e or Meg-01 cells, or 2 × 10⁶ HEL cells. Mo7e cells in IMDM with 10% FCS, and Meg-01 and HEL cells in medium with 1× Nutridoma Hu, were cultured for 72 h in the presence or absence of TNF-α or other cytokines. After that, the cells were labeled with 4 μCi/ml [3H]thymidine for an additional 4 h. The radioactivity incorporated into DNA (counts per minute) was determined by a liquid scintillation counter according to a previously described protocol (14). All assays for [3H]thymidine incorporation were repeated at least three times. Some of our data from [3H]thymidine incorporation assays also were compared with those obtained from the Non-radioactive Cell Proliferation Wst-1 kit (Roche Molecular Biochemicals). Both in vitro and ex vivo experiments yielded consistent results, but only the data from [3H]thymidine incorporation assays are presented.

**MAP Kinase Kinase Activity and Western Blotting Analysis**—The functional activity of MAP kinase kinase (MEK) was detected by the kinase-induced phosphorylation of a kinase-defective p42/p44 MAP kinase (K52R) labeled with [32P]ATP, following a published protocol (35) (K52R) and anti-phosphorylated-STAT3 antibodies (New England Biolabs), according to the methods suggested by the manufacturer. For comparison, total STAT1 and STAT3 (both phosphorylated and unphosphorylated) also were examined with anti-STAT1 and -STAT3 antibodies. Western blotting was visualized with enhanced chemiluminescence.

**Preparation of Nuclear Extracts and Electrophoretic Gel Mobility Shift Assays**—Preparation of nuclear extracts and gel mobility shift assays were performed according to methods described previously (40, 41). The sequence of the NF-κB-binding oligonucleotide used for the radioactive DNA probe was 5′-TGGACAGAGGGAGACTTTCCAGAGGC-3′. Equal amounts of nuclear proteins (5–10 μg) for each sample were incubated with 1 ng of 32P-labeled probe. The DNA binding reaction was...
performed at room temperature in a volume of 25 μl, which contained binding buffer (10 mM Tris-HCl, pH 7.5, 1 μM EDTA, 100 mM NaCl, 20 μg/ml bovine serum albumin, and 0.2% Nonidet P-40, 1.8 μg/ml salmon sperm DNA), 1 ng of 3'-labeled probe, and 5–10 μg of nuclear proteins. After incubation for 15 min, the samples were electrophoresed on native 6% acrylamide, 0.25 × Tris borate-EDTA gels. The gels were dried and exposed to x-ray film. Competition was performed by adding 50–100 μl excess of each unlabeled DNA fragment along with the 32P-labeled probe. For gel mobility supershift assays, nuclear extracts were co-incubated with the indicated specific anti-NF-κB subunit antibodies and 32P-labeled oligonucleotide probes. The DNA-protein complexes and unbound probe were separated electrophoretically on 6% native polyacrylamide gels in 0.25 × TBE buffer (44.5 mM Tris, pH 8.0, 1 mM EDTA, and 44.5 mM boric acid). The gels were fixed and dried, and the DNA-protein complexes were visualized by autoradiography at −70 °C with Kodak X-Omat film and a DuPont Cronex Lightning Plus intensifying screen.

Flow Cytometry Analysis—A FACScan flow cytometer was used to examine Mo7e cell cycle status, intracellular localization of SN50 peptides, and apoptosis. Mo7e cells were incubated with 50 μg/ml FITC-labeled SN50 peptide for various periods or with various amounts of FITC-SN50 for 30 min to investigate the intercellular incorporation of the peptides. The status of apoptosis was analyzed by incubating cells with FITC-labeled annexin V and propidium iodide (PI), following the manufacturer’s suggested procedure. For the analysis of cell cycle status and cellular DNA ploidy, the cells were fixed with methanol and stained with PI according to the published method (14).

