Tumor necrosis factor-α (TNF) is a multifunctional cytokine affecting a wide range of biological activities of many cell types, including endothelial cells, fibroblasts, and hematopoietic cells (1–6). Its unique killing effect on malignant cells has been well established (7), which is mediated by the mechanism of programmed cell death (apoptosis) and necrosis (8). During early apoptosis, a family of cysteine proteases, the caspases, is activated. The pathway from TNF receptor to initial caspase activation is reasonably well characterized. Unlike apoptosis, necrosis is not an energy-requiring process and is a less ordered event resulting in cell swelling or shrinkage and disruption of the plasma membrane (9–10).

The growth inhibitory effect of TNF on hematopoietic progenitor cells has been widely observed in various systems (3–6). Although the growth inhibition has been reported to be mediated by TNF p55 receptor (11), the cytoplasmic and nuclear events in response to TNF is not clear at all. There is no evidence suggesting that the growth inhibition of these hematopoietic cells is a result of TNF-induced apoptosis or necrosis.

Cyttoplasmic protease systems have recently been identified as important regulators of intracellular activities including programmed cell death, protein kinase activities, and cell-cycle progression (12–14). A typical example is the degradation of IκBα during TNF-induced apoptosis. Degradation of IκBα leads to activation of transcription factor NF-κB, which is required for TNF-regulated gene expression and downstream activities (15–17). These previous studies prompted us to investigate the mechanism by which TNF suppresses proliferation of myeloid leukemia cells. We used two myeloid leukemia cell lines, TF-1 and MV4–11, as model systems in our studies, since they are very sensitive to TNF treatment. TF-1 is a growth factor-dependent human erythroid cell line that originally was isolated from the bone marrow cells of a patient with erythroleukemia. Since the TF-1 cell line expressed various cytokines and cytokine receptors and is sensitive to TNF inhibitory effect, this cell line has been a well established model for studying apoptosis, differentiation, and cytokine-regulated gene expression. MV4–11 is a growth factor-independent myeloid leukemia cell line isolated from a child with acute myeloid leukemia. Our previous studies demonstrated that this cell line is very sensitive to TGF-β-induced G1 arrest (18). We have now found that this cell line is also sensitive to TNF-induced cell cycle arrest. Our data showed that TNF caused a significant reduction of D-type cyclins and induced G1 arrest in both TF-1 and MV4–11 cells. The down-regulation of the D-type cyclins is a result of proteasome-dependent degradation.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies used and their sources were as follows: antibodies to IκBα, cyclin-dependent kinase 2 (cdk2), cdk4, cdk6, cyclin A, cyclin E, cyclin D1, cyclin D2, cyclin D3, p27, and actin (Santa Cruz Biotechnology, Santa Cruz, CA); pRb, phosphorylated pRb, and luciferase (Promega, Madison, WI).

Recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) and TNF were purchased from Immunex (Seattle, WA) and R&D Systems (Minneapolis, MN), respectively. D-type cyclin probe template and ribonuclease protection assay (RPA) kit were purchased from BD PharMingen (San Diego, CA). Protein G-agarose was obtained from Amersham Biosciences (Piscataway, NJ). [γ-32P]ATP and [35S]methionine were supplied from ICN (Costa Mesa, CA) and PerkinElmer (Boston, MA), respectively. Propidium iodide and RNase A were purchased from Sigma. Culture media, fetal bovine serum, and TRIzol reagent were purchased from Invitrogen (Grand Island, NY). Proteasome and calpain inhibitors were obtained from Calbiochem Corp. (San Diego, CA).
TNFa-mediated Degradation of D-type Cyclins

Diego, CA) and BioMol Research Laboratories (Plymouth Meeting, PA), respectively. Caspase inhibitor (z-VAD-FMK) was purchased from Biochem (La Jolla, CA). Ubiquitin Protein Conjugating and 26 S Protein Degradation Kits were obtained from Calbiochem Corp. TNT Transcription/Translation kits were purchased from Promega (Madison, WI). pRF/cyclin D3 was a generous gift of Dr. Armando Felsani (CNR, Istituto Tecnologico Biomediche, Roma, Italy).

