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Effect of Cell Cycle on the Regulation of the Cell Surface and Secreted Forms of Type I and Type II Human Tumor Necrosis Factor Receptors

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Abstract The cell cycle has been shown to regulate the biological effects of human tumor necrosis factor (TNF), but to what extent that regulation is due to the modulation of TNF receptors is not clear. In the present report we investigated the effect of the cell cycle on the expression of surface and soluble TNF receptors in human histiocytic lymphoma U-937. Exposure to hydroxyurea, thymidine, etoposide, bisbensimide, and demecolcine lead to accumulation of cells primarily in G_1/S, S, S/G_2/M, G_2/M, and M stages of the cell cycle, respectively. While no significant change in TNF receptors occurred in cells arrested in G_1/S or S/G_2 stages, about a 50% decrease was observed in cells at M phase of the cycle. Scatchard analysis showed a reduction in receptor number rather than affinity. In contrast, cells arrested at S phase (thymidine) showed an 80% increase in receptor number.

The decrease in the TNF receptors was not due to changes in cell size or protein synthesis. The increase in receptors, however, correlated with an increase in total protein synthesis (to 3.8-fold of the control levels). A proportional change was observed in the p60 and p80 forms of the TNF receptors. A decrease in the surface receptors in cells arrested in M phase correlated with an increase in the amount of soluble receptors. The cellular response to TNF increased to 8- and 2-fold in cells arrested in G_1 and S phase, respectively; but cells at G2/M phase showed about 6-fold decrease in response. In conclusion, our results demonstrate that the cell cycle plays an important role in regulation of cell-surface and soluble TNF receptors and also in the modulation of cellular response.

Key words: TNF, human histiocytic lymphoma, protein synthesis, receptors, p60, p80

Tumor necrosis factor (TNF) is a macrophage-derived cytokine that plays an important role in inflammatory and immunological reactions, septic shock, and autoimmune diseases [see Aggarwal and Vilcek, 1992; Beutler, 1992]. It has antiproliferative effect against tumor cells; proliferative effects on normal cells; and antiviral, antimicrobial, and immunomodulatory effects. TNF exerts its biological effects via two distinct high-affinity receptors, one with a molecular mass of 60 kD (p60, TNFR type I, or TNFRα) and the other of 80 kD (p80, TNFR type II, or TNFRβ) [Hohmann et al., 1989; Brockhaus et al., 1990]. Both receptors have been cloned recently in man [Loetscher et al., 1990; Smith et al., 1990] and in mouse [Lewis et al., 1991; Rothe et al., 1993], and monoclonal antibodies against both have also been developed [Brockhaus et al., 1990]. The cDNA sequences of the extracellular domains of the TNF receptors revealed significant homology between the p60 and p80 forms of the receptors and also with other receptors or cell surface antigens, e.g., nerve growth factor receptor, CD40, CD27, and Fas antigen [Akira et al., 1990; Benton, 1991]. There is, however, no similarity between the cytoplamic domains of the TNF receptors. The

Abbreviations: FCS, fetal calf serum; GM-CSF, granulocyte-macrophage colony stimulating factor; LT, lymphotoxin; p60 (also referred to as p55), TNF receptor I or TNF receptor type B; p80 (also referred to as p75), TNF receptor II or TNF receptor type A; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF, tumor necrosis factor.

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tissue distribution of the two forms of the TNF receptors differs; p60 receptor is expressed ubiquitously on various types of cells, but mainly on epithelial cells, while the p80 form is expressed predominantly on cells of hematopoietic origin [Hohmann et al., 1989; Brockhaus et al., 1990; Dembic et al., 1990; Gehr et al., 1992; Ryffel and Mihatsch, 1993].

The functions of the two forms of TNF receptors are still controversial. The p60 receptor was identified as the major transducing component in the induction of cytotoxic activity, proliferative effects, and expression of class II MHC antigens on tumor cells [Thoma et al., 1990; Van Ostade et al., 1993], as well as in the induction of leukocyte adhesion to human umbilical vein endothelial cells and expression of cell adhesion molecules (intercellular adhesion molecule type 1, ICAM-1, E-selectin, vascular cell adhesion molecule type 1C, VCAM-1, CD44) in man [Mackay et al., 1993]. However, the p80 receptor has also been shown to be involved in TNF cytotoxicity [Heller et al., 1992; Higuchi and Aggarwal, 1993b]. Other data support the notion that both receptors mediate proliferative signals in human mononuclear cells [Gehr et al., 1992]. Increasing evidence shows that the two forms of the TNF receptors mediate distinct biological responses to TNF, namely, that p60 is responsible for the cytotoxic and p80 for the proliferative response [Tartaglia et al., 1991; Waage et al., 1992]. Similarly, the p80 receptor exclusively mediates up-regulation of TGFα mRNA, whereas the p60 form regulates the epidermal growth factor receptor (EGFR) mRNA in epithelial cell lines [Kalthoff et al., 1993]. A role for the p60 receptor in endotoxic shock in mice has recently been described [Pfeffer et al., 1993]. Thus, the two type of TNF receptors appear to mediate both overlapping and nonoverlapping activities.

Cell surface expression of TNF receptors is controlled by multiple factors, including cytokines, protein kinases, phosphatases, oncogenes, and proteases, which act at the transcriptional and posttranscriptional level [Tsujimoto and Oku, 1992; Higuchi and Aggarwal, 1993a, 1994; Zhang et al., 1994]. Recently, the effect of cell density on the expression of TNF receptors has also been described as a function of protein synthesis and glutathione levels in epithelial and myeloid cell lines [Pocsik et al., 1994].

An association between cell cycle and sensitivity of cells to TNF can be assumed from studies that show that drugs inhibiting RNA or protein synthesis, like actinomycin D and cycloheximide, as well as protein kinase C activators and inhibitors, can cause cell cycle arrest of cells [Pardee and Keyomarski et al., 1992] and can change the sensitivity of target cells to the cytotoxic effect of TNF [Higuchi and Aggarwal, 1993b; Zhang et al., 1994; Kirstein et al., 1986; Ruggiero et al., 1987]. Previous studies have also shown that the cellular response of TNF is dependent on the cell cycle [Darzynkiewicz et al., 1984; Watanabe et al., 1987]. Whether this is due to the changes in the cell surface expression of TNF receptors is not clear. In this study we investigated the effect of the cell cycle on the expression of cell surface and soluble forms of the TNF receptor. Our results show that expression of the TNF receptors in U-937 cells is regulated by the cell cycle, namely, it is down-regulated in cells arrested in M phase and up-regulated in cells arrested in S phase.

