The TF-1 human erythroleukemic cell line exhibits opposing physiological responses toward tumor necrosis factor-α (TNF) treatment, dependent upon the mitotic state of the cells. Mitotically active cells in log growth respond to TNF by rapidly undergoing apoptosis whereas TNF exposure stimulates cellular proliferation in mitotically quiescent cells. The concentration-dependent TNF-induced apoptosis was monitored by cellular metabolic activity and confirmed by both DNA epifluorescence and DNA fragmentation. Moreover, these responses could be detected by measuring extracellular acidification activity, enabling rapid prediction (within ~1.5 h of TNF treatment) of the fate of the cell in response to TNF. Growth factor resupplementation of quiescent cells, resulting in reactivation of cell cycling, altered TNF action from a proliferative stimulus to an apoptotic signal. Expression levels of the type II TNF receptor subtype (p75TNFR) were found to correlate with sensitivity to TNF-induced apoptosis. Pretreatment of log growth TF-1 cells with a neutralizing antibody to p75TNFR monoclonal antibody inhibited TNF-induced apoptosis by greater than 80%. Studies utilizing TNF receptor subtype-specific TNF mutants and neutralizing antisera implicated p75TNFR in TNF-dependent apoptotic signaling. These data show a bifunctional physiological role for TNF in TF-1 cells that is dependent on mitotic activity and controlled by the p75TNFR.

Cells have the capability of responding to a multitude of signals that it encounters in its extracellular environment. One such signal with widespread pleiotropic actions is the cytokine tumor necrosis factor-α (TNF) (1). TNF has been shown to modulate proliferation, differentiation, and apoptotic or necrotic cell death in a number of different cell types (2–4). These disparate responses to TNF are mediated by TNF binding to specific cell surface receptors. Two distinct TNF receptors, type I (p55TNFR) and type II (p75TNFR) (M, 55,000–60,000 and 70,000–80,000 in human cells, respectively), have been identified (5, 6), although it remains unclear which of the many responses reported for TNF can be attributed to a specific receptor subtype (4). Moreover, the precise signal transduction pathways for each of these receptor subtypes have yet to be fully delineated.

One action of TNF, the induction of apoptosis, is characterized by a discrete set of cellular events regulated by gene expression (7, 8). The physiological events accompanying apoptosis include condensation of the chromatin, degradation of DNA through the activation of endogenous nuclease, and dissolution of the cell into small membrane-bound apoptotic vesicles (9, 10). In vivo, these vesicles are phagocytosed by macrophages or other phagocytic cells. Cell death by apoptosis is essential in many physiological processes, including embryonic development of the nervous system (11), oncogenic pathology (12), and clonal selection of hematopoietic cells (13).

Conversely, TNF has also been shown to stimulate cellular proliferation in a variety of systems (14, 15). Although apoptosis as a physiological phenomenon has only recently received widespread attention, it is apparent that proper development, organismal homeostasis, and oncogenic transformation are all thought to be contingent on a delicate balance between these opposing processes. The importance of both can be seen in a number of pathological diseases that are a consequence of either uncontrolled proliferation or apoptosis. Whereas proliferation is clearly linked to cell cycle progression, the role of the cell cycle and cell cycle-related proteins, such as cyclins, in apoptosis is less clear. Whether TNF-induced cell proliferation or apoptosis demonstrated in different model systems is also connected to the cell cycle apparatus has yet to be determined.

We have used the human erythroleukemic cell line TF-1 as a model system to study TNF actions in early hematopoietic progenitor cells. TF-1 cells are a CD34+ myeloid progenitor stem cell line originally isolated from a human erythroleukemia patient (16). These cells are factor-dependent and thus require a cytokine such as granulocyte-macrophage colony

receptor subtype; p75TNFR, type II 75-kDa TNF receptor subtype; R1-TNF, R232W/S86T human TNF mutant protein; R2-TNF, D143N/A145R human TNF mutant protein; XTT, sodium 3-(1-phenylamino-carbonyl)-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate.

Gregory T. Baxter§§, Richard C. Kuo¶¶, Orla J. Jupp**, Peter Vandenabeele***, and David J. MacEwan****§§
From the §Cornell Nanofabrication Facility, Cornell University, Ithaca, New York 14853, the ¶Neurosciences Program, Stanford University School of Medicine, Stanford, California 94305, the ¶¶Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom, and the **Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology, University of Gent, Ledeganckstraat 35, B-9000 Ghent, Belgium

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Mitotic Activity and TNF Receptor Subtypes in TNF Action

stimulating factor (GM-CSF) or interleukin-3 for survival and proliferation. In this study, we demonstrate that the TF-1 cell line displays differential sensitivity toward TNF depending on the growth state of the cell. We show that mitotic activity is required for TNF-induced apoptosis, whereas conversely, mitotically quiescent cells respond to TNF by cellular proliferation. In addition, we demonstrate an important role for the p75TNFR in addition to the p55TNFR in mediating apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—Unless otherwise noted, chemicals were purchased from Sigma. Recombinant human TNF was obtained from R & D Systems (Minneapolis, MN). Recombinant human GM-CSF was procured from Boehringer Mannheim. Sea Kem GTG agarose was purchased from FMC Corp. (Rockland, ME). TF-1 cells were cultured as described previously in the presence of 1.0 ng/ml GM-CSF unless otherwise indicated (17). Log growth or stationary cultures were grown from a starting cell density of 2.0 × 10^6 cells/ml. Cell counts were performed daily over a period of 8 days to generate the TF-1 cell growth curves seen in Fig. 1, A and B (inset).