Cell Culture and Cytokine/Drug Treatment—Human TF-1 and MV4–11 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were routinely maintained in suspension culture in RPMI 1640 (for TF-1) and Iscove’s modified Dulbecco’s medium (for MV4–11) supplemented with 10% fetal heat-inactivated bovine serum in the absence (for MV4–11) or presence (for TF-1) of 5 ng/ml GM-CSF. The cultures were incubated at 37 °C under 5% CO2 in a humidified atmosphere. Before treatment with TNF, exponentially growing cells were collected by centrifugation and resuspended in RPMI 1640 with 1 ng/ml GM-CSF (for TF-1) or Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum at a density of 5 × 10^6 cells/ml. In some experiments, exponentially growing cells were pretreated with inhibitors of caspase 3, proteasome, or calpain for 30 min at 37 °C, after which TNF was added and the incubation was continued for the times required.

Cell Cycle Analysis—Cells in log-phase growth were harvested by centrifugation, washed once with phosphate-buffered saline, and collected in RPMI 1640 supplemented with 10% fetal bovine serum at the times required in a humidified atmosphere containing 5% CO2 at 37 °C. The cells were then harvested, washed once with phosphate-buffered saline, and resuspended in 1 ml of phosphate-buffered saline at a concentration of 2 × 10^6 cells/ml. Subsequently, 2 ml of methanol were added and the cells were incubated for 30 min on ice. The cells were then collected by centrifugation and resuspended in 500 μl of propidium iodide (0.1 mg/ml) and 100 μl of RNase A (2 mg/ml) for 30 min in the dark at room temperature, after which DNA content was determined using a flow cytometer (BD Pharmingen).

Immunoprecipitation and Western Blotting Assays—Cells were collected by centrifugation and washed once with phosphate-buffered saline. Whole lysates and nuclear extracts were prepared as described previously (19). For immunoprecipitation, lysates containing 500 μg of total proteins were incubated with an appropriate antibody (1–2 μg/ml) for 2 h at room temperature or overnight with agitation at 4 °C. About 30 μl of protein A- or protein G-agarose beads were added to the lysates and the incubation continued for another 2 h at the same conditions. Immune complexes were then collected by centrifugation, washed three times with lysis buffer (18), and resuspended in 2 × SDS sample buffer (125 mM Tris-HCl, pH 8.8, 4% SDS, 20% glycerol, 150 mM diithiothreitol, 0.2% bromphenol blue). Lysates in sample buffer were analyzed for the expression of proteins by Western blotting (18) and detected by a chemiluminescence (New England BioLabs, Beverly, MA). For direct Western blotting, lysates containing 30–50 μg of total proteins were loaded on SDS-PAGE gels followed by Western blotting procedure (18).

In Vitro Kinase Assay—Preclarified lysates (200 μg of total protein) extracted from cells treated with or without TNF were immunoprecipitated with an antibody against cyclin D2 or D3 followed for 2 h at 4 °C with an antibody against cyclin or cdk followed by addition of protein G-agarose beads for 2 h. The lysates were collected by centrifugation and washed. After three washes in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and washes in kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl2, 10 mM dithiothreitol), the kinase reaction was carried out in a total volume of 10 μl, which included 5 μl of 2 × kinase buffer containing 10 μCi of [γ-32P]ATP and 2 μg of GST-Rb at 37 °C for 30 min. The reaction was stopped by adding 10 μl of 2 × sample buffer and boiling for 4 min before separation by SDS-PAGE. The gel was then dried and exposed to film (Kodak X-AR-5).

Measurement of D-type Cyclin Biosynthesis and Turnover Rate—Cells were treated with or without TNF for different times. One hour prior to harvesting, culture medium was replaced with methionine-free medium supplemented with 10% fetal bovine serum and 300 μCi/ml [35S]methionine. Cells were then collected and cell lysates were prepared in lysis buffer. An equal amount of protein from each sample was immunoprecipitated with an antibody against cyclins D2 or D3 followed by addition of protein G-agarose. Subsequently, the immunoprecipitates were resolved by SDS-PAGE, and visualized by autoradiography. For pulse-chase experiments, cells treated with or without TNF for 48 h were collected and resuspended in normal culture medium containing [35S]methionine for 1 h, at 37 °C, after which the labeling medium were removed and cells were incubated in the presence of an excess of nonradioactive methionine for 1–4 h. Cells were then collected, cell lysates were prepared, and immunoprecipitated with an antibody against cyclins D2 or D3 following the procedure described above.

In Vitro Protease Activity Assays—In vitro protease activity assays were performed as described previously (20), with slight modification. Protease substrates (200 μg of total protein) extracted from cells treated with or without TNF were incubated with peptide-AMC substrate (Calbiochem, San Diego, CA)—containing reaction buffer (20 mM Tris-HCl, 1 mM ATP, 2 mM MgCl2) in the presence or absence of the indicated protease inhibitor (30 min, 30 °C). Fluorescent product, AMC, released from the reaction, were quantitated by measuring fluorescence emission intensity at 440 nm.