MATERIALS AND METHODS

Materials

Gentamicin, RPMI 1640, and fetal calf serum (FCS) were obtained from GIBCO, Grand Island, NY. Carrier-free $Na^{125}I$ and L-[4,5-^{3}H] leucine were purchased from Amersham Buchler GmbH, Braunschweig, Germany; [6-^{3}H] thymidine was from UVVVR, Prague, Czechoslovakia. Tetrachlorodiphenylglycouril (Iodogen), bovine serum albumin, gelatin, hydroxyurea, thymidine, etoposide, bisbenzimide (Hoechst No. 33342), demecolcine, nocodazole, Triton X-100, Tween 20, Tris, 3,3′,5,5′-tetramethylbenzidine (TMB), and MTT were obtained from Sigma Chemical Co., St. Louis, MO. Propidium iodide was obtained from Calbiochem (La Jolla, CA), and vinblastine sulfate from Richter Gedeon Chemical Co., Budapest, Hungary. *Escherichia coli*-derived recombinant human TNF purified to homogeneity with a specific activity of 5×10^7 units/mg was kindly provided by Dr. Günther Adolf (Bender, Vienna, Austria). The purified monoclonal antibodies htr-9, htr-20, utr-1, and utr-4, specific to the human TNF receptors p60 and p80 (TNFR-I and TNFR-II), respectively, the highly purified form of the baculovirus-derived recombinant extracellular domains (aa 1-182) of the p60 and CHO cell-derived recombinant extracelluar form (aa 1-180) of the p80 form of the TNF receptor, biotinylated human recombinant TNF, and kathon (Christ AG,

Aesch, Switzerland) were gifts from Hoffmann-La Roche, Basel, Switzerland.

Cell Culture

U-937 cells (histiocytic lymphoma), HeLa (epithelioid cervical carcinoma) cells, and K562 (erythroleukemia) cell lines were purchased from the American Type Cell Culture Collection (Rockville, MD). All the cell lines were grown in RPMI 1640 medium supplemented with 10% FCS and 50 μg/ml gentamicin. The cells were seeded regularly at 1–3 × 10^5 cells/ml (nonadherent cell lines) or 0.5 × 10^5 cells/ml (adherent cell lines) in 175-cm^2 tissue culture flasks at 5.7 × 10^3 cells/cm^2 cell density and grown at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged routinely every third day. For receptor-binding assay the adherent cells were detached from the flasks by a short treatment with 0.05% trypsin/0.5 mM EDTA mixture, which had no effect on the TNF receptors [Kirstein et al., 1986]. For treatment with cell cycle inhibitors, nonadherent cells seeded at 2–5 × 10^5/ml were used the following day. Subsequently, cell density was readjusted to 4–5 × 10^5 cells/ml for one-day treatments and to 2–3 × 10^5 cells/ml for two-day treatments of cells, cell cycle inhibitors were added, and the cultures were incubated at 37°C for 24 h or 48 h, respectively.

Receptor-Binding Assay

Recombinant human TNF was labeled with $Na^{125}I$ using the Iodogen procedure as described previously [Aggarwal et al., 1985]. The specific activity of labeled TNF was 11 μCi/μg. Binding assays were performed in 96-well, U-shaped bottom, flexible microplates (Dynatech, Cat. No. 1-220-24, Alexandria, VA) as described [Pocsik et al., 1994; Higuchi and Aggarwal, 1992a]. Briefly, 1 × 10^6 cells/well were incubated in a binding buffer (RPMI 1640-10% FCS) with ^{125}I-labeled ligand (1 × 10^5 cpm for U-937 cells, 2 × 10^5 cpm for all the other cells) with or without a 100-fold excess of unlabeled ligand (100 or 200 nM, respectively) for 1 h at 4°C in a total volume of 0.1 ml. Thereafter, cells were washed three times with ice-cold PBS-0.1% BSA, and cell-bound radioactivity was measured by a Beckman gamma counter (model Gamma 5500). To determine the dissociation constant (Kd) and receptor number on cells, Scatchard analysis was performed by adding increasing amounts of cold TNF to 1 × 10^6 U-937 cells in the presence of ^{125}I-labeled ligand (1 × 10^5 cpm). The Kd and receptor numbers were calculated according to the method described by Scatchard [1949].

In order to determine the cell surface expression of p60 and p80 form of the TNF receptors, cells were preincubated at 37°C for 30 min in the presence or absence of 2 μg/ml purified monoclonal anti-p60 (htr-9), or anti-p80 (utr-1) antibody and then standard TNF binding assay was performed as described above. Nonspecific binding was measured in the presence of 100 nM unlabeled TNF. The percent of p60 and p80 receptors were calculated by subtracting the specific binding obtained in the presence of htr-9 or utr-1 from specific binding observed without antibodies (set to be 100%).

Determination of Soluble TNF Receptors

U-937 cells were treated in culture flasks with cell cycle inhibitors at 37°C for 24 h or 48 h at cell densities of 0.2 × 10^6 and 0.4 × 10^6 cells/ml, respectively. The culture supernatants were collected, centrifuged, filtered on a membrane with 22 μm pore size, aliquoted, and stored at −80°C until use. Determination of soluble TNF receptors in culture supernatants was performed by an enzyme-linked immunological, biological assay (ELIBA; Hoffmann-La Roche) as described elsewhere [Kern et al., 1992]. Briefly, 96-well ELISA plates (Costar, Cat. No. 9018, Cambridge, MA) were coated for 24 h at room temperature with 0.2 ml of 10 μg/ml monoclonal anti-p60 (htr-20) or 5 μg/ml monoclonal anti-p80 (utr-4) antibodies in 0.1 M sodium phosphate, pH 6.5. Thereafter, the coating solution was decanted, and the plates were washed three times with deionized water. The remaining protein-binding capacity of the coated plates were saturated with 0.2 ml of saturation buffer (1% BSA in 200 mM Tris/HCl, 0.02% Kathon, pH 7.5) for 24 h at room temperature. After withdrawing the saturation buffer, serial dilutions of the soluble TNF receptor standards and the samples were added in a 0.2 ml volume. Subsequently, 50 μl of 0.4 μg/ml and 50 μl of 2 μg/ml human recombinant TNF-peroxidase conjugate were added into the wells of assays for soluble p55 and p75 TNF receptors, respectively. A test buffer containing 5% heat-inactivated FCS in 0.1 M sodium phosphate, 0.1% w/v phenol, 0.1% Tween 20, 0.02% v/v Kathon, pH 7.25 and pH 6.0, was used for dilution of the samples, TNF-peroxidase conjugates, and soluble p55 and p75 TNF receptors. Thereafter, the plates were incu-

bated for 24 h at room temperature, washed six times with 0.05% Tween 20, and then 0.2 ml of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (500 nM TMB, 4 mM H_2O_2 in 30 mM potassium citrate, pH 4.1, 0.5% acetone, 4.5% ethanole, 0.015% kathon) was added. After 10 min, 0.1 ml of 1 M sulfuric acid was added to the wells, and absorbance was measured at 450 nm. The concentration of soluble TNF receptors was calculated from the corresponding standard curve. With the measurements scale of 0–5 ng/ml soluble TNF receptors, the detection limit for both types of soluble TNF receptors was about 0.1 ng/ml.

Flow Cytometric Analysis of DNA Content

Flow cytometric analysis of the DNA content of cells was performed with the method described [Nicoletti et al., 1991]. Briefly, 4–6 × 10^5 cells in 100 μl of culture medium were lysed by adding 0.9 ml of a mixture of 0.1% Triton X-100 and 0.1% Na-citrate. This was followed by staining the nuclei with 30 μg/ml propidium iodide. Samples were analyzed by Ortho Cytoron Absolute flow cytometer. The percentage of cells in S phase were analyzed by means of DNA cell-cycle analysis software (Ortho Cell Program, simple methods).