RESULTS

TNF-α Induces Activation of NF-κB in Human Leukemic Cell lines—When the electrophoretic gel mobility shift assay (EMSA) was used to examine the activation of NF-κB in Mo7e, HEL, and K562 human leukemic cell lines, no detectable activation of NF-κB was observed in the cells without cytokine exposure (Fig. 1a, CTL). However, exposure of the cells to 5 ng/ml TNF-α for 30 min significantly induced the activation of NF-κB in Mo7e, HEL, and K562 cells (Fig. 1a, TNF). The Mo7e cells were used to further investigate the dynamics of the activation of NF-κB. When Mo7e cells were treated with 5 ng/ml TNF-α for 0–120 min, the activation of NF-κB was first detected within 5 min, attained a maximal level at 30 min, and declined thereafter (Fig. 1b). Supershift EMSA was then used to examine the components of the DNA-protein complexes. The results showed that both anti-p50 and anti-p65 antibodies induced a supershift of the DNA-protein complex in nuclear extracts from Mo7e, HEL, and K562 cells treated with 5 ng/ml TNF-α, indicating that the TNF-α-induced DNA-protein complexes in these cells contained both p50 and p65 NF-κB subunits. When the nuclear extracts from cells treated with TNF-α were incubated with anti-p52, anti-RelB, or anti-c-Rel antibodies, no supershift was observed (data not shown). Furthermore, the formation of the NF-κB-DNA complex was inhibited competitively by an unlabeled NF-κB DNA probe in a dose-dependent manner. A 100-fold molar excess of nonradioactive DNA containing the NF-κB binding site completely prevented binding of the activated NF-κB to the 32P-labeled NF-κB-binding probe (Fig. 2c), but did not affect the nonspecific DNA-protein complexes (Fig. 2, bands marked as NS). The results, therefore, demonstrate clearly that the NF-κB p65/p50 heterodimer is activated in these leukemic cell lines in response to TNF-α.

TNF-α Treatment Results in a Rapid Translocation of NF-κB to the Nucleus of Mo7e Cells—We then examined the effect of TNF-α treatment on the localization of NF-κB in Mo7e cells by Western blotting with specific antibodies against the p65 NF-κB subunit. Before stimulation, p65 was detected exclusively in the cytoplasmic lysates (Fig. 3a, lane 1). When Mo7e cells were treated with 5 ng/ml TNF-α for 5–120 min, the level of cytoplasmic NF-κB p65 declined gradually (Fig. 3a, lanes 2–7). When nuclear extracts were used in Western blotting to analyze the level of nuclear NF-κB p65, there was no detectable NF-κB p65 in the nucleus of Mo7e cells without TNF-α exposure (Fig. 3b, lane 1). However, NF-κB was detectable in the nucleus within 5 min, and the level of nuclear NF-κB p65 increased markedly in cells exposed to TNF-α, with a maximal level within 30–60 min of the treatment (Fig. 3b, lanes 4–7). When nonimmune immunoglobulins were used as the first antibody, no NF-κB p65 was detected in the nuclear extracts from Mo7e cells (data not shown). The results from Western blotting analysis of cytoplasmic lysates and nuclear extracts confirm and extend the EMSA findings of activation and nuclear translocation of NF-κB in Mo7e cells treated with TNF-α.

![Fig. 2. Identification of the components of activated NF-κB in the DNA-protein complex by EMSA.](image-url)
Degradation of IκBα Is Associated with the Activation of NF-κB in Mo7e Cells—A common feature of the regulation of NF-κB is its sequestration in the cytoplasm as an inactive complex with IκBα in the basal state. To investigate the role of IκBα in the activation of NF-κB by TNF-α, phosphorylation and the total level of IκBα were analyzed by Western blotting, and the results were compared with the dynamics of NF-κB activation in Mo7e cells after TNF-α stimulation. When nuclear extracts from Mo7e cells treated with TNF-α were analyzed by Western blotting with an antibody specific for phosphorylated IκBα, the phosphorylation of IκBα became detectable in cells treated with 5 ng/ml TNF-α for 1 min, reached the maximum level at 5 min, and then declined rapidly (Fig. 4a, lanes 2–6). Western blotting analysis of cytoplasmic lysates showed that the rapid decline of nuclear IκBα in TNF-α-treated Mo7e cells was due to a decrease of total IκBα rather than dephosphorylation of IκBα, since the level of total IκBα (phosphorylated and unphosphorylated) also was decreased markedly upon TNF-α treatment (Fig. 4b). Comparison of these results to those in Fig. 3, which showed that the activation of NF-κB induced by TNF-α was first detected within 5 min and maximal at 30 min, indicate clearly that the degradation of IκBα coincides with the activation and nuclear translocation of NF-κB in Mo7e cells.

