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The Regulation of TNF Receptor mRNA Synthesis, Membrane Expression, and Release by PMA- and LPS-Stimulated Human Monocytic THP-1 Cells *in Vitro*

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The regulation of the 55-kDa TNF receptor (TNF-R) mRNA synthesis, membrane expression, and TNF binding factor (BF) release was examined in resting and activated human monocytic THP-1 and human promyelocytic leukemia HL-60 cells *in vitro*. Cells were activated with phorbol myristate acetate (PMA) and bacterial lipopolysaccharide (LPS). TNF α cytolytic activity in the supernatant of THP-1 cells stimulated by PMA began to appear at 4 hr, reached a peak at 8 hr, and declined by 12 hr. For THP-1 cells stimulated with LPS, the peak of TNF α activity appeared at 4 hr and then declined. TNF α -binding sites on the cell membrane were down-regulated within 1 hr after PMA and LPS treatment and then reappeared 12 hr later. Fifty-five-kilodalton TNF-R mRNA expression during this time period did not correlate with the level of membrane TNF-binding site expression. Additional studies indicated the presence of a 30-kDa TNF-BF in the supernatants which appeared after 24 hr. These data suggest that activated THP-1 and HL-60 cells are capable of releasing TNF-BF into the supernatant and this material may be involved in the control of secreted TNF α activities. © 1991 Academic Press, Inc.

INTRODUCTION

Native tumor necrosis factor (TNF α) is a 55,000-MW trimer comprised of 17,350 MW peptides (1). TNF α is produced by activated macrophages, lymphocytes, and other cell types in special situations (2). This cytokine has a spectrum of effects on cells *in vitro* and tissues *in vivo* and has important roles in various phases of cell-mediated immune reaction (3).

The mechanism through which TNF α mediates its bioactivity is initiated by first binding to specific cell membrane receptors (TNF-R) (4). Subsequent to human TNF α binding to its membrane receptor on cells *in vitro*, the complex disappears from the membrane and the receptor can be internalized, degraded, or recycled (5-7). Binding is specific; however, the response which this cytokine elicits is dependent on the type of target cell or tissue to which it has attached. Cells from many different tissue sources express low numbers of TNF-R (3-10,000/cell) with dissociation constants (K_d) ranging from 1.3×10^{-9} M to 7.0×10^{-10} M (8, 9). Most cells express a single class of receptor; however, myeloid cells express both high- and low-affinity receptors (10). A recent report suggests that the high- and low-affinity receptors are distinct and differ in size, glycosylation, and their peptide maps (11). TNF α and lymphotoxin (LT or TNF β)

have been shown to bind to the same receptors on the human histiocytic cell lines U-937 (11). Estimates of the size of the TNF-R, as determined by affinity-labeling studies from several groups, range from 55 to 310 kDa (12, 13). Recently, several groups, including ours, have cloned two distinct human TNF α receptors which were 55 kDa and 68–75 kDa (14–16).

In vitro studies reveal different agents and treatments can rapidly up- and down-regulate the expression of TNF-R on both lymphoid and nonlymphoid cells. Stimulation with bacterial lipopolysaccharide (LPS) (6), phorbol myristate acetate (PMA), IL-1 and TNF α itself rapidly downregulates TNF-R expression on human monocytes, macrophages, and promyelocytic leukemia cell line HL-60 (17). Most investigators have thought that downregulation in these cultures was due to internalization and degradation of TNF α receptor; however, release is an important alternative which was not considered.

Several groups have identified two soluble TNF-R in human urine and we have identified one of these receptors, in the serum of human cancer patients, that is capable of specifically binding to and inactivating human TNF cytolytic activity *in vitro* (18–22). These soluble TNF receptors have molecular weights of 28 to 30 kDa and appear to be derived from the N-terminal extracellular region of the 55- and 75-kDa TNF cell membrane receptors (23). Other groups have identified cell surface receptors in biological fluids that are released and specifically bind to other biological response modifiers such as IL-2, IL-4, and growth factors (24–26). Collectively, these studies have led to the concept that release of soluble receptors may represent a new mechanism to specifically control cytokine activity. This concept is supported by the recent report of Gatanaga *et al.* (23) that human soluble TNF-R can block the anti-tumor activity of recombinant human TNF α when injected into Meth-A tumor-bearing BALB/c mice. It is not yet clear what the cell and tissue source of these BF may be; however, a recent report by Porteu and Nathan (27) indicates that activated human neutrophils can release TNF-R derived from both 55 and 68–75 kDa receptors. The present study was initiated to examine some of the stages in the synthesis of TNF-R message, membrane expression, and release of TNF-BF in the LPS- and PMA-stimulated macrophage-like cell lines THP-1 and HL-60.