Antiproliferative Assays

The antiproliferative assay was done using the modified tetrazolium salt (MTT) assay as previously described [Hansen et al., 1989]. Briefly, 2 × 10^4 U-937 cells were incubated in the presence or absence of a serial dilution of TNF in a final volume of 0.2 ml for 48 h at 37°C. Thereafter, 25 μl of MTT solution (5 mg/ml in PBS) was added to each well. After 2 h incubation at 37°C, 50 μl of the extraction buffer (20% sodium dodecyl sulphate, 50% dimethyl formamide) was added. After an overnight incubation at 37°C, the optical densities at 570 nm were measured using a 96-well multiscanner autoreader (Anthos 2001, Anthos Labtec Instruments GmbH, Salzburg, Austria), with the extraction buffer as a blank. Percent relative cell viability was calculated as optical density in the presence of TNF divided by optical density in the absence of the cytokine multiplied by 100. The dose of TNF inducing 50% cell viability (LD_{50}) was calculated from the dose-response curve of TNF by linear regression.

Determination of [^{3}H] Leucine Incorporation Into Proteins

U-937 cells were treated in culture flasks with cell cycle inhibitors at 37°C for 24 h or 48 h at cell densities of 0.2 × 10^6 and 0.4 × 10^6 cells/ml, respectively. During the last 6 h of incubation, 100 μl of cell cultures were seeded into 96-well plates and cells were pulsed with 1 μCi of tritiated leucine (specific activity, 152 Ci/mmol), washed with the medium, and then solubilized with 0.2% sodium dodecyl sulfate. To determine the amount of leucine incorporated into the protein, trichloroacetic acid (TCA) precipitation was performed with 10% TCA at 4°C for 30 min. The TCA-insoluble fraction was harvested on a glass fiber filter (Skatron MCH1, Lierbyen, Norway), washed, and counted on a Beckman LS 6000SE scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

[^{3}H] Thymidine Incorporation Assay

U-937 cells were treated in culture flasks with cell cycle inhibitors at 37°C for 24 h or 48 h at densities of 0.2 × 10^6 and 0.4 × 10^6 cells/ml, respectively. During the last 8 h of incubation, 100 μl of cell cultures were seeded into 96-well plates and cells were pulsed with 0.5 μCi of tritiated thymidine (specific activity: 25.9 Ci/mmol). Thereafter, the cells were harvested and lysed with distilled water. Radioactivity bound to the filter was measured in a liquid scintillation counter (Beckman LS 6000SE).

RESULTS

Effect of Cell Cycle Inhibitors on the Cell Cycle Distribution of Cells

To study the effect of cell cycle on the expression of TNF receptors in U-937 cells, we first examined the cell cycle distribution by using various inhibitors. The distribution was characterized by measuring the DNA content of the cells. Optimal concentrations of the inhibitors and incubation times were selected (data not shown) to avoid toxicity and to obtain maximal effect on the DNA content of cells, and maximal inhibition of cell growth. A 24 h incubation period was found to be optimal for almost all the drugs investigated except for thymidine, which needed a 48 h incubation to exert its growth inhibitory effect on U-937 cells (Table I).

As indicated in Figure 1, a 24 h incubation with 2 mM hydroxyurea, an inhibitor of ribonucleoside diphosphate reductase [Pfeiffer and

TABLE I. Effect of Cell Cycle Inhibitors on the Distribution of U-937 Cells*

Inhibitor	Cell density ($\times 10^6$ cells/ml)	% Cell viability	% Cells		
			G_1	S	G_2 + M
Treatment for 24 h					
None	1.22	98.1	49.1	36.0	13.0
Hydroxyurea	0.78	98.8	70.5[a]	24.4	4.6
Etoposide	0.47	97.2	20.7	55.6	21.7
bisBenzimide	0.55	95.0	5.4	33.2	56.0
Demecolcine	0.43	94.4	6.0	19.3	71.2
Nocodazole	0.45	87.8	19.6	10.6	69.8
Vinblastine	0.39	75.5	18.1	12.7	69.2
Treatment for 48 h					
None	0.97	99.3	56.0	10.0	34.0
Thymidine	0.38	99.2	8.1	83.9	7.7

*U-937 cells were treated at 0.4×10^6 cells/ml with 2 mM hydroxyurea, 1 μM etoposide, 1.25 μg/ml bisbenzimide, 0.2 μg/ml demecolcine, 50 ng/ml nocodazole, or 10 ng/ml vinblastine for 24 h at 37°C, or at 0.2×10^6 cells/ml with 1 mM thymidine for 48 h. After two washings, cell density and cell viability were determined by trypan blue dye exclusion. Determination of DNA content of cells was performed by propidium iodide staining as described in Materials and Methods.
[a]Percentage of cells in G_1/S interphase.

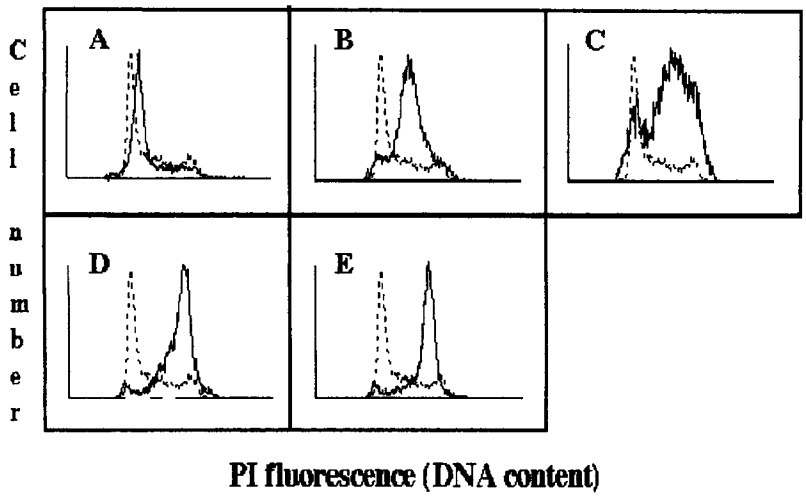

Fig. 1. Effect of cell cycle inhibitors on the cell cycle distribution of U-937 cells. U-937 cells were incubated at 0.4×10^6 cells/ml and treated with 2 mM hydroxyurea (**A**), 1 μM etoposide (**C**), 1.25 μg/ml bisbenzimide (**D**), or 0.2 μg/ml demecolcine (**E**) for 24 h, and incubated at 0.2×10^6 cells/ml and treated with 1 mM thymidine (**B**) for 48 h at 37°C. Determination of the DNA content of untreated (dotted line) and treated (continuous line) cells was performed by propidium iodide staining as described in Materials and Methods. The cell cycle distribution of 24 h and 48 h control cells did not differ from each other. The distribution of 24 h control cells is represented on the figure.