TNF-α Specifically Induces the Activation of NF-κB, but Not MAPK or STAT5, Which Are Activated by IL-3 and GM-CSF—MAPK and STAT signal transduction pathways have been reported to be activated by several hematopoietic growth factors and to be involved in growth factor-induced Mo7e cell proliferation (14, 42). To investigate whether these signal transduction pathways are activated by TNF-α and whether they are involved in TNF-α-induced cell proliferation, we examined the effect of TNF-α on the activation of MAPK and STAT signal transduction pathways. When Mo7e cells were treated with 5 ng/ml IL-3 or GM-CSF for 30–60 min, no significant activation of NF-κB was observed (Fig. 5a, lanes 3–6), although TNF-α treatment induced significant activation of NF-κB (Fig. 5a, lane 2). When the activation of the STAT5 signal transduction pathway in Mo7e cells was examined by EMSA, the results, which were similar to those that we reported previously (14), showed that STAT5 activation was observed in Mo7e cells treated with GM-CSF, IL-3, TPO, and IL-6, but not in cells treated with 5 ng/ml TNF-α for 10–60 min (data not shown). When the activation of STAT1 and STAT3 was examined by EMSA, supershift EMSA, or Western blotting with specific anti-phosphorylated STAT1 or STAT3 antibodies, no significant activation or phosphorylation of STAT1 or STAT3 was observed in Mo7e cells treated with 5 ng/ml TNF-α, IL-3, or GM-CSF for 10–60 min (data not shown). However, a significant activation of STAT1 and STAT3 was detected in control SK-BR-3 cells treated with 2 ng/ml epidermal growth factor for 30 min, which we reported previously as the positive control for detecting activation of STAT1 and STAT3 (42).

When functional MEK activation was investigated in Mo7e, HEL, or Meg-01 human leukemic cell lines after exposure to TNF-α, we found no significant activation of MEK in any of these cell lines exposed to 5 ng/ml TNF-α for 5–30 min (data not shown). For example, in Mo7e or HEL cells treated with 5 ng/ml TNF-α for 10 min, no significant MEK activation was observed (Fig. 5b, Mo7e and HEL, TNF). However, activation of MEK was observed in these cells after exposure to 5 ng/ml GM-CSF for 10 min (Fig. 5b, GM). In separate experiments to examine phosphorylation of p44/p42 MAPK, our data also showed that phosphorylation of MAPK was barely detectable (not different from untreated control cells) in all of these cell lines after exposure to 5 ng/ml TNF-α for 5–30 min, but increased phosphorylation was readily detectable in cells treated with 5 ng/ml IL-3 or GM-CSF for 10 min and reached the...
SN50 Peptide Inhibits Nuclear Translocation of Activated NF-κB—SN50 is a synthetic peptide containing signal sequences of Kaposi's fibroblast growth factor and the nuclear localization sequence of NF-κB p50. It has been reported to have the capacity to specifically block the nuclear translocation of activated NF-κB (39). To investigate the membrane permeability, dynamics of cellular uptake, and intracellular incorporation of the synthesized peptide, FITC-labeled SN50 was incubated with Mo7e cells. Flow cytometry analysis showed that the peptide penetrated into Mo7e cells rapidly. When the cells were incubated with 50 μg/ml amounts of the peptide, the maximal level of FITC-SN50 was detected within 15 min and longer incubation with FITC-labeled peptide did not further increase fluorescence (Fig. 6a). When Mo7e cells were incubated with 1–100 μg/ml FITC-SN50 for 30 min, a dose-dependent intracellular accumulation of SN50 was observed (Fig. 6b). Furthermore, our data showed that SN50 is quite stable after penetrating into the cells, with a half-life more than 12 h (data not shown).