Measurement of Metabolic Activity by Microphysiometry—Description of the Cytosensor microphysiometer (Molecular Devices Corp., Sunnyvale, CA) have been detailed elsewhere (18, 19). Briefly, TF-1 cells were collected from cultures of either 5.0 × 10^6 to 1.0 × 10^7 cells/ml (log growth) or 1.0–1.2 × 10^6 cells/ml (stationary). Approximately 2.5 × 10^5 cells were confined between two microporous polycarbonate membranes separated by a 50-μm-thick annular spacer immobilized within a fibrin clot described previously (20). The microporous membranes and annular spacer form a disk-shaped chamber of 6 mm diameter and 50 μm height (volume = 2 μl) that, together with the fibrin immobilizing reagent, traps cells during the fluid perfusion of microphysiometer experiments. This cell capsule was placed in a flow- and temperature (37 °C)-regulated sensing chamber of the microphysiometer so that the lower membrane was in diffusive contact with the surface of the light-addressable potentiometric sensor chip, and pH changes resulting from the metabolic activity of the cells could be monitored at the sensor surface in the center of the chamber. Cells were perfused with low phosphate-buffered RPMI medium (Irvin Scientific, Irvine, CA) in a cyclic manner automatically controlled by a peristaltic pump. The pump cycle was 150 s long, comprising a flow-on period (100 μl/min for 110 s) followed by a flow-off period (40 s). The 110 s duration of the flow-on period was necessary to allow for complete renewal of the medium in the sensing chamber by a flow-off period (40 s). The 110 s duration of the flow-on period was necessary to allow for complete renewal of the medium in the sensing chamber by a flow-off period (40 s). The 110 s duration of the flow-on period was necessary to allow for complete renewal of the medium in the sensing chamber by a flow-off period (40 s).

Analysis of (3H)Thymidine Incorporation—(3H)Thymidine incorporation was performed as described (21). Briefly, equal numbers of TF-1 cells were pulsed with 1 μCi/ml [methyl-3H]thymidine (5 Ci/mmol, Amersham Pharmacia Biotech) for 1 h at 37 °C. Cells were collected onto glass fiber filters under vacuum and washed three times with 5 ml of ice-cold PBS. Filters were then washed with ice-cold 5% trichloroacetic acid followed by a wash with 10 ml of ice-cold absolute ethanol. Dry filters were placed in scintillation vials, and radioactivity was determined by counting in a Beckman LS1801 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Analysis of Cell Number—In order to assess proliferation or cell death from within the same assay, measurement of cell activity (and thus proportionately cell number) was determined by using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 3-(1-phenyl-a-mino-carbonyl)-3-tetrazolium-bis-(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) (Boehringer Mannheim) colorimetric assays. Basically, TF-1 cells (100 μl of growth medium/well) were seeded into a round-bottomed 96-well plate at the indicated density. After 18–24 h of recovery, cells were treated with an additional 100 μl/well of growth medium containing drug and/or TNF and incubated for 24 h more. Each well then received a further 20 μl of growth medium with 5 mg/ml of MTT or XTT. The cells were then allowed to incubate for 3–4 h to allow the metabolism of substrate to form the colored metabolic product. Cells in the 96-well plates were pelleted by centrifuging for 5 min at 1500 rpm (800 RCF). The supernatant medium was carefully aspirated off with a bent 24 gauge needle under low vacuum. To each well was added 100 μl of DMSO, and after 15–30 min, the absorbance for the MTT-formazan product was read at 540 nm (450 nm for XTT) with background subtraction at 690 nm using a Thermomax microplate reader (Molecular Devices Corp.). The results are expressed in arbitrary absorbance units or as a percentage of the absorbance in control-treated cells.

Epifluorescence Apoptosis Analysis—TF-1 cells had various agents added directly to the culture medium for the indicated time. Cells were then washed once by resuspension-centrifugation in culture medium (4 °C), and DNA was stained by inclusion of 5 μg/ml Hoechst 33342 stain as described (22). A 5-μl aliquot of the stained cell sample was examined by epifluorescence using a Leitz Aristoplan microscope equipped with an A cube, and photographed at a magnification of ×320. Fields with large numbers of cells (>150) were used to ascertain the percentage of cells that were intensely fluorescing under ultraviolet excitation (characteristic of nuclear condensation due to the chromosomal compaction and nuclear karyorrhexis seen in apoptosis). The existence of nonfluorescent cells was confirmed by visible light microscopy. Poorly fluorescing cells that had passed through the greatest nuclear condensation were not included in calculations.