Tolmach, 1967], resulted in an accumulation of 70% of the U-937 cells at the G_1/S boundary (Table I). Etoposide, an inhibitor of eukaryotic DNA topoisomerase II [Constantinou et al., 1992], increased number of cells in S and G_2/M phase to approximately a 56% and 22% from 36% and 13% in the control, respectively, at a concentration of 1 μM. It also decreased the cells in G_1 phase from 49% in the control down to 21%. Bisbenzimide (1.25 μg/ml), another inhibitor of DNA topoisomerase II [Tobey et al., 1990], induced an increase to 56% cells in G_2/M while depleting the cells in G_1 phase to 5% over the control. Demecolcine (0.2 μg/ml), a potent inhibitor of microtubule spindle formation and mitosis [Kung et al., 1990], resulted in the accumulation of 71.2% of the cells in G_2/M phase. Similar results were obtained with 50 ng/ml nocodazole and 10 ng/ml vinblastine which are also known to inhibit microtubule formation and arrest the cell cycle in M phase [Kung et al., 1990] (Table I). An excess of thymidine, which inhibits cell growth [Ashihara and Baserga, 1979], arrested 83.9% of the cells in S phase at the concentra-

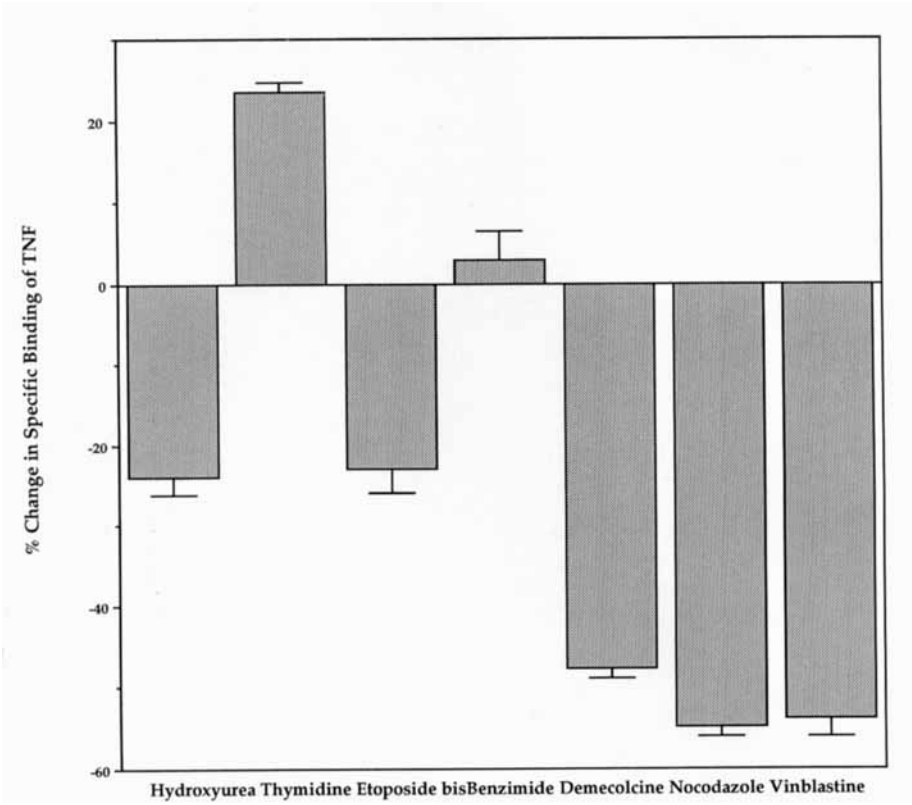

Fig. 2. Effect of cell cycle inhibitors on specific binding of TNF to U-937 cells. U-937 cells were treated at 0.4×10^6 cells/ml with 2 mM hydroxyurea, 1 μM etoposide, 1.25 μg/ml bisbenzimide, 0.2 μg/ml demecolcine, 50 ng/ml nocodazole, or 10 ng/ml vinblastine for 24 h, and at 0.2×10^6 cells/ml with 1 mM thymidine for 48 h at 37°C as described in legend to Figure 1. The cells were then washed three times, and specific TNF binding to 1×10^6 cells/well was determined in the absence or presence of a 100-fold excess of unlabeled ligand as described in Materials and Methods. All measurements were carried out in triplicate. Each bar represents the % change of specific binding and standard error of three determinations.

tion of 1 mM during a 48 h incubation period. Thus the cell cycle inhibitors used above at growth inhibitory concentrations induced a change of the distribution of randomly growing U-937 cells from late G_1/early S (hydroxyurea) through middle S (thymidine) and late S (etoposide) towards late S/G_2 (bisbenzimide) and M (demecolcine, nocodazole, vinblastine).

Effect of Cell Cycle on the Specific Binding of TNF

In order to study cell cycle-dependent expression of TNF receptors, we investigated the specific binding of ^{125}I-labeled TNF to U-937 cells treated with inhibitors for 24 h (hydroxyurea, etoposide, bisbenzimide, demecolcine, nocodazole, vinblastine) or 48 h (thymidine). Untreated cells served as controls. As indicated in Figure 2, hydroxyurea (2 mM) and etoposide (1 μM) induced about a 20% decrease in the specific binding of TNF to U-937 cells, bisbenzimide (1.25 μg/ml) had no effect on TNF binding, and thymidine induced about a 20% increase in the specific binding of TNF to U-937 cells. All the three inhibitors of microtubule spindle formations resulted in a marked (about 50%) reduction of specific binding of TNF. A close inverse correlation was found between the dose-response curve of TNF binding and the number of cells in G_2/M in vinblastine- and nocodazole-treated cell cultures (data not shown).

To determine whether the changes in TNF binding were due to changes in the number of TNF receptors or to changes in receptor affinity, we performed Scatchard analysis on U-937 cells treated with various cell cycle inhibitors (Fig. 3, Table II). We found an 1.8-fold increase in the number of TNF receptors concomitantly with a 1.4-fold increase in the K_d value in cells treated with thymidine, and a 17% decrease in the number of TNF receptors with about a 2-fold increase in the K_d value in cells treated with etoposide. Demecolcine induced a 1.9-fold reduction in the number of TNF receptors without any change in the K_d. Bisbenzimide did not have significant effect on the number and affinity of the TNF receptors in U-937 cells.