In Vitro Transcription and Translation—Transcription and translation of pRC/CMV/cyclin D3 using TNT reticulocyte lysate (Promega) were performed following the manufacturer’s instruction without 35S labeling.

In Vitro Ubiquitin Conjugation/Translation—Protein products were incubated in 1 × ubiquitin reaction buffer, which contained ATP, E1, E2, and E3, at 37 °C for 2 h, after which the reaction product was transferred to quenching buffer (10% trichloroacetic acid, 200 μl of 5% bovine serum albumin) followed by addition of 26 S proteasome fractions (Calbiochem, San Diego, CA). The degradation reaction was performed at 37 °C for 1.5–1 h following the manufacturer’s instructions. After reaction, aliquots of reaction mixture were loaded to SDS-PAGE separated gels and analyzed by Western blot with a specific antibody against cyclin D3.

Total RNA Isolation and Ribonuclease Protection Assay—Total RNA was isolated from cells treated with or without TNF using TRIZol reagent following the manufacturer’s protocol. RNA concentrations were determined using a spectrophotometer. RNA preparations were then performed using the RPA kit following the manufacturer’s instructions. Briefly, a labeled RNA probe was synthesized by T1 RNA polymerase in the presence of RPA template and [α-32P]UTP. The RPA template contains the cDNAs encoding cyclin B, cyclin C, cyclin D1, cyclin D2, cyclin D3, L32, and GAPDH, respectively. The labeled probe was then mixed with the sample RNA at 56 °C for 14 h followed by an RNase A treatment (30 °C for 15 min. After hybridization, RNA was added and incubated at 30 °C for 45 min. After RNase digestion was completed, the samples (target RNAs) were separated on a polyacrylamide gel, and protected RNAs were visualized by autoradiography.

Statistical Analysis—Quantities of proteins detected by in vitro kinase assay, Western blot, and immunoprecipitation were quantitated by image reading, using ImageQuant (Molecular Dynamics, Sunnyvale, CA). The numbers produced were proportional to the degree of protein expression. Percent decreases or increases in protein levels were then calculated. Cell cycle data were expressed as the mean from two to four separate experiments. The statistical significance of differences between group means or protein quantities was determined using the Student’s t test.

RESULTS

TNF Arrests TF-1 and MV4–11 Cells in G1 Phase—When studying the apoptotic effect of TNF-α on TF-1 human erythroid leukemia cells, we observed that the cells also showed cell cycle arrest features. Cell cycle analysis by flow cytometry showed that TNF induced a G1 arrest. In normal culture conditions, 43% of TF-1 cells in G1, and 44% of the cells in S phases. TNF, at a concentration of 30 ng/ml for 24 h, increased the proportion of G1 cells to 67% and decreased the proportion of S cells to 14%. Longer incubation time (48 h) of cells with TNF slightly increased G1 population and decreased S population (Fig. 1A). TNF-induced G1 arrest is not limited to this particular cell line, since the same concentration of TNF also brought about significant G1 arrest in MV4–11 cells. Approximately, 55% of cells were distributed in G1 and 41% of cells in S in control MV4–11 cells. Treatment of cells with TNF caused a marked increase of G1 cells to 76% and a decrease of S cells to 11% by 24 h. Since the phosphorylation status of pRB (the retinoblastoma gene product) and the expression of “cdk inhibitor” p27 have been linked to cell cycle status, we next investigated whether TNF-induced G1 arrest accompanied with an alteration in pRB phosphorylation or p27 expression. In exponentially growing phase, TF-1 and MV4–11 cells (0 time) expressed both underphosphorylated (bottom band) and phospho-
FIG. 1. TNF inhibits TF-1 and MV4-11 cell growth and arrests the cells in G_1 phase. A, cells were cultured in 6-well culture plates at a concentration of 5 x 10^5/ml in the presence or absence of TNF (30 ng/ml) for 24 or 48 h at 37 °C in humidified air containing 5% CO_2. The cells then were collected and incubated with propidium iodide for 30 min, and DNA content was measured by flow cytometry. B, cells treated with or without of TNF for 24 and 48 h were collected, lysed, and the expression of pRb, phosphorylated pRb, and p27 were detected by Western blotting with antibodies against pRb, phosphorylated pRb, or p27, respectively. Similar results were obtained from three independent experiments. Expression of pRb from TGFβ-treated cells was used as a control.
TNFα-mediated Degradation of D-type Cyclins