DNA Fragmentation Assay—TF-1 cultures of either 8.0 × 10^6 to 1.0 × 10^7 cells/ml (log growth) or 1.0–1.2 × 10^6 cells/ml (stationary) were collected and treated with or without 30 ng/ml TNF for 3 and 18 h, respectively. Cells were pelleted at 500 × g for 5 min and washed twice in ice-cold PBS. Total DNA was extracted using the G NOME DNA kit from Bio 101 (La Jolla, CA) as per the manufacturer’s instructions. DNA concentrations were calculated using 260 nm absorbance with a Hewlett-Packard 8451 spectrophotometer (Hewlett-Packard, Palo Alto, CA). Equal amounts of DNA (10 μg) were loaded per well on a 1.2% agarose gel and run at 7 V/cm for 1 h. DNA bands were visualized and photographed using a Phosphorima (Molecular Dynamics, Sunnyvale, CA) to monitor the extent of DNA fragmentation. Extent of DNA fragmentation was quantitated using the cellular DNA fragmentation enzyme linked-immunoassay kit by Boehringer Mannheim. Briefly, TF-1 cells were labeled with 5-bromo-2’-deoxyuridine for 24 h prior to treatment with TNF at the indicated concentrations for 3 h. Cells were lysed, and fragmented DNA was separated from genomic DNA by centrifugation at 250 × g for 10 min. The lysate was removed and tested by the enzyme-linked immunoassay as described previously (23).

TNF Radiolabeling and Binding Measurements—Munitual analysis of human TNF revealed that certain mutations of the wild-type sequence could enable the mutated protein to selectively bind to either of the TNF receptor subtypes. The specific double mutation of R32W/S86T (termed R1-TNF) allows selective activation by this mutant protein (“mutein”) of the p55TNFR only, whereas the D143N/A145R (termed R2-TNF) double mutation allows selective activation of the p75TNFR subtype only (24, 25). The TNF receptor-subtype-selective muteins were 125I-radiolabeled essentially as described (26) for use in radioligand binding studies to determine the TNF receptor subtype composition of TF-1 cells under varying conditions. Essentially, 5 μg of mutein was dissolved in 10 μl of 20 mM phosphate buffer in a siliconized tube. To the protein solution was added 1 μl (100 μCi) of 125I (Amerham Pharmacia Biotech) plus 10 μl of chloramine T (freshly dissolved in 10 mM phosphate buffer), which was then mixed and incubated for 30 s at room temperature before the addition of 10 μl of sodium metabisulfite (freshly dissolved in 10 mM phosphate buffer) and 100 μl of 1% albumin in PBS. The mixture was then loaded onto a G25 Sephadex column (equilibrated in 1% albumin in PBS). Radiolabeled protein was eluted from the column using 1 ml of 100 mM sodium phosphate buffer and 1 ml of 100 mM NaCl. The radiolabeled protein was then further purified by collecting 1-ml fractions, which were tested for incorporation. Specific radioactivity of the muteins was 1–5 μCi/μg. TNF binding occurred in 200 μl of culture medium (4 °C) for 4 h before separation of unbound label in a Brandel filtration system (Seast, St. Albans, United Kingdom) by washing in 50 mM Tris (pH 7.4) plus 1 mg/ml bovine serum albumin (4 °C). Nonspecific binding accounting for up to 80% of the total binding as determined in the presence of 200 mM (4 μg/ml) excess unlabeled wild-type human TNF. Saturation (0.01–30 nM label) binding measurements were performed to allow Scatchard analysis of the TNF receptor subtypes on whole TF-1 cells (3 × 10^5 cells/tube).
The Metabolic Effects of TNF on TF-1 Cells Differ Depending upon Growth State—To investigate the functional responses of TF-1 cells to TNF, we monitored changes in metabolic activity during continuous TNF exposure with a Cytosensor microphysiometer. Based on a pH-sensitive, light-addressable potentiometric sensor (18), the Cytosensor renders a general representation of ligand-induced receptor activation by monitoring the rate of extracellular acidification (27). Basal extracellular acidification rates depend on the energy-producing metabolic pathways, glycolysis, and aerobic respiration. Both metabolic pathways produce protons through formation of the acidic breakdown products lactic acid and CO₂, respectively. Thus, to a first approximation, extracellular acidification rate represents the sum of cellular glycolytic and respiratory activity and is therefore a measure of cellular metabolic activity. Stimulation by TNF or any growth factor, if sufficiently coupled to energy dependent cellular processes, perturbs extracellular acidification rates and is thus detected by the Cytosensor (27).

Preliminary experiments indicated a differential sensitivity of TF-1 cells to TNF depending on culture condition. Therefore, we investigated TNF effects on cells at the extremes of both log growth and stationary phase. As seen in Fig. 1A, TF-1 cells obtained from a culture undergoing log growth (8 × 10⁴ to 1.0 × 10⁵ cells/ml, see Fig. 1A, inset) responded to TNF with a concentration-dependent decrease in metabolic activity over a period of 3 h. At 30 ng/ml TNF, an initial transient increase in metabolic activity (~5%) was followed by a decrease of greater than 30% when compared with untreated control cells (Fig. 1A, Table I). Continuous exposure to 30 ng/ml TNF for 18 h decreased metabolic activity by approximately 90% of the initial basal activity (data not shown).