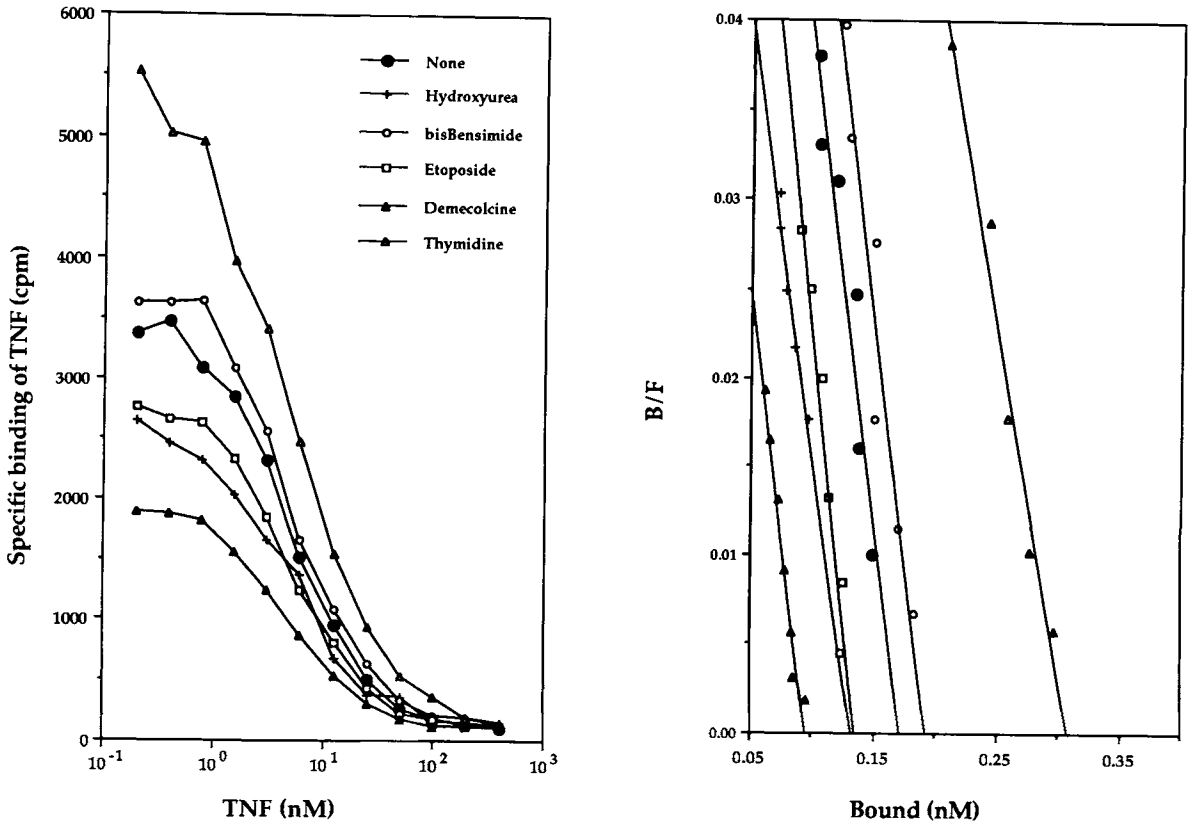

Fig. 3. Effect of cell cycle inhibitors on the number and affinity of TNF receptors on U-937 cells. Cells were treated with 2 mM hydroxyurea, 1 μM etoposide, 1.25 μg/ml bisbenzimide, or 0.2 μg/ml demecolcine for 24 h, or with 1 mM thymidine for 48 h at 37°C as described in the legend to Figure 1. The cells were then washed three times and Scatchard analysis was performed by adding increasing amounts of unlabeled TNF to 1×10^6 cells in the presence of ^{125}I-labeled ligand (1×10^5 cpm) as described in Materials and Methods. All measurements were carried out in triplicate. Each point represents the average of three determinations. Competitive TNF receptor binding analysis (**left panel**) and Scatchard analysis (**right panel**).

We also investigated the effect of cell cycle inhibitors on the expression of TNF receptors on various other cell lines. Hydroxyurea (2 mM) did not result in significant changes in the specific binding of TNF to HeLa, K562, and Jurkat cells (Table III), even if it was growth inhibitory for these cells at the concentration used. Thymidine was growth inhibitory at 1 mM concentration only for K562 cells, and it resulted in an increase in TNF binding to K562 cells. Etoposide (1 μM) inhibited growth of all the three cell lines and induced an increase in specific binding of TNF. Bisbenzimide did not produce significant changes in TNF binding even though it was growth inhibitory for Jurkat and HeLa cells. All three inhibitors of microtubule spindle apparatus induced a decrease in the specific binding of TNF, but only in the cell lines whose growth they inhibited (Table III). These results thus demonstrate that TNF receptors were expressed throughout the cell cycle, but an increase in middle S phase and a characteristic, marked

TABLE II. Effect of Cell Cycle Inhibitors on the Number and Affinity of TNF Receptors*

Inhibitor	Density	Viability	Receptors/ cell	Kd (nM)
None (24 h)	1.06	99.1	10,257	1.81
None (48 h)	1.06	98.1	10,878	1.96
Hydroxyurea (G_1/S)	0.60	99.5	8,551	3.67
Thymidine (S)	0.36	98.2	18,487	2.54
Etoposide (S/G_2)	0.50	96.2	7,855	1.40
bisBenzimide (late S/G_2)	0.48	99.3	11,524	1.81
Demecolcine (G_2/M)	0.47	97.3	5,521	1.59

*U-937 cells were treated with cell cycle inhibitors as described in the legend to Figure 1. Scatchard analysis was performed as described in legend to Figure 3. All measurements were carried out in triplicate.

decrease in M phase was observed in the TNF receptor number.

Effect of Cell Cycle Inhibitors on Cell Size, Cell Growth, DNA and Protein Synthesis

One of the possible explanations of the differences in the specific binding of TNF during the cell cycle is that cell cycle inhibitors may affect cell size and protein synthesis. Therefore, the effect of drugs on protein synthesis and cell size was investigated in parallel with DNA synthesis and changes in the cell number. Our results indicate (Table IV) that none of the drugs affected the size of cells. However, protein synthesis was affected by some of the drugs as indicated in Figure 4. Thymidine (1 mM), bisbenzimide (1.25 μg/ml), nocodazole (50 ng/ml), hydroxyurea (2 mM), and demecolcine (0.2 μg/ml) induced about a 178%, 218%, 125%, 42%, and 43% increase in the protein synthesis, respectively. Etoposide (1 μM) and vinblastine (10 ng/ml) did not affect protein synthesis. Cell numbers did not increase significantly after addition of the drugs as compared to the initial input of the cells (Fig. 4). A 40–98% decrease was observed in DNA synthesis by all drugs except hydroxyurea and bisbenzimide, which induced a 50% and 90% increase in DNA synthesis, respectively. These data show that in the case of thymidine, the increase in the TNF receptor number can be due to an increase in the overall protein synthesis, but in the case of the other drugs metabolic perturbation of the cells by the drugs cannot be responsible for changes in the TNF receptor number.

TABLE IV. Effect of Cell Cycle Inhibitors on Cell Size of U-937 Cells*

Treatment	Cell density ($\times 10^6$ cells/ml)	Cell size (mode channel number)
For 24 h		
None	0.61	126.2 ± 20.7
Hydroxyurea	0.38	133.5 ± 20.1
Etoposide	0.37	136.2 ± 20.7
bisBenzimide	0.43	139.6 ± 20.8
Demecolcine	0.36	132.3 ± 24.7
Nocodazole	0.32	135.1 ± 24.1
Vinblastine	0.37	138.5 ± 20.1
For 48 h		
None	0.52	128.7 ± 20.5
Thymidine	0.19	136.2 ± 19.9

*U-937 cells were treated with cell cycle inhibitors as described in legend to Table I. Viability of cells exceeded 90% except for cells treated with hydroxyurea, etoposide, and thymidine in which it was 87%, 85%, and 82%, respectively. Determination of cell size was performed after formalin fixation of the cells by cytofluorograph gating for the living populations of cells. Cell size is characterized by the mean channel number (±SD) obtained plotting the distribution of cells in forward scatter.