Fig. 2. TNF inhibits the expression of D-type cyclins and their kinase activities. TF-1 and MV4–11 cells treated with or without TNF (30 ng/ml) were collected and lysed in lysis buffer. A, aliquots of lysates were analyzed for the expression of cyclins and cdks by Western blotting with the antibodies indicated in the figure. B, TF-1 cells treated with TNF for 48 h were collected and lysed in lysis buffer. Aliquots of lysates containing 200 μg of total proteins were immunoprecipitated with antibodies against cyclins D2, D3, cdk4, or IgG. The immunoprecipitates were then incubated with GST-Rb in kinase buffer in the presence of 10 μCi of [γ-32P]ATP and the expression of phosphorylated GST-Rb was detected by autoradiography. Expressed proteins were quantitated by image reading, using ImageQuant software, and percent increase or decrease was calculated as described under "Experimental Procedures."

The expression of phosphorylated GST-Rb was detected by autoradiography. High levels of cyclins D2- and D3-dependent kinase activities were observed in TF-1 cells in the absence of TNF. In contrast, cells treated with TNF for 48 h showed very low cyclin D2- and D3-dependent kinase activities, based on the expression of phosphorylated GST-Rb, with decreases of 55% and 87% of being detectable, respectively. As negative controls, IgG immunoprecipitates either from proliferating TF-1 cells or from the cells treated with TNF lacked kinase activity (Fig. 2B). Since D-type cyclins are mainly associated with cdk4, and the cyclin-cdk complexes play a critical role in G1 progression, we also examined cdk4 kinase activity. As expected, exposure to TNF for 48 h caused a dramatic decrease of cdk4 kinase activity with ∼90% decrease being detected. Cyclin-cdk4 complexes are assumed to modify pRb, thereby promoting cell cycle progression toward DNA replication. By using a combination of immuno-
precipitation and Western blot, we detected that association of D-type cyclins with pRb was significantly decreased in the cells treated with TNF for 48 h due to a decrease of total D-type cyclins (data not shown).

TNFα-induced G1 Arrest Is Apoptosis Pathway-independent—Hypodiploid DNA (see sub-G1 population) was observed in both TF-1 and MV4–11 cells treated with TNF (Fig. 1A), suggesting that TNF caused apoptotic cell death in these cells, which is consistent with previous observations (23). One might suggest that TNF caused apoptotic cell death in these cells, by using both anti-caspase (denced by appearance of 19-, 17-, and 14-kDa proteins, detected absence of TNF expressed 32-kDa proenzyme and barely acti- vated caspase-3 consists of multiple subunits, including 19 kDa, 17 kDa, and possible other smaller subunits, depending on the activity of caspase 3 and the cell type. TF-1 cells in the absence of TNF expressed 32-kDa proenzyme and barely activated caspase 3. However, a 24-h incubation with TNF caused a significant expression of activated caspase 3 (Fig. 3A), evi- denced by appearance of 19-, 17-, and 14-kDa proteins, detected by using both anti-caspase (top panel) and anti-specific acti- vated caspase 3 (the second panel from top) antibodies. The specificity of caspase 3 activation was confirmed by using caspase inhibitor, zVAD-FMK, since the inhibitor at a concentra- tion of 100 μM completely inhibited TNF-induced activation of caspase 3. In contrast, the inhibitor was not able to block TNF-induced down-regulation of the D-type cyclins and G1 arrest at any dose up to 200 μM. However, zVAD-FMK partially reduced the TNF-induced sub-G1 population (Fig. 3, A and B). To further ensure that the inhibition of D-type cyclins was not secondary to TNF-induced apoptosis, we analyzed patterns of cyclin D3 inhibition and cell death. The inhibition of cyclin D3 was dose-dependent with a maximal inhibition of 85% being detected at a concentration of 30 ng/ml TNF, above which the level of cyclin D3 was not further decreased. In contrast, the cell death caused by TNF increased linearly with increasing TNF concentration, with maximal cell death being observed at a concentration of 50 ng/ml TNF. In addition, TNF at a concentra- tion of 10 ng/ml induced activation of caspase 3 but not inhibition of D-type cyclins (Fig. 3C). These results demon- strated that TNFα-induced G1 arrest is caspase 3 pathway-independent.