When Mo7e cells were pretreated with various amounts of SN50 for 15 min and then incubated with 5 ng/ml TNF-α for an additional 30 min, there was a marked SN50 dose-dependent reduction of TNF-α-induced NF-κB-binding activity in Mo7e nuclei, as determined by EMSA. For example, in cells treated with 50–100 μg/ml SN50, the nuclear NF-κB-DNA complexes were almost undetectable (Fig. 7a). When Western blotting with a specific anti-NF-κB p65 antibody was used to analyze effects of SN50 on nuclear translocation of NF-κB in Mo7e cells treated with TNF-α, the results showed that there was a marked SN50 dose-dependent reduction of the amount of nuclear NF-κB p65. For example, in cells treated with 5 ng/ml TNF-α and 100–200 μg/ml SN50, almost no nuclear NF-κB P65 subunit was detected (Fig. 7b). However, no significant decrease of the level of nuclear NF-κB p65 was observed in nuclear extracts from the cells treated with 5 ng/ml TNF-α and 10–200 μg/ml SN50mt, a nonfunctional mutant of SN50 (Fig. 7c). These results demonstrate the specific inhibitory effect of SN50 on the nuclear translocation of activated NF-κB.

SN50 Specifically Inhibits TNF-α-induced Survival and Proliferation of Mo7e Cells—To investigate the role of NF-κB activation in TNF-α-induced Mo7e cell proliferation, SN50 and SN50mt were used to examine the effect of SN50-induced inhibition of NF-κB nuclear translocation on TNF-α-induced Mo7e cell survival and proliferation. When Mo7e cells were treated with various amounts of SN50 with 1 ng/ml TNF-α, TNF-α-induced Mo7e cell proliferation was inhibited in a dose-dependent manner. For example, treating Mo7e cells with 1

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**Fig. 6. Cellular uptake of SN50 in Mo7e cells.** The cells were incubated with 50 μg/ml FITC-labeled SN50 peptide for 0–60 min (a) and 1–100 μg/ml FITC-SN50 for 30 min (b). The cells were washed three times and analyzed by flow cytometry. The solid line peaks represent untreated control cells, and broken line peaks indicate the cells treated with various amounts of SN50. Similar results were obtained in three separate experiments.
with 5 ng/ml TNF-α probed with anti-NF-κB. Western blotting was performed on 10% SDS-PAGE, and the blots were analyzed by Western blotting in nuclear extracts from Mo7e cells (lanes 3–6). The autoradiographs show the location of DNA-protein complexes and unbound probe were separated on 6% native polyacrylamide gels. The autoradiographs show the location of NF-κB p65 was analyzed by Western blotting in nuclear extracts from Mo7e cells (lane 1), with 5 ng/ml TNF-α alone (lane 2), or 5 ng/ml TNF-α plus indicated amounts of SN50 (lanes 3–6). c, the effect of SN50mt on the level of NF-κB p65 was analyzed by Western blotting in nuclear extracts from Mo7e cells treated without TNF-α (lane 1), with 5 ng/ml TNF-α alone (lane 2), or 5 ng/ml TNF-α plus indicated amounts of SN50 (lanes 3–6). c, the effect of SN50mt on the level of NF-κB p65 was analyzed by Western blotting in nuclear extracts from Mo7e cells treated without TNF-α (lane 1) or with 5 ng/ml TNF-α alone (lane 2) or with 5 ng/ml TNF-α plus indicated amounts of SN50 (lanes 3–6). Western blotting was performed on 10% SDS-PAGE, and the blots were probed with anti-NF-κB p65 antibody. Similar results were obtained in two separate experiments.

ng/ml TNF-α and 50 μg/ml SN50 completely blocked TNF-α-induced cell proliferation (Fig. 8a, lane 5). In contrast, incubation of Mo7e cells with the same concentration of SN50mt showed no effects on TNF-α-induced cell proliferation (data not shown). Exposure of Mo7e cells to 50 μg/ml SN50 alone did not show significant effects on cell proliferation (Fig. 8a, lane 7).