In contrast, TF-1 cells obtained from the same culture, but in the stationary phase of their growth curve (1.0–1.2 × 10⁶ cells/ml; see Fig. 1B, inset) responded to TNF with a stimulation of metabolic activity (Fig. 1B, Table I). At high concentrations (>3 ng/ml), the response was biphasic: a dramatic initial burst followed by a secondary sustained elevation of metabolic activity (Fig. 1B; Table I). This response was reminiscent of the metabolic response seen with GM-CSF (28), a cytokine required for TF-1 cell proliferation, suggesting that TNF may induce cellular proliferation under these conditions. Maximal effects of TNF were seen at 30 ng/ml TNF (data not shown) consistent with other known responses (22, 29).

Differential Effects of TNF upon Cell Viability and Proliferation Depend on Cell Growth State—We hypothesized three possible mechanisms of decreased metabolic activity resulting from TNF exposure. These included (i) a general suppression of cellular metabolism; (ii) a conversion from glycolytic to aerobic metabolism, resulting in reduced proton production per ATP turnover; and (iii) a cytotoxic mode of action. We observed that TNF exhibited dramatically different effects on cell viability as a function of the growth state. Log growth cells responded to a 48-h treatment with TNF by a concentration-dependent decrease in viable cell number as compared with untreated cells (Fig. 2A). Alternatively, cells initially in a stationary growth state showed a concentration-dependent increase in viable cell number following a 48-h TNF treatment when compared with untreated controls (Fig. 2B).

Increased cell number in response to TNF could either be due to rescue from cell death as a result of growth factor depletion or actual induction of cellular proliferation. To distinguish between these possibilities, we measured cell growth over a period of 3 days in the presence and absence of TNF. The data indicate that the number of viable TF-1 cells derived from a stationary culture decreased slightly over time in the absence of TNF (Fig. 3), but with 1 ng/ml TNF treatment, the number of viable cells increased over the course of the experiment (Fig. 3). This appeared to represent a TNF stimulation of cellular proliferation with TF-1 cells collected from a stationary culture. In addition, the ability of TNF to induce proliferation in quiescent TF-1 cells is due to its actions directly on the cells and not through its induction of other secondary mitogenic factors. This was confirmed in conditioned medium experiments in which the medium from 24 h, 50 ng/ml TNF-treated cells in the extra presence of 0.1 ng/ml of a neutralizing anti-TNF polyclonal antibody was unable to elicit proliferation of quiescent TF-1 cells as measured by MTT assay (104 ± 5% of control cell number). Medium with neutralizing antibody alone
resulted in 97 ± 8% of control, whereas medium conditioned for 24 h with TNF alone (no neutralizing antibody included) still partly retained its ability to induce proliferation (122 ± 6% of control cell levels (mean ± S.D. from representative experiments repeated three other times with similar findings)). Thus, the proliferative effect of TNF is directly on quiescent TF-1 cells and not via induction of some other TNF-induced mitogenic factor.

Mitotically Active TF-1 Cells Undergo TNF-dependent Apoptosis—TF-1 cells in log growth responded to TNF by both a decrease in metabolic activity (Fig. 1A) and a decrease in viable cell number (Fig. 2A). These results suggested a cytotoxic mode of action for TNF, which we further examined by using inhibitors of protein synthesis and gene transcription. Treatment with 2 μg/ml cycloheximide or actinomycin D completely eliminated the decrease in metabolic activity seen with TNF (data not shown), implying that the cytotoxic action of TNF required active macromolecular synthesis consistent with an apoptotic mechanism. Also intriguing was the transient increase in metabolic activity during the first 30 min of treatment with high concentrations of TNF (Fig. 1A). This was indicative of an energy-requiring process consistent with other work showing that the progression of apoptosis requires cellular energy (30). Taken together with previous reports of TNF action, this suggests that in mitotically active cells, TNF may be inducing apoptosis.

To more directly evaluate the possibility of TNF-induced apoptosis, we employed two independent assays. Chromatin condensation and DNA fragmentation, two physiological stages in the progression of apoptosis (8), were assessed by analysis of Hoechst 33342 staining and gel electrophoresis, respectively. As seen in Fig. 4A, a concentration-dependent increase in the percentage of highly fluorescent (“epifluorescent”) Hoechst-stained apoptotic cells occurred following TNF stimulation. The time course of TNF-mediated apoptosis is shown in Fig. 4B. These results indicated that 40% of the cell population was apoptotic after only 3 h of treatment with 50 ng/ml TNF as measured by Hoechst stain. Decreases in the percentage of apoptotic cells seen at later time points were due to advancement beyond the state of chromatin condensation, and cells beyond this stage of apoptosis are not scored by the Hoechst stain assay (see under “Experimental Procedures”). As an independent assay of apoptosis, we also determined the extent of DNA fragmentation by agarose gel electrophoresis after TNF treatment. As seen in Fig. 4C, cells treated with 30 ng/ml TNF exhibited significant DNA fragmentation after 3 and 18 h of treatment when compared with untreated controls. Both the time course and concentration dependence of DNA fragmenta-