Down-regulation of D-type Cyclins Is linked to Proteasome-dependent Degradation—The TNF-induced down-regulation of D-type cyclins could be potentially regulated at mRNA or pro- teins levels. To test the first possibility, RPA were performed. As illustrated in Fig. 4A, cells expressed cyclin D3, cyclin D2, and little cyclin D1 mRNAs. Addition of TNF (30 ng/ml) had no effect on the levels of mRNA encoding cyclins D1 and D2, but caused modest reduction in the levels of mRNA for cyclin D3 with a decrease of 22% being detected by 48 h measured using ImageQuant software. Control and TNF-treated cells showed the same levels of mRNAs for housekeeping genes, L32 and GAPDH, which were used as controls and references for normalizing samples. Unexpected, TNFα dramatically down-regulated the expression of mRNA for cyclin B, which is involved primarily in regulation of the G2–M transition of the cell cycles. Thus, the specific inhibitory effects of TNF on G1 regulatory molecules appear to be mainly at post-transcriptional levels. Two possibilities may account for the down-regulation of D- type cyclins at post-transcriptional levels: 1) TNF induces a decrease of biosynthesis rate of D-type cyclins; or 2) TNF causes a degradation of D-type cyclins. To clarify this question, MV4–11 cells were treated with or without TNF for 24 and 48 h, after which cells were labeled with [35S]methionine for 1 h and the expression of cyclins D2 and D3 were detected by autoradiography. As showed in Fig. 4B, control cells (TNF−) expressed labeled cyclins D2 and D3, representing new synthesis of these cyclins. Addition of TNF for 24 h caused ~50% decreases in the quantities of cyclins D2 and D3. However, further increase of incubation time (48 h) with TNF did not further down-regulate the levels of cyclins D2 and D3, suggesting that TNF induced down-regulation of D-type cyclins is most likely not related to biosynthesis rate. However, if cells were chased for different times with normal culture medium containing unlabeled methionine after they released from medium containing [35S]methionine, we observed a chase time-depend- ent reduction in the amounts of labeled cyclins D2 and D3 in the cells treated with TNF, with maximal reductions of 65 to 70% being detected by 4 h (Fig. 4, B and C). In contrast, the expression of cyclin E was not affected by TNF treatment. Thus, TNF-induced down-regulation of D-type cyclins appears to be a result of degradation. This was confirmed by the approach of using degradation inhibitors. Since degradation of IxBo by TNF has been well established, we wondered if degradation of IxBo was linked to D-type cyclin expression. To clarify this question, the expression of IxBo and D-type cyclins in the absence and presence of TNF and several protein degradation inhibitors was examined by Western blotting. ALLN is a calpain-dependent inhibitor, lactacystin is both a protea- some and calpain-dependent inhibitor, and zVAD-FMA is a caspase 3 inhibitor. In the absence of TNF, MV4–11 cells expressed high levels of IxBo. Addition of TNF (30 min) led to degradation of IxBo, with a decrease of 70% of the molecule being found. If cells were pretreated with ALLN, zVAD-FMA, or lactacystin followed by TNF treatment, the degradation of IxBo was eliminated or significantly reduced. The degree of recovering was dependent on the type of inhibitor. Lactacystin almost completely blocked IxBo degradation whereas zVAD- FMA prevented ~50% IxBo degradation. The effect of ALLN was intermediate between lactacystin and zVAD-FMA with 80% of IxBo that was degraded by TNF being prevented (Fig. 5A). These effects were confirmed by examining NF-xB nuclear translocation. It has been reported that the NF-xB complex is composed of two proteins of molecular weight 50,000 and 65,000, referred to as p50 and p65, respectively. Since the p50/p65 combination leads to marked transcriptional activation, while p50 alone does not, the transactivation activity of NF-xB seems to be provided primarily by the p65 subunit or at least to require the p65 subunit (24). Therefore, p65 nuclear translocation was investigated in response to TNF in the ab- sence or presence of the inhibitors. As seen in Fig. 5A, control cells (lane 1, panel 2, from top) did not show a significant amount of nuclear p65. After addition of TNF, the levels of nuclear p65 were markedly accumulated with a 7.3-fold increase being detected. In contrast, cytoplasmic p65 was dra- matically decreased (data not shown). ALLN and lactacystin blocked more than 85% TNF-induced nuclear translocation of p65, whereas zVAD-FMK had a less blocking effect. Thus, the results described above suggest that the degradation of IxBo by TNF involves multiple pathways. With these data, we next investigated if any of these inhibitors can block TNF-induced degradation of D-type cyclins. The results in Fig. 5A showed that control MV4–11 cells expressed significant quantities of cyclins D2 and D3. Addition of TNF for 48 h led to considerable degradation of cyclin D2 (65%) and cyclin D3 (72%). If cells were pretreated with lactacystin for 30 min followed by addi- tion of TNF, more than 85% of the D-type cyclin degradation induced by TNF was prevented. This effect is dose-dependent, with the maximal blocking effect being detected at a concen-
FIG. 3. TNF-induced down-regulation of D-type cyclins is caspase-3 pathway independent. A, TF-1 cells were pretreated with or without zVAD-FMK (100 μM), followed by addition of TNF (20 ng/ml) for 24 h. As a negative control, growing cells were treated with dimethyl sulfoxide (the vehicle for zVAD-FMK) alone at the same condition. Subsequently, cell lysates were prepared and aliquots of the lysates were analyzed by Western blotting with antibodies against cyclin D2, cyclin D3, caspase 3, or activated caspase 3. B, cells were treated with or without TNF in the presence or absence of zVAD-FMK for 24 h, after which cells were collected and stained with propidium iodide for cell cycle analysis by flow cytometry. C, cells in log phase treated with different concentrations of TNF for 24 h were collected and lysed, and an aliquot of lysate was analyzed for the expression of cyclin D3 by Western blotting with anti-cyclin D3 antibody. The relative inhibition was calculated from quantitation of individual bands after subtraction from control bands. In a parallel experiment, some cells after treatment with TNF were incubated with trypan blue for 15 min at room temperature. A drop of the cell suspension was loaded on a hemocytometer, and stained (blue) and unstained cells were counted under a light microscope. Cell viability was calculated as described under “Experimental Procedures.” Some cells treated with 10 ng/ml TNF were collected, lysed, and analyzed for the expression of cyclin D3 and activated caspase 3 with antibodies against cyclin D3 or caspase 3, respectively.
FIG. 4. TNF accelerates proteolytic degradation of D-type cyclins. 