Effect of Cell Cycle on the Expression of p60 and p80 Forms of the TNF Receptors

Because U-937 cells are known to [Higuchi and Aggarwal, 1992b] express both p60 and p80

TABLE III. Effect of Cell Cycle Inhibitors on Specific Binding of TNF to Various Cell Lines*

Treatment	HeLa		K562		Jurkat	
	SB[a]	Density[b]	SB[a]	Density[c]	SB[a]	Density[c]
None, 24 h	638 ± 73	8.55	2,286 ± 16	0.69	205 ± 12	0.65
None, 48 h	600 ± 27	10.32	2,674 ± 83	0.49	296 ± 21	0.59
Hydroxy-urea	669 ± 23	4.35	2,616 ± 69	0.58	198 ± 20	0.34
Thymidine	634 ± 26	11.26	3,350 ± 100	0.42	237 ± 26	0.79
Etoposide	1,128 ± 115	5.04	3,362 ± 22	0.47	312 ± 32	0.38
bisBenzimide	534 ± 21	6.90	2,616 ± 69	0.60	163 ± 30	0.25
Demecolcine	299 ± 7	4.08	2,257 ± 58	0.57	113 ± 24	0.42
Nocodazole	250 ± 35	4.12	1,797 ± 50	0.48	163 ± 42	0.38
Vinblastine	619 ± 72	7.40	1,209 ± 107	0.47	128 ± 26	0.46

*HeLa cells were treated at 3.5×10^6/30 ml/culture flask; K562 and Jurkat cells at 0.5×10^6 cells/ml density for 24 h with 2 mM hydroxyurea, 1 μM etoposide, 1.25 μg/ml bisbenzimide, 0.2 μg/ml demecolcine, 50 ng/ml nocodazole, 10 ng/ml vinblastine, and at 2×10^6/30 ml/culture flask and 0.3×10^6 cells/ml density for 48 h with 1 mM thymidine as described in legend to Figure 1. The cells were trypsinized (HeLa cells) and/or washed, and TNF binding assay was performed by adding 0.2×10^6 cpm of ^{125}I-TNF to 1×10^6 cells in the presence or absence of a 100-fold excess of unlabeled ligand as described in Materials and Methods.

[a]Specific binding (SB) was calculated by subtraction of nonspecific binding from the total binding. All measurements were carried out in triplicate. Each point in the table represents the average ± standard error of three determinations.

[b]$\times 10^6$ cells/culture flask.

[c]$\times 10^6$ cells/ml.

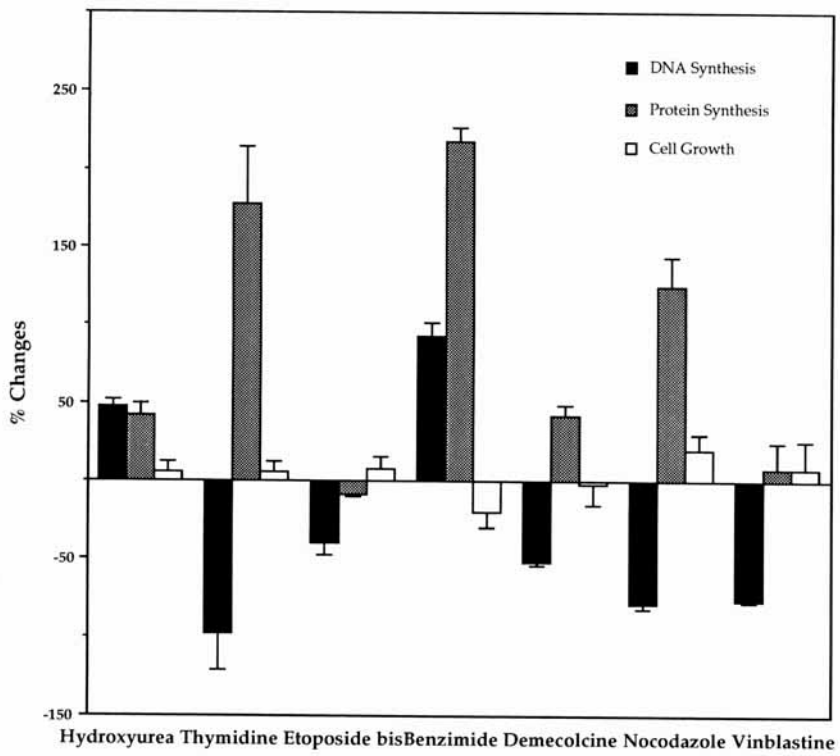

Fig. 4. Effect of cell cycle inhibitors on cell growth, DNA, and protein synthesis of U-937 cells. Cells were treated at 0.4 or 0.2 × 10^6 cells/ml with cell cycle inhibitors for 24 or 48 h at 37°C as described in Figure 1. For the last 6 h [^{3}H] leucine (1 μCi) or [^{3}H] thymidine (0.5 μCi) was added to 100 μl of the cell cultures, concomitantly with determination of cell number. Results of DNA (black columns) and protein synthesis (hatched columns) are expressed as percent of increase or decrease of incorporation (cpm)/1 × 10^5 cells as compared to that of untreated cultures (100%). In the case of cell growth, changes of cell numbers in treated cultures (empty columns) was compared to the initial cell number plated into the wells (100%). Each value represents the average and standard deviation of six determinations.

forms of the TNF receptors, we investigated the effect of cell cycle inhibitors on both kinds of receptors. By using ligand competing monoclonal antibodies, specific to p60 and p80 (htr-9 and utr-1, respectively), to inhibit specific binding of TNF to U-937 cells, contribution of the two kinds of receptors in the specific binding was calculated. As indicated in Figure 5, in untreated control cells 27% and 70% of the TNF receptors were p60 and p80, respectively. No significant changes in the proportion of the two receptors were observed in cells treated with cell cycle inhibitors. These data suggest whatever mechanism is responsible for induction of changes in the expression of TNF receptors, it does not differentially affect the two forms of the TNF receptor.

Effect of Cell Cycle on Shedding of TNF Receptors

Because inhibitors of microtubule spindle formation induced a down-regulation of the cell surface expression of the TNF receptors in U-937 cells, we investigated the possibility of shedding of the receptors into the culture medium. The culture medium of cells treated with cell cycle inhibitors was tested for the appearance of soluble forms of both TNF receptors using ELIBAs specific to the p60 and p80 receptors. As indicated in Figure 6, about a twofold increase was found in the soluble form of the p80 receptor in cell culture supernatants treated with hydroxyurea, etoposide, and bisbenzimide as compared to untreated cells. Demecolcine induced a sixfold, nocodazole and vinblastine about a threefold, and thymidine a 2.5-fold increase in the amount of soluble p80 receptors in the culture medium. Appearance of the soluble p60 receptor was detectable only in the culture supernatants of cells treated with microtubule spindle formation. These results show that M phase-arrested cells shed both TNF receptors in association with a decrease in the cell surface expression of TNF receptors. Low amounts of soluble receptors (mainly the p80 form) are released constitutively throughout the cell cycle.