### Table I

| TNF (ng/ml) | Log growth cells | Stationary cells |
|-------------|------------------|-----------------|
|             | 0.5 h | 3 h | 0.5 h | 3 h |
| 0.03        | 0.3 ± 0.6 | −9.0 ± 3.8 | 8.3 ± 2.9 | 12.7 ± 3.2 |
| 0.3         | 1.3 ± 1.5 | −14.6 ± 4.5 | 41.0 ± 9.0 | 19.0 ± 11.0 |
| 3           | 2.7 ± 0.6 | −23.0 ± 1.2 | 61.3 ± 14.5 | 14.0 ± 7.8 |
| 30          | 3.7 ± 2.1 | −30.3 ± 1.0 | 78.2 ± 7.4 | 14.2 ± 8.2 |

Fig. 2. The effect of TNF on TF-1 cell viability. TF-1 cells either in a state of log growth (A) or stationary (B) were cultured in a 96-well microtiter plate at a starting cell density of either 1 × 10^6 (A) or 1 × 10^6 (B) cells/well in the presence of TNF at the indicated concentrations, and viable cell number was determined after 48 h using the XTT colorimetric assay described under “Experimental Procedures.” Data represent the means ± S.E. of at least three independent experiments performed with triplicate determinations.

Mitotically Active TF-1 Cells Undergo TNF-dependent Apoptosis—TF-1 cells were seeded at 1 × 10^6 cells/ml in the presence (shaded columns) or absence (open columns) of 1 ng/ml TNF in 96-well microtiter plates, and viable cell number was determined at the indicated time points using the XTT colorimetric assay described under “Experimental Procedures.” Data represent the means ± S.E. of at least three independent experiments performed with triplicate determinations.
tion and Hoechst stain were consistent with measurements of cell viability (Fig. 2A) and changes in metabolic activity (Fig. 1A).

**DNA Synthesis and the Effects of TNF**—The previous data demonstrate that TF-1 cells in a state of log growth respond to TNF by induction of apoptosis, whereas induction of proliferation occurs with the same cell population when allowed to grow to a stationary phase. This suggested that only a mitotically active cell, i.e. one undergoing active DNA synthesis, was sensitive to TNF-induced apoptosis. Similarly, a quiescent cell not actively undergoing DNA synthesis should not be sensitive to TNF-induced apoptosis. To test these predictions we induced mitogenesis in a stationary culture by GM-CSF supplementation and then compared the extent of TNF-induced apoptosis in the proliferating culture relative to a non-GM-CSF stimulated stationary culture.

Mitotic activity was determined by measuring [³H]thymidine incorporation into DNA. TF-1 cells initially obtained from a stationary culture, when supplemented with 1 ng/ml GM-CSF for 18 h, showed similar levels of [³H]thymidine incorporation into DNA when compared with cells from a log growth culture (Fig. 5A). Both of these conditions were significantly greater than that seen with control cells from a stationary culture (Fig. 5A). This indicates that the addition of GM-CSF to mitotically quiescent cells induces mitogenesis to levels comparable to log growth cultures. The extent of TNF-induced apoptosis was determined under these culture conditions by quantitating the amount of DNA fragmentation as described under “Experimental Procedures.” Results for log growth cultures showed approximately a 6–7-fold increase in DNA fragmentation with TNF concentrations greater than 3 ng/ml (Fig. 5B). We did not detect a significant amount of TNF-induced DNA fragmentation in the stationary cultures (Fig. 5B). In contrast, GM-CSF supplementation of these cells caused a dramatic enhancement of TNF-induced DNA fragmentation to levels comparable to log growth cultures (Fig. 5B). Furthermore, experiments utilizing drugs that block mitosis in HeLa cells: nocodazole (31) and aphidicolin (32) preincubated for 1 h before 1 ng/ml GM-CSF treatment for 24 h, prevented the ability of GM-CSF to render TF-1 cells susceptible to TNF-induced apoptosis. The added inclusion of 20 μM nocodazole or 3 μg/ml aphidicolin reduced control TNF-induced apoptosis levels after 15 h from 59 ± 2% to 18 ± 5 and 17 ± 3%, respectively (apoptosis levels measured by epifluorescence in cells without 50 ng/ml TNF for 15 h were 9 ± 2% (data are mean ± S.E., n = 5)). These results provide strong evidence supporting the claim that active mitogenesis is required for TNF to induce apoptosis.

**p75TNFR Is Involved in TNF Signaling for Apoptosis**—To begin assessing which receptor subtype(s) mediates the TNF apoptotic effect, we performed immunoblot analysis on whole