A, total RNA (10 μg/reaction) extracted from MV4–11 cells treated with or without TNF were hybridized with RNA probes containing the specific mRNAs for the different cyclins as indicated in the figure. After hybridization and RNase treatment, the protected RNAs were separated by SDS-PAGE and detected by autoradiography. The housekeeping gene probes, L32 and GAPDH, are included among the RNA probes for normalizing sampling and technique errors and to permit comparison of individual mRNA species between samples. Identical results were obtained from four separate experiments.

B, MV4–11 cells were treated with or without 30 ng/ml TNF for 24–48 h. The cells were metabolically labeled with [35S]methionine and immunoprecipitated cyclins or cdks were resolved by SDS-PAGE and visualized by autoradiography. In parallel experiments, MV4–11 cells were treated with or without 30 ng/ml TNF for 24–48 h. The cells were then labeled with [35S]methionine for 1 h and subsequently chased by RPMI containing unlabeled methionine for various times as indicated in the figure. Immunoprecipitated cyclins were resolved by SDS-PAGE and visualized by autoradiography. C, the quantitation of individual bands under "degradation" in B was calculated as percent expression after subtraction of background by image reading, using ImageQuant software.

FIG. 5. TNF-induced down-regulation of D-type cyclins is via proteasome/proteolysis-dependent degradation. MV4–11 cells were pretreated without or with ALLN (1 μg/ml), MG132 (1 μM), lactacystin (2 μM), or zVAD-FMA (100 μM) for 30 min, followed by addition of TNF for 30 min (for detection of IκBα and NF-κB) or 48 h (for detection of D-type cyclins), after which cells were collected and lysed in lysis buffer. Lysates (nuclear lysates for NF-κB and whole lysates for IκBα and D-type cyclins) containing 50 μg of total protein were separated on 10–12% SDS-PAGE and analyzed for the expression of IκBα, NF-κB (p65), cyclin D2, and cyclin D3 with an antibody against each of these molecules (A). In parallel experiments, lysates containing 200 μg of total protein were immunoprecipitated with antibodies against cyclins D2 and D3, after which the immunoprecipitates were measured for their kinase activities by in vitro kinase assay, using GST-Rb as a substrate. The relative kinase activity is calculated from quantitation of individual bands after subtraction of background by image reading using ImageQuant software (B).
TNFα-mediated Degradation of D-type Cyclins