We then investigated the effect of these peptides on the proliferation of Mo7e cells promoted by GM-CSF and IL-3, which stimulate Mo7e proliferation via the activation of MAPK and STAT5 signaling pathways. The results showed that incubation of Mo7e cells with 1–100 μg/ml SN50 with 1 ng/ml GM-CSF or IL-3 for 72 h showed no significant effects of SN50 on [3H]thymidine incorporation promoted by GM-CSF (Fig. 8b) or IL-3 (data not shown). SN50mt also did not show the inhibitory effect on GM-CSF- or IL-3-induced Mo7e cell proliferation (data not shown).

When flow cytometric analysis was used to analyze the effect of SN50-induced inhibition of nuclear translocation of NF-κB on cell cycle status, the results showed that TNF-α treatment enhanced the progression of Mo7e cells into S-phase of the cell cycle, but preincubating Mo7e cells with 100 μg/ml SN50 for 3 days blocked TNF-α-induced progression of the cells into S-phase (Fig. 9a). SN50mt (100 μg/ml) had no inhibitory effect on TNF-α-induced progression of Mo7e cells into S-phase (Fig. 9a, lane 2), SN50nt (100 μg/ml) showed no significant effects on cell growth (data not shown).

We previously reported that TNF-α stimulated the proliferation of the Mo7e human leukemic cell line (14). In addition to TNF-α, numerous other hematopoietic growth factors, including GM-CSF, IL-3, IL-6, and TPO, also support the survival and proliferation of Mo7e cells (14, 35). Considering our previous report and present data, we conclude that the signaling for TNF-α-induced Mo7e cell proliferation occurs via a different mechanism from that of other cytokines. Cytokines like IL-3, GM-CSF, IL-6, and TPO mainly cause activation of MAPK and JAK2/STAT5 signal transduction pathways (42), but TNF-α specifically induces activation of NF-κB in Mo7e cells. Our data are also consistent with prior reports that phosphorylation and degradation of 1αB2 play an important role in the activation of NF-κB. TNF-α rapidly induces the degradation of 1αB2, and then the release of activated NF-κB, which translocates to the nucleus of Mo7e cells. Western blotting analysis further showed that the nuclear translocation of activated NF-κB, we provide clear evidence showing the direct

FIG. 7. Effects of SN50 and SN50mt on TNF-α-induced activation and translocation of NF-κB. a, identification of the effect of SN50 on the activity of NF-κB by EMSA. Nuclear extracts were prepared from cells treated without TNF-α (lane 1) or with 5 ng/ml TNF-α alone (lane 2) or 5 ng/ml TNF-α plus 1–100 μg/ml SN50 (lanes 3–6). The DNA-protein complexes and unbound probe were separated on 6% native polyacrylamide gels. The autoradiographs show the location of NF-κB and the nonspecific DNA-binding band (NS). Similar results were obtained in two separate experiments. b, the effect of SN50 on the level of NF-κB p65 was analyzed by Western blotting in nuclear extracts from Mo7e cells treated without TNF-α (lane 1), with 5 ng/ml TNF-α alone (lane 2), or 5 ng/ml TNF-α plus indicated amounts of SN50 (lanes 3–6). c, the effect of SN50mt on the level of NF-κB p65 was analyzed by Western blotting in nuclear extracts from Mo7e cells treated without TNF-α (lane 1) or with 5 ng/ml TNF-α alone (lane 2) or with 5 ng/ml TNF-α plus indicated amounts of SN50mt (lanes 3–6). Western blotting was performed on 10% SDS-PAGE, and the blots were probed with anti-NF-κB p65 antibody. Similar results were obtained in two separate experiments.