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**Fig. 4.** TF-1 cells in log growth respond to TNF by induction of apoptosis. A, apoptosis was determined after treating log growth TF-1 cells with TNF at the indicated concentrations for 15 h, staining with Hoechst 33342, and scoring for condensed chromatin as described under “Experimental Procedures.” Percentage of apoptosis was calculated by dividing apoptotic cells by the total number of cells scored. Data represent the mean ± S.D. of triplicate determinations from a representative experiment that was repeated at least twice with similar results. A, inset, a representative field of highly fluorescent Hoechst-stained cells scored in the Hoeschst stain apoptosis assay as described under “Experimental Procedures” is shown. The arrows point to TF-1 cells undergoing apoptosis, whereas the arrowhead points to a cell undergoing mitosis. B, log growth TF-1 cells were treated with 50 ng/ml TNF, and apoptosis was determined using Hoechst stain at the indicated time points as described. Percentage of apoptosis was calculated by dividing apoptotic cells by the total number of cells scored. Data represent the mean ± S.D. of triplicate determinations from a representative experiment that was repeated at least twice with similar results. C, log growth TF-1 cells were treated with or without 30 ng/ml TNF for the indicated time periods, and total DNA was purified as described under “Experimental Procedures.” DNA size standards are shown. 10 μg of DNA was loaded per well and separated on a 1.2% agarose gel. DNA was visualized by UV illumination following staining with ethidium bromide. This figure shows representative data from three independent experiments.
activity. Cells under each of these conditions were subjected to \[^3H\]thy-

GM-CSF (A or B, inset). Cells in stationary phase were supplemented with 1 ng/ml 

GM-CSF (striped column) for 18 h prior to treatment with TNF at the 

indicated concentrations for 3 h before DNA fragmentation was quan-

titated by the enzyme-linked immunosorbent assay described under “Experimental 

Procedures.” The data (means ± S.D. of triplicate deter-

minations) are from an determination that is representative of at least 

four independent experiments.

FIG. 5. TNF induced apoptosis in mitotically stimulated TF-1 

cells. A, TF-1 cells were collected from cultures either in log growth 

(open column) or stationary (shaded column) growth phase (see Fig. 1, 

A or B, inset). Cells in stationary phase were supplemented with 1 ng/ml 

GM-CSF (striped column) for 18 h prior to treatment with TNF at the 

indicated concentrations for 3 h before DNA fragmentation was quanti-

tated by the enzyme-linked immunosorbent assay described under “Experimental 

Procedures.” The data (means ± S.D. of triplicate deter-

minations) are from an determination that is representative of at least 

four independent experiments.

FIG. 6. Immunoblot analysis of p55TNFR and p75TNFR. Cells 

were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer, and immunoblot analysis was performed as described under “Experimental Procedures” using a mouse monoclonal antibody to the human p55 TNF receptor subtype (left panel) or a rat monoclonal antibody to the human p75 TNF receptor subtype (both antisera from Genzyme Corp., Cambridge, MA) (right panel). Left lanes, U937 cells 

known to express both p55TNFR and p75TNFR (22); middle lanes, TF-1 

cells obtained from a culture in log growth (see Fig. 1, inset); right 

lanes, TF-1 cells obtained from a stationary culture. Immunoblotting 

was repeated three times with similar results, and a representative 

experiment is shown. Shown at the left are the locations of molecular 

mass standards.

for R2-TNF were 3516 receptors/cell for log growth TF-1 cells 

and 2873 receptors/cell for stationary phase cells. These data, 
taken together, indicate that p75TNFR subtype levels are mod-

ulated in mitotically active TF-1 cells; however, the p55TNFR 

subtype remains relatively constant.

Because p75TNFR receptor levels seemed to parallel 

changes in TNF-mediated cell death, we next sought to examine 

its individual role in mediating apoptosis. We utilized a 

neutralizing anti-p75TNFR monoclonal antibody (33) and 

quantitated TNF-mediated apoptosis in the presence and ab-

sence of anti-p75TNFR by Hoechst staining. Immunoblot anal-

ysis indicated no cross-reactivity with p55TNFR by this anti-

body (Fig. 6), so inhibition of TNF responses by this antibody 

should be specific for p75TNFR. Fig. 8 shows that pretreatment 

with anti-p75TNFR antibody inhibits TNF-induced apoptosis 

of TF-1 cells in log growth by approximately 80%. Nonspecific 

rat IgG had no significant effect on TNF-induced apoptosis of 

TF-1 cells, and neither antibody activated apoptosis in the 

absence of TNF (Fig. 8). In addition, the cytotoxic action of TNF 

as seen by the Cytosensor assay was significantly inhibited by 

the anti-p75TNFR neutralizing antibody (data not shown).

These results again implicated a role for p75TNFR in TNF-

induced apoptotic death in log growth TF-1 cells.

Further detailed analysis using the p55TNFR-selective mu-

tein R1-TNF and the p55TNFR-selective mutein R2-TNF (as 

well as unmutated wild-type TNF and TNF procured from a 

commercially available source) indicated a role for both the 

TNF receptor subtypes in TNF-induced 

TF-1 cell proliferation or death (Fig. 9). Activation of each TNF 

receptor subtype individually could lead to a partial prolifera-

tive response or cytotoxic response, which was, once again, 

dependent on metabolic status of the cells (Fig. 9). The ability 

of each individual receptor to partially perform the TNF-medi-

ated proliferative or death actions in TF-1 cells does not extend 

to all lymphoid cells that express both TNF receptor subtypes.
and die in response to TNF. For example, the U937 cell line, which expresses approximately equal proportions of TNF receptor subtypes (Fig. 6), seems to die in response to TNF treatment through only a p55TNFR-mediated mechanism (Table II). These investigations again implicated a partial role for both TNF receptor subtypes in TNF-induced apoptotic death in log growth TF-1 cells. Taken together, these data strongly implicate a role for p75TNFR in mediating the TNF-induced apoptosis observed in TF-1 cells.