**DISCUSSION**

The balance between cell growth and differentiation is a tightly regulated process that is controlled by complex interactions between growth stimulatory and inhibitory cytokines. Many interleukins and cytokines have been found to stimulate hematopoietic cell proliferation, whereas only a few cytokines (e.g. TNF, interferon γ, and TGFβ) are thought to be physiologic negative regulators of hematopoiesis. TNF-mediated growth inhibition has been observed in a number of different types of hematopoietic progenitors. However, the mechanism that is responsible for the growth inhibition induced by TNF is not quite understood. In this study, we demonstrated that TNF induced G1 arrest in two myeloid leukemia cell lines, TF-1 and MV4-11, while simultaneously causing apoptosis. The G1 arrest is indicated by an increase of cells in G1 and a decrease of cells in S phase after initiating TNF treatment. These results suggest that TNF, like TGFβ, can suppress G1 progression and prevent G1-S transition. Additional support for the G1 arrest is the observation that cells treated with TNF showed accumulation of p27 and dephosphorylated pRb. It is generally agreed that p27 accumulates when cells stop cycling and arrested in G1, although the real role of p27 in cell cycle is an unresolved issue. pRb, which acts as a signal transducer connecting the cell cycle clock with the transcriptional machinery, becomes dephosphorylated as cells are arrested in G1. Subsequently, we demonstrated that TNF-induced G1 arrest is linked to down-regulation of D-type cyclins as evidenced by the facts that: 1) addition of TNF led to a marked decrease in the amounts of cyclins D2 and D3 in both TF-1 and MV4-11 cells, and 2) TNF significantly down-regulated cyclin D2 and D3-dependent kinases activities and formation of pRb-cyclin D complexes. The critical role of D-type cyclins in promoting G1 progression and exit has been well established (21, 22, 26, 27). Although a critical role of cyclin D1 in cell cycle control in fibroblasts has been widely reported (29, 30), we have found that cyclins D3 and D2, but not cyclin D1, are major cyclins expressed in human myeloid leukemia cells, at least, in TF-1 and MV4-11 cells. These data suggest that cyclins D3 and D2, but not cyclin D1, may play an important role in cell cycle control in human hematopoietic cells. Our results are consistent with some previous findings in which overexpression of cyclin D3 in 32D myeloid precursor cells caused an increase in the fraction of cells in the S phase, apparently related to a shortening of the G1 phase (31). Granulocyte differentiation was inhibited by cyclin D2 and cyclin D3, but not cyclin D1 (32).

Since TNF also induced apoptosis, it is possible that TNF-induced G1 arrest is a result of apoptosis. By using apoptosis inhibitors, we excluded this possibility, because zVAD-FMK blocked TNF-induced caspase 3 activation and apoptosis partially, but failed to prevent TNF-induced down-regulation of D-type cyclins and G1 arrest. In addition, TNF-induced activation of caspase 3 and cell death did not parallel inhibition of D-type cyclins. It is reasonable that both apoptosis and G1 arrest may occur in cells in response to TNF treatment, but whereas cyclin D3 conjugated with ubiquitin expressed smear bands (different sizes of ubiquitinated cyclin D3) with a predominant band being detected at sizes of 35–37 kDa in SDS-PAGE. Addition of 26 S proteasome had no effect on the expression of cyclin D3 that was not treated with ubiquitin but significantly decreased the amounts of ubiquitinated cyclin D3 by as much as 75% (quantitated using ImageQuant software) (Fig. 7B). Our data suggest that: 1) the degradation of cyclins D2 and D3 by TNFα is not caused by caspase or calpain-dependent mechanism; 2) TNF-induced G1 arrest is IκBα pathway-independent; and 3) the degradation of cyclins D2 and D3 is proteasome proteolysis-dependent.

**FIG. 6. TNF-induces increase of proteasome activity.** Lysates containing 200 μg of total protein extracted from MV4-11 cells treated with or without TNF (24 h) in the presence or absence of proteasome inhibitors were subjected to in vitro assays of proteasome activity as described under “Experimental Procedures,” after which fluorescence emission were measured. The results shown are mean ± S.D. of three independent experiments.