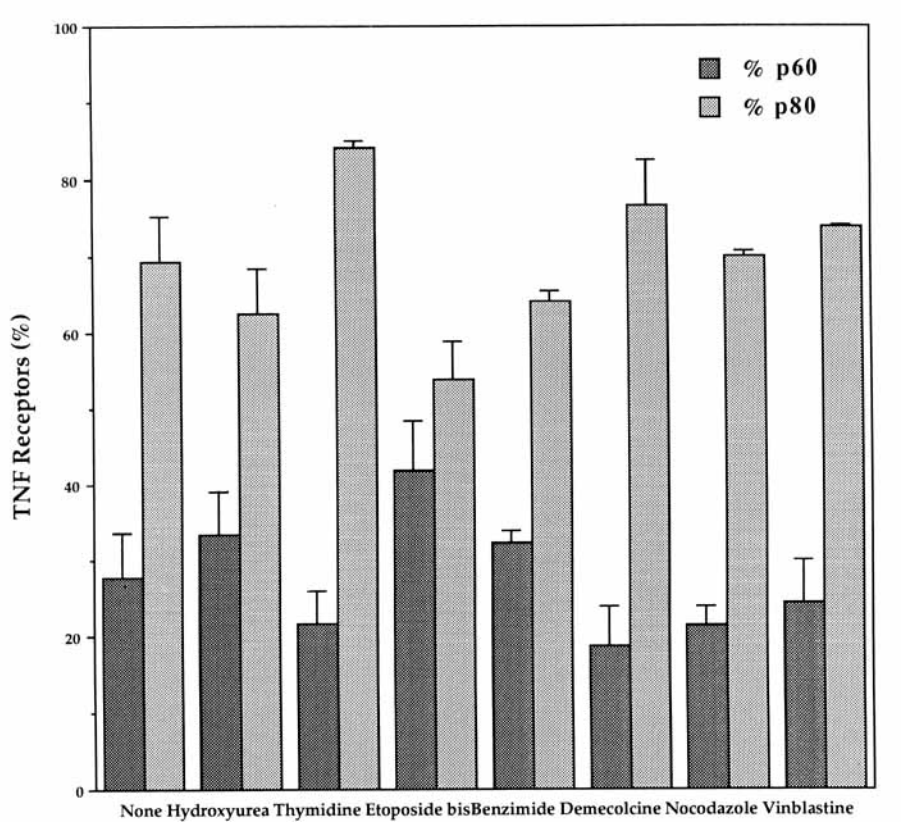

Fig. 5. Effect of cell cycle inhibitors on the expression of p60 and p80 forms of TNF receptors on U-937 cells. Cells were treated with cell cycle inhibitors as described in legend to Figure 1. A standard TNF binding assay was performed as described in Materials and Methods in the presence or absence of 2 μg/ml purified monoclonal anti-p60 (htr-9), or anti-p80 (utr-1). Nonspecific binding was measured in the presence of 100 nM unlabeled TNF. Percent of p60 (black columns) and p80 receptors (hatched columns) were calculated by subtracting the specific binding obtained in the presence of htr-9 or utr-1 from specific binding obtained in the absence of the antibodies (set to be 100%). Each value represents the average of three determinations.

Effect of Cell Cycle on the Sensitivity of U-937 Cells to TNF Cytotoxicity

In order to investigate whether changes in the expression of receptors induced by cell cycle inhibitors affects the biological response to TNF, we treated U-937 cells with the cell cycle inhibitors for 24 h or 48 h as described above, and then determined the sensitivity of the cells to TNF-mediated cytotoxicity in a 48 h incubation period in the presence of a serial dilution of the cytokine. As indicated in Figure 7, hydroxyurea, thymidine, and etoposide induced a 87%, 54%, and 56% decrease in LD_{50} of TNF, i.e., an increase in sensitivity of U-937 cells to TNF, respectively. In contrast, bisbenzimide induced a 470%, and demecolcine a 560% increase in the LD_{50}. Thus the sensitivity of U-937 cells to TNF-mediated cytotoxicity correlated with the expression of cell surface TNF receptors in M phase arrested cells but not in others.

DISCUSSION

In the present report, we demonstrate that cell cycle-specific inhibitors can modulate the expression of TNF receptors and sensitivity of cells to TNF. Hydroxyurea, an inhibitor of ribonucleoside diphosphate reductase, frequently used to synchronize cells at the G_1/S interphase [Pfeiffer and Tolmach, 1967; Ashihara and Baserga, 1979; Mironescu and Ellem, 1976], resulted in an accumulation of cells in G_1/S boundary without any significant effect on the expression of cell surface and soluble TNF receptors. Similarly, etoposide, an inhibitor of eukaryotic DNA topoisomerase II [Constantinou et al., 1992], had no effect on TNF receptors. Bisbensimide, another inhibitor of DNA topoisomerase II with G_2 phase specificity [Constantinou et al., 1992; Hirschberg et al., 1980], enriched cells in G_2/M but had minimal affects on the expression of cell surface and soluble TNF receptors. Thy-

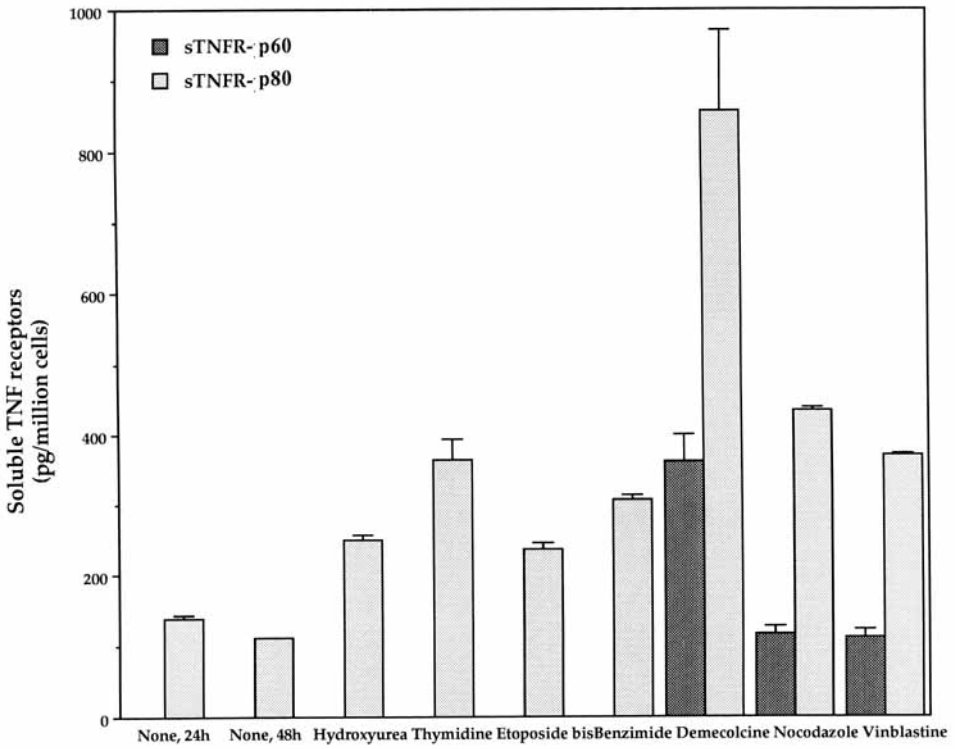

Fig. 6. Effect of cell cycle inhibitors on the production of soluble forms of p60 and p80 forms of TNF receptors. U-937 cells were treated with cell cycle inhibitors for 24 or 48 h at 37°C as described in legend to Figure 1. Afterwards, cell culture supernatants were collected and assayed for soluble forms of p60 and p80 of TNF receptors by ELIBA as described in Materials and Methods. The concentrations of p60 (black column) and p80 (hatched column) per 1×10^6 cells are expressed. Each value represents the average and standard error of three independent experiments.

midine [Ashihara and Baserga, 1979] induced accumulation of cells in S phase that coincided with an increase in the expression of cell surface and soluble TNF receptors. However, the involvement of the increase in overall protein synthesis induced by thymidine cannot be excluded. Demecolcine, vinblastine, and nocodazole, potent inhibitors of microtubule spindle assembly and mitosis, used often for cell synchronization procedures for M phase arrest [Pardee and Keyomarski, 1992; Kung et al., 1990], resulted in the accumulation of cells in G_2/M phase concomitantly with a 50% reduction in the specific binding of TNF. We also found that treatment of cells with hydroxyurea (2 mM for 24 h) to block the cells in G1 phase, followed by removal of the drug, lead to the decrease of TNF receptors as cells approached G2M phase.