FIG. 8. Effects of SN50 on DNA synthesis in TNF-α- or GM-CSF-treated Mo7e cells. a, the cells were treated without cytokine (lane 1), with 1 ng/ml TNF-α alone (lane 2), 50 μg/ml SN50 alone (lane 7), or 1 ng/ml TNF-α plus 1–100 μg/ml SN50 (lanes 3–6) for 72 h and labeled with 4 μCi/ml [3H]thymidine for additional 4 h. In b, Mo7e cells were pretreated without cytokine (lane 1), or with 1 ng/ml GM-CSF alone (lane 2) or 1 ng/ml GM-CSF plus 1–100 μg/ml SN50 (lanes 3–6) for 72 h and labeled with 4 μCi/ml [3H]thymidine for an additional 4 h. The [3H]thymidine incorporation was determined from triplicate samples and expressed as the mean ± S.E. of counts per minute.

DISCUSSION

We previously reported that TNF-α stimulated the proliferation of the Mo7e human leukemic cell line (14). In addition to TNF-α, numerous other hematopoietic growth factors, including GM-CSF, IL-3, IL-6, and TPO, also support the survival and proliferation of Mo7e cells (14, 35). Considering our previous report and present data, we conclude that the signaling for TNF-α-induced Mo7e cell proliferation occurs via a different mechanism from that of other cytokines. Cytokines like IL-3, GM-CSF, IL-6, and TPO mainly cause activation of MAPK and JAK2/STAT5 signal transduction pathways (42), but TNF-α specifically induces activation of NF-κB in Mo7e cells. Our data are also consistent with prior reports that phosphorylation and degradation of 1αB2 play an important role in the activation of NF-κB. TNF-α rapidly induces the degradation of 1αB2, and then the release of activated NF-κB, which translocates to the nucleus of Mo7e cells. Western blotting analysis further showed that the nuclear translocation of activated NF-κB, we provide clear evidence showing the direct
linkage of TNF-α-induced NF-κB activation and Mo7e cell survival and proliferation.

TNF-α is an extremely pleiotropic factor, which can induce growth inhibition or death in some malignant cell lines but promotes the survival and proliferation of other cell lines (1, 3–5). The different mechanisms responsible for its pleiotropic functions are not well understood. In this study, we examined TNF-α-induced signal transduction pathways in Mo7e, Meg-01, K562, and HEL leukemic cell lines. We found that TNF-α-induced activation of signal transduction pathways is very similar in all these cell lines, although they behave differently in response to other cytokines. In Mo7e cells, TNF-α induces the activation of NF-κB but not MAPK or STAT signal transduction pathways. In HEL and Meg-01 cells, although there is constitutive activation of STAT5, as we reported previously (42), TNF-α-induced activation of NF-κB was observed (Figs. 1, 2, and 5). The activation of NF-κB is specifically derived from the binding of TNF-α to TNF receptors, as the activation can be blocked by anti-TNF-α neutralizing antibody (data not shown). Based on the published observation that TNF-α stimulates a number of other human leukemic cell lines such as CMK, HU-3, and M-MOK megakaryocytic leukemic cell lines (3–5, 2010).
14), the role of NF-κB in preventing apoptosis and promoting cell proliferation is likely to be a more general phenomenon.