DISCUSSION

Although initially recognized and named for its ability to cause necrotization of tumor masses, TNF has since been shown to also have dramatic systemic effects. Chronic TNF production occurs in cachexia, a wasting disease of cancer (1). TNF also plays a primary role in inflammation and at extreme physiological concentrations is responsible for septic shock (1). Understanding how TNF can have such extreme physiological consequences requires an understanding of the cellular basis of its action. At the cellular level, TNF has been shown to modulate the fundamental processes associated with development, including cell proliferation, differentiation, and apoptosis. However, the molecular mechanisms by which TNF induces these events are only now beginning to be understood, involving a complex array of TNFR-associating factors. Indeed, although both TNF receptors are believed to be involved in TNF-mediated signaling, it has been widely thought that the majority of the responses are mediated by the p55TNFR subtype (34).

In assessing the affects of TNF, we initially used the Cyto-sensor microphysiometer, an instrument that measures cellular metabolic activity by detecting changes in rate of extracellular acidification. We found that metabolic activity decreased after TNF treatment of log growth phase cells. From this find-

Fig. 7. TNF receptor subtype-specific radioligand binding. Scatchard analyses of the radioligand saturation binding to TF-1 cells of mutant TNF ligands that specifically recognize either the p55TNFR (R1-TNF) (A) or the p75TNFR (R2-TNF) (B). Whole TF-1 cells in either log growth (open symbols) or stationary (closed symbols) phase had the TNF receptor subtype composition measured as described under “Experimental Procedures.” The data represent the binding from experiments, which is representative of at least three independent determinations (all experiments gave similar results). In the experiment shown, the Kd values for R1-TNF and R2-TNF binding were 50 and 117 nM, respectively, for log growth TF-1 cells and 49 and 127 nM in stationary phase TF-1 cells. The Bmax values for R1-TNF and R2-TNF binding were 2438 and 3516 receptors/cell, respectively, for log growth TF-1 cells and 2356 and 2873 receptors/cell in stationary phase TF-1 cells.

Fig. 8. p75TNFR is involved in mediating apoptosis in TF-1 cells. Cells derived from a culture in log growth (see Fig. 1A, inset) were treated with or without 30 ng/ml TNF in the presence of either 10 μg/ml rat monoclonal anti-p75TNFR (Genzyme) or 10 μg/ml nonspecific rat IgG (Calbiochem, San Diego, CA) as indicated. Hoechst stain analysis was performed after 15 h of treatment as described under “Experimental Procedures.” Percentage of apoptosis was calculated by dividing apoptotic cells by the total cell number. Data represent the means ± S.E. of four independent experiments.

Fig. 9. TNF receptor subtype action in TNF-mediated TF-1 cell proliferation or cell death. TF-1 cells were seeded into a 96-well culture plate at the indicated density to allow cells to be in a quiescent or log growth state (Fig. 1A or B, inset) 18 h after seeding. The cells were then treated with a maximal (50 ng/ml) concentration of TNF (from a commercial source, R & D Systems), wild-type TNF, or the R1-TNF and R2-TNF mutants alone or in combination. After 24 h, the cell number was determined with the MTT cell assay as described under “Experimental Procedures.” The data represent the mean ± S.D. of eight determinations from an experiment that is typical of findings in at least three other individual experiments.
ing, we predicted a cytotoxic mode of action for TNF on cells in log growth. TNF treatment of stationary phase cells resulted in a dramatic increase in metabolic activity; thus, we were able to predict a proliferative response to TNF that was subsequently confirmed. Although changes in metabolic activity above or below initial basal levels are not definitive for apoptosis or proliferation, in this study, we have shown that they can be predictive. The microphysiometer system correlated well with more traditional indexes of apoptosis, which confirmed the validity of this method for monitoring progression of apoptosis in this system. Hoechst staining and analysis of DNA fragmentation by gel electrophoresis, although definitive, only measure the end stages of apoptosis; early signaling events of apoptosis are not observed. Other measurements such as intracellular calcium have also been used (35). Some forms of apoptosis may be independent of changes in intracellular calcium and thus may not be a clear indicator of apoptosis (29).

Extracellular acidification rate or metabolic activity reflects homeostasis in ATP-generating pathways. Activation of either apoptosis or proliferation should also concomitantly change cellular ATP homeostasis because these processes require energy. Indeed, using microcalorimetry, Wallen-Ohman et al. (36) have obtained similar results in examining apoptosis in the KM-3 pre-B acute lymphocytic leukemia cell line. Microcalorimetric measurements revealed an increase in metabolic activity, which preceded detectable DNA fragmentation by several hours. Clearly, as cells undergo death, their contribution to the metabolic activity of a population of cells disappears and metabolic activity decreases. Rapid, continuous monitoring of metabolic activity may offer a way of screening agents that affect early mechanisms in the apoptotic pathway.