Our data show that TNF receptors are expressed throughout the cell cycle. The correlation between the TNF binding and the numbers of cells accumulated in G_2/M as a function of the dose of microtubule-disassembling agents suggests that the reduction in the TNF binding and M phase block are closely related to each other. Our data support the recent finding that vinblastine induced about a 50% decrease in the specific binding of TNF to the human osteogenic sarcoma cell line Saos-2 [Galeotti et al., 1993]. However, unlike our results, in Saos-2 cells maximal inhibition was achieved after a 2 h incubation, and the effect was reversed almost completely after 10 h [Galeotti et al., 1993]. Also, the optimal dose of vinblastine used for treatment of Saos-2 cells (10 μM) was 1,000-fold higher than that of ours (11 nM). It is possible that total disassembly of microtubules was achieved by shorter incubation with a much higher dose of the drug.

Our results also support the observations of Ding et al. [1990], who showed that exposure of human endothelial and myosarcoma cells to micromolar concentrations of various microtubule-depolymerizing agents, including colchicine, nocodazole, podophyllotoxin, vincristine, and vinblastin, resulted in loss of TNF binding sites by 40–60% in 1 h and by about 75% in 2–4 h. In our studies we found that the effects of hydroxy-

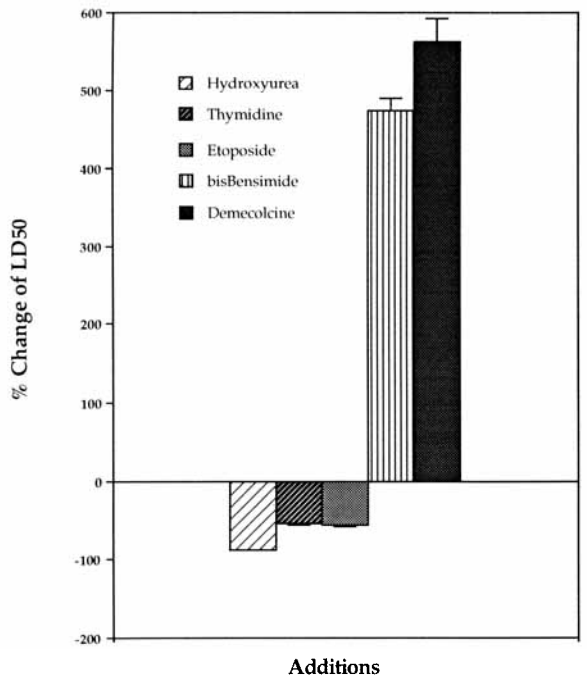

Fig. 7. Effect of cell cycle inhibitors on the biological response of U-937 cells to TNF. U-937 cells were treated with cell cycle inhibitors for 24 or 48 h at 37°C as described in the legend to Figure 1. Afterwards, the cells were washed three times, and 1×10^4 treated and untreated U-937 cells were plated into wells of 96-well microplates. The cells were then incubated in the presence or absence of a serial dilution of TNF (10,000–0.001 U/ml) for 48 h at 37°C in 0.2 ml final volumes. Thereafter, the percentage of viable cells was determined by MTT assay as described in Materials and Methods. The LD_{50} of TNF was calculated in treated and control cultures as described in Materials and Methods. The LD_{50} was expressed as percent of that of the control cells (100%). All determinations were made in triplicate.

urea, thymidine, bisbensimide, and nocodazol on the TNF receptors were reversible, whereas that of demecolcine and vinblastin were irreversible. These results are also consistent with the studies of Ding et al. [1990], who showed that the effects of nocodazole are reversible, whereas that of colchicine were irreversible.

Our data show that sensitivity of U-937 cells increased in G_1 and S phase, and decreased in the G_2/M phase of the cell cycle. This is correlated with the changes in the expression of TNF receptors only after treatment with inhibitors of mitotic spindle formation, suggesting that cells in M phase are most resistant to TNF cytotoxicity. Watanabe et al. [1987] showed that in synchronized mouse fibrosarcoma L-M cells, TNF binding was the highest in M phase, which supported their and others' finding that cells in M phase were most sensitive to the cytotoxic effect of TNF [Darzynkiewicz et al., 1984; Watanabe et al., 1987]. Another report showed that resistance to TNF cytotoxicity of mouse L929 cells is associated with G_1 phase [Belizario and Dinarello et al., 1991]. Whether the differences in the results from different laboratories are related to cell line used or to the procedure employed for analysis is not clear.

Our hypothesis is also supported by the recent finding that the human histiocytic and monocytic leukemia derived U-937, HL-60, and THP-1 cell lines are most sensitive to TNF cytotoxicity and monocyte-mediated cytotoxicity in G_1, while most resistant in G_2/M phases of the cell cycle [van de Loosdrecht et al., 1993]. Some other cell surface molecules, like β_2-integrins (CD11a, CD11b, CD18), have also shown their highest expression in the G_1 and lowest in the G_2/M phases [van de Loosdrecht et al., 1993]. Based upon their and our finding we assume that distinct mechanisms confer cell cycle-dependent changes in mouse L929 and human U-937 sensitivity to TNF cytotoxicity. This is supported by the data on cell line-specific differences in the control of cell cycle progression [Kung et al., 1990]. Human HeLa S3 cells remain arrested in mitosis after treatment with mitotic spindle-disrupting agents with an elevated level of cyclin B and $p34^{cdc2}$ kinase activity, while rodents cell lines are only transiently inhibited when spindle assembly is disrupted [Kung et al., 1990]. Differences in the regulation of protein synthesis during the cell cycle have also been described [Kung et al., 1993].

The mechanism of cell cycle-dependent changes in the sensitivity and expression of both cell surface and soluble forms of TNF receptors is not understood. Our finding that soluble forms of both the p60 and p80 TNF receptors appear in the culture supernatant after treatment with microtubule-disassembling drugs supports the hypothesis that cell surface TNF receptor expression is down-regulated by mitosis blockers via shedding of the receptors. It is unlikely, however, that modulation of sensitivity of cells to TNF by various drugs is solely due to shedding of TNF receptors. It has been shown that colchicine, besides depolymerizing the microtubule, can also activate protein kinase A, destabilize polyribosomes and affect mRNA stability [for references see Ding et al., 1990]. Some of these mechanisms may be involved in modulation of TNF response. How do various drugs employed in our studies induce receptor shedding is not clear but different signals have been shown to induce release of cell surface TNF receptors,

including TNF itself [Lantz et al., 1990], the protein kinase C activator phorbol ester [Aggarwal and Eessalu, 1987, Aggarwal et al., 1993], the inhibitor of protein phosphatases 1 and 2A okadaic acid [Higuchi and Aggarwal, 1993a], and elastase in azurophil granules of polymorphonuclear granulocytes [Porteu and Nathan, 1990]. The activation of lymphocytes has also been shown to cause TNF receptor shedding [Ware et al., 1991]. It is possible that in our system microtubule-disassembling drugs induce secretion of TNF which in turn is responsible for shedding of TNF receptors. However, when examined, none of these drugs caused the production of TNF (data not shown). This suggests that an intact microtubule network is important for the maintenance of TNF receptors on the cell surface. Cell cycle progress is regulated by a complex mechanism involving cyclic changes in cellular regulatory proteins and kinases [reviewed in Pines, 1992; Pardee, 1989]. These mechanisms may also play an important role in regulating the expression of TNF receptors and their cellular responses.

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