To provide direct evidence that proteasome are involved in the degradation of D-type cyclins, an in vitro degradation assay system was applied in which cyclin D plasmid was transcribed and translated followed by ubiquitination and degradation. Since cyclin D3 may play a critical role in cell cycle control in these human myeloid leukemia cells,2 cyclin D3 plasmid was used for these experiments. The results shown in Fig. 7 indicated that translation of pRC/CMV/cyclin D3 expressed a 35-kDa protein, which is consistent with published data, whereas translation with pRC/CMV alone did not produce any visible protein band. As a positive control, translation of luciferase DNA expressed an expected 62-kDa protein (Fig. 7A). Cyclin D3 without ubiquitination expressed a single band of 35 kDa, whereas cyclin D3 conjugated with ubiquitin expressed smear bands (different sizes of ubiquitinated cyclin D3) with a predominant band being detected at sizes of 35–37 kDa in SDS-PAGE.

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**FIG. 6. TNF-induces increase of proteasome activity.** Lysates containing 200 μg of total protein extracted from MV4-11 cells treated with or without TNF (24 h) in the presence or absence of proteasome inhibitors were subjected to in vitro assays of proteasome activity as described under “Experimental Procedures,” after which fluorescence emission were measured. The results shown are mean ± S.D. of three independent experiments.

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through different mechanisms. In many cases of apoptosis, the G₁ arrest may be masked due to a dominant finding of cell death and sensitivity of activation of apoptotic signals induced by TNF.

Using [35S]methionine-labeled pulse-chase experiments revealed that degradation of D-type cyclins was significantly accelerated in TNF-treated cells, with a maximal decrease of D-type cyclins being detected by 4 h, suggesting that a cyclin D degradation mechanism plays an important role in TNF-mediated cell cycle arrest. Protein degradation has been shown to be an efficient mechanism in various cell activities. For example, following binding to its receptors on the plasma membrane, TNF initiates transcription of genetic networks, in part through activating nuclear translocation of NF-κB. NF-κB is sequestered in the cytoplasm by association with a binding protein called IκB, which masks the nuclear localization signals of NF-κB. TNF activates NF-κB by phosphorylation of IκB on serine residues and subsequent degradation of IκB (15–17). Protein degradation is also involved in the transition from G₁ to S-phase in mammalian cells. Recent work revealed that an important component of cyclin D1 regulation was through alteration in protein stability (33, 34). These investigators found that cyclin D1 was degraded in an ubiquitin/proteasome-dependent manner. Other studies have more directly demonstrated that protein degradation is essential for the initiation of DNA replication in vertebrates (25, 28). Our primary observation is that TNF-induced degradation of D-type cyclins is consistent with these previous data and might be essential for TNFα-mediated G₁ arrest. Finally, we detected a proteasome pathway involved in TNFα-mediated growth inhibition. There are two cytoplasmic protease systems: ubiquitin-proteasome pathway that mediates targeted turnover of misfolded and unstable proteins and the calcium-activated neutral protease (calpain)-calpastatin system, which initially was thought to be important in regulating turnover of protein kinases and key structural proteins in the cell (12). More recently, inducible proteolysis has also been shown to be important in the mechanisms for intracellular signaling produced by TNF. In our system cells treated with calpain-dependent inhibitor ALLN and apoptosis inhibitor zVAD-FMK suppressed degradation of IκBα, NF-κB nuclear translocation, and activation of caspase 3, respectively. However, these two inhibitors were not able to prevent TNF-induced degradation of D-type cyclins, which suggests that TNF-induced degradation is not calpain protease system related. In contrast, proteasome inhibitors, MG132 and lactacystin blocked TNF-induced degradation of IκBα and both D-type cyclins. Consistent with these data, an increasing activity of proteasome was observed in TNF-treated cells but not in control cells. Direct evidence of proteasome on cyclin D was from in vitro degradation assays in which the translation product of cyclin D3 plasmid was greatly degraded by 26 S proteasome.

Taken together, the available data now suggest that TNF induces G₁ arrest while simultaneously causing apoptosis. TNF induced G₁ arrest is not due to apoptosis, but is a result of degradation of D-type cyclins. Both apoptosis and G₁ arrest may co-exist in cells in response to TNF treatment. By using multiple approaches, we have demonstrated that the TNFα-induced degradation of D-type cyclins occurs through a proteasome-proteolysis dependent mechanism. To our knowledge, this is the first report in the field.

Since TNF also modestly affected expression of mRNA encoding cyclin D3 but not cyclin D2 and D1, we cannot exclude the possibility that instability of mRNA for cyclin D3 may be a factor affecting TNFα-mediated cell cycle control and G₁ arrest. Future study on stability of mRNAs for D-type cyclins in response to TNF treatment may clarify this question.

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