It has been reported that TNF-α treatment activates MAPK and STAT signaling pathways in several human cell lines (20–23, 43). TNF-α-induced MAPK activation was also reported to be involved in TNF-α-induced apoptosis (27, 31). The data presented here show that TNF-α treatment did not activate STAT signal transduction pathways in the Mo7e cell line, although the functional STAT5 signaling pathway was detected in this cell line treated with IL-3, GM-CSF, or TPO. Our data also indicated that TNF-α treatments failed to activate STAT1 and STAT3 in Mo7e cells, confirmed by either Western blotting, EMSA, or supershift EMSA analyses (data not shown). When the DNA probes, which bind to STAT2, STAT4, and STAT6, were used in EMSA to examine the activation of these STATs, no significant DNA-protein complexes were detected in Mo7e cells treated with 1–5 ng/ml TNF-α for 5–30 min (data not shown). Furthermore, we provided clear evidence showing that TNF-α treatment also failed to activate the MAPK signal transduction pathway. If the activated MAPK signal transduction pathway is involved in TNF-α-induced apoptosis in other reported cell lines (27, 31), the lack of TNF-α-induced MAPK activation may be one of the reasons why TNF-α promotes cell survival and proliferation, rather than apoptosis in Mo7e cells.

There is considerable evidence that the activation of NF-κB promotes survival and proliferation of certain cell types. In RelA (p65) knockout mice, Beg et al. (44) showed that a considerable degree of apoptosis occurred in hepatocytes in RelA-deficient embryos. They also reported that TNF-α-induced apoptosis of fibroblasts and macrophages derived from RelA knockout mice, and transfection of RelA into the cells rescued optosis of fibroblasts and macrophages derived from RelA knockout mice, and transfection of RelA into the cells rescued apoptosis. The MAPK signal transduction pathway is involved in TNF-α-induced apoptosis in other reported cell lines (27, 31), the lack of TNF-α-induced MAPK activation may be one of the reasons why TNF-α promotes cell survival and proliferation, rather than apoptosis in Mo7e cells.

In contrast, several reports demonstrated that activation of NF-κB induces apoptosis in certain cells (32–34). Beesho et al. (33) showed that treatment of human leukemia cells and thymocytes with PDTC, an antioxidant inhibitor of NF-κB activation, prevented their apoptosis. In addition, radiation or agents such as lipopolysaccharides and TNF-α triggered the hydrolysis of membrane phospholipids, which produced ceramide, a pro-apoptotic role of NF-κB using cell lines stably transduced with dominant negative IkBa. Liu et al. (30) also showed that three different responses to TNF-α were mediated by activation of the TNF receptor complex: activation of JNK, activation of NF-κB, and induction of apoptosis. The activation of NF-κB protected the cells against TNF-α-induced apoptosis. Ozaki et al. (48) also reported that NF-κB inhibitors pyrroldine dithiocarbamate (PDTC) and chloromethyl ketone stimulated apoptosis of mature rabbit osteoclasts, which resulted in the inhibition of bone resorption. These findings, together with the present report, indicate that the NF-κB signaling cascade is closely involved in the prevention of apoptosis of cells.

Using SN50 to block the nuclear translocation of activated NF-κB, we demonstrated that NF-κB has an essential role in TNF-α-induced Mo7e cell proliferation, thus confirming that NF-κB has an anti-apoptosis function in Mo7e cells stimulated by TNF-α. Our experiments demonstrated a direct linkage of activated NF-κB and TNF-α-induced cell proliferation. Furthermore, we find that TNF-α treatment not only prevents apoptosis but also actually stimulates DNA synthesis and promotes the progression of Mo7e cells into S-phase (Fig. 9). Thus, the Mo7e cell line seems to be a good model system to investigate the signal transduction pathways involved in TNF-α-induced cell proliferation.

Studying the control mechanisms of survival and proliferation of leukemia cells would be important for understanding the regulation of leukemia cell survival, not only in vitro, but also possibly in vivo. Treatment of Mo7e cells with TNF-α for 1 h could support their survival, which was evaluated 32–48 h later (data not shown). This indicates that NF-κB stimulates expression of gene products that have long term effects on the survival of Mo7e cells. Identification of such activated survival and proliferation-promoting proteins in Mo7e cells will be an important focus of future studies. Further studies are required...
to determine a more detailed mechanism of the involvement of NF-κB in the survival and proliferation of Mo7e cells and the expression of specific gene products promoted by TNF-α that exert anti-apoptotic and pro-proliferative effects.

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