Our results demonstrate that TNF stimulates proliferation of TF-1 cells when cells are in a growth-arrested state. Increased cellular number was due to true cellular proliferation and not rescue or prevention of cell death as compared with control cultures. The stimulation of proliferation by TNF was not as potent as GM-CSF, a growth factor known to promote growth and survival of TF-1 cells (37). This suggests that the mitogenic signal of TNF may be insufficient for long term survival of TF-1 cells, although this was not established in this study. In contrast, our data also indicate that the TF-1 cell line is only sensitive to TNF-mediated apoptosis when mitotically active or actively progressing through the cell cycle, although we have yet to explore the effects of TNF sensitivity on cells arrested in specific phases of the cell cycle, such as G1/S phase-arrested cells. These data suggest a common link between proliferation and apoptosis. Both may intersect at the level of mitosis and the cell cycle, as our results indicate that the biological effect of TNF is strongly influenced by cell cycle progression. This argues that cell cycle-related proteins may be involved in governing the physiological consequences of TNF.

Although a direct relationship between TNF and cell cycle-related molecules has not been reported, some studies have shown either an acceleration or inhibition of the cell cycle in the presence of TNF (38, 39).

The intracellular second messenger ceramide is a possible mechanism by which TNF may affect cell cycle progression and apoptosis (40–42). Signaling by the p55TNFR stimulates a neutral sphingomyelinase, which in turn generates ceramide and sphingosine. A role for ceramide in inducing apoptosis was originally shown in HL-60 myeloid leukemia cells. Subsequent work has shown that ceramide also induces cell cycle arrest, which may activate apoptosis in some cell types. Ceramide then may be an effector molecule in the cell cycle and apoptotic effects seen with TNF.

More generally, a number of recent studies have reported a connection between the cell cycle and induction of apoptosis (43–45). Meikrantz et al. (44) showed that agents that promote premature mitosis in HeLa cells induce apoptosis and that sensitivity for induction of apoptosis paralleled the activation of cyclin A-dependent kinases. In another study, premature activation of p34cdc2 kinase was sufficient for induction of apoptosis of cytotoxic T lymphocytes (45). Clearly, an association exists between the cell cycle and apoptotic sensitivity. The c-myc gene, in addition to its well-established role in mitogenesis, is a potent inducer of apoptosis and that expression of Bcl-2 inhibits c-myc-induced apoptosis (46). Bcl-2 and other proteins known to participate in apoptosis may be important in defining a physiological role for c-myc (47–51). Although the involvement of c-myc in TNF-mediated cytotoxicity has been suggested (52), TNF-induced mitogenesis via a c-myc mechanism has yet to be established.

Our results have also demonstrated that p75TNFR is involved in inducing apoptosis in the TF-1 cell line. A role for p75TNFR in mediating cytotoxicity is controversial (26, 29, 53–56). Our data neither rule out a role for p55TNFR nor contradict the ligand-passing model proposed by Tartaglia et al. (57). Indeed, the role for p75TNFR in TF-1 cell apoptosis or proliferation may be only as a “ligand-passing” role, because we have not attempted to demonstrate a signaling function of p75TNFR here. Although a “death domain” has been defined for p55TNFR (54) and not for p75TNFR, this does not rule out the involvement of associated signaling proteins. In fact, a signaling role for p75TNFR has been demonstrated by the discovery of TNFR-associated factor proteins that associate with the receptor (58, 59). Whether these proteins are involved in apoptotic signaling remains to be fully determined. The finding that p75TNFR is involved in apoptosis and our results demonstrating that levels of p75TNFR expression correlate with sensitivity to TNF-induced apoptosis may explain why TNF induction of cell proliferation in mitotically quiescent TF-1 cells does not lead to sensitivity to apoptosis. However, the continued presence of p75TNFR subtype in conditions where TNF does not induce cell death (i.e. stationary phase cells) is obvious from this study, suggesting that its mere presence does not dictate sensitivity of TF-1 cell to TNF-induced cell death. Therefore, just as the role of the p55TNFR solely to induce apoptosis in this study cannot be ruled out, neither can the role of p75TNFR in inducing cell death be proven as exclusive. Indeed, it seems that TNF-induced cell proliferation or cell death in TF-1 cells does not occur exclusively through one of the TNF receptor subtypes, unlike TNF-mediated cell death in U937 cells (see Table II). It is now becoming clear that, in some cell systems at least, the relative proportion of p75TNFR subtype (acting in co-operation with the p55TNFR) may be decisive in predetermining the apoptotic cell death that occurs in response to TNF (26, 60, 61). Clearly, there are other cellular

### Table II

| Treatment | Apoptosis |
|-----------|-----------|
|           | Log growth TF-1 cells | U937 cells |
| Control   | 4 ± 2      | 21 ± 10    |
| TNF       | 31 ± 8     | 52 ± 14    |
| Wild-type TNF | 36 ± 9     | 55 ± 16    |
| R1-TNF    | 21 ± 8     | 50 ± 13    |
| R2-TNF    | 19 ± 9     | 28 ± 9     |
| R1-TNF + R2-TNF | 32 ± 6   | 53 ± 15    |

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factors that are regulated and contribute to the distinct TNF effects that we see here, and it will be important to distinguish the subtle cellular changes that occur in order to fully understand the principle or cell life or death.

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