MATERIALS AND METHODS

Reagents

Recombinant TNF α was supplied by Genentech Corp. (South San Francisco, CA). Lipopolysaccharide (LPS) was purchased from Difco (Detroit, MI). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Media and Cell Lines

RPMI 1640 media and heat-inactivated fetal bovine serum (FBS) were purchased from GIBCO Laboratories Life Technologies, Inc. (Grand Island, NY). The human monocytic cell line THP-1 and HL-60 were obtained from ATCC and maintained over many passages in RPMI 1640 medium, supplemented with 10% (v/v) FCS. Cells were passed biweekly and incubated at 37°C in 5% CO₂.

L929 Cytolytic Assay

Cytolytic activity of TNF α in culture supernatants was assayed with L929 mouse fibroblast by the *in vitro* method of Gatanaga *et al.* (28). Briefly, L929 cells (8×10^4 cells/well) were established as monolayer in flat-bottom 96-well microplates. They were then incubated with dilutions of standard TNF α (Genentech Corp.), culture supernatant, and actinomycin D (0.2 μ g/well) to a final volume of 200 μ l at 37°C in 5% CO $_2$ for 18 hr. Then the cells were stained with 1% crystal violet for 5 min, washed with water, and solubilized with 100 μ l of acidified methanol (100 mM HCl in methanol). The absorbance at 600 nm was then measured.

TNF α and 55-kDa TNF-Receptor Specific Oligonucleotide Probe

The oligonucleotide probes, 5'-GGGGCAGCCTTGGCCCTTGAAGAGGAC CTGGGAGTAGATGAGGTACAGGCCCTCTGATGG-3' for human TNF α (amino acid Nos. 52–71) (29) and 5'-GCCACACACGGTGTCCCGGTCCACTGTGCAA-GAAGAGATCTCCACCTGACCCATTCCT-3' for human 55 kDa TNF α receptor (amino acid Nos. 67–86) (14) were synthesized by Biosearch 8600 DNA synthesizer. Oligonucleotide probes were phosphorylated by [γ - 32 P]ATP (30) (New England Nuclear, Boston, MA).

RNA Isolation and Northern Blotting Analysis

Total cellular RNA was isolated from THP-1 and HL-60 cells by the guanidinium isothiocyanate/cesium chloride method and quantitated spectrophotometrically as previously described (31). Ten micrograms RNA was electrophoresed in 2.2 M formaldehyde in 1.2% agarose gels, transferred to nitrocellulose filter (Schleicher & Schuell, Keene, NH), and baked at 80°C for 2 hr as described (32). Filters were hybridized with 32 P-labeled TNF α and TNF α receptor-specific synthetic probes. After hybridization, filters were washed at 25°C with $2 \times$ SSC, 0.1% SDS for 30 min then at 25 or 42°C with $0.1 \times$ SSC, 0.1% SDS for 30 min. Filters were then exposed to X-Omat XAR-5 (Kodak, Rochester, NY) X-ray films at -70°C . The amount of the RNA was confirmed by ethidium bromide (0.5 mg/ml) staining.

Iodination of rTNF α and Binding Assay

Two hundred micrograms of rTNF α were equilibrated in PBS(–) by exhaustive dialysis against PBS(–) in 6–8000 MW cutoff Spectra-por dialysis tubing (Spectrum Medical, Inc, Los Angeles, CA). After the dialysis, rTNF α was concentrated using Speed Vac Concentrator (Savant, NY). The final concentration of rTNF α was 2 mg/ml. The rTNF α iodination procedure was performed as described by Bolton *et al.* (33). The rTNF α (100 μ g) in 20 μ l of 0.1 M sodium borate buffer, pH 8.5, was added to the dried iodinated ester and the reaction mixture (Bolton–Hunter Reagent, Monoiodo, ^{125}I , DuPont, Wilmington, DE). Free iodine was removed by gel filtration over a NAP-5 column (Pharmacia, Sweden) with PBS(–) containing 5 mg gelatin. Fractions of 150 μ l were collected and 1- μ l aliquots were assayed by Clinigamma counter (Pharmacia, LKB, Uppsala, Sweden). Peak fractions were pooled, and the specific activity was determined to be 5400 cpm/ng. The labeled material was stored at 4°C and used within 1 month.

Binding Studies and Scatchard Analyses

One million cells were incubated with binding buffer (RPMI 1640 + 10% FBS) in 24-well plates and then incubated with 0.5 ng–30 ng of ^{125}I -labeled $\text{TNF}\alpha$ in the presence or absence of a 100-fold excess of unlabeled $\text{rTNF}\alpha$. After a 3- to 4-hr incubation at 4°C , binding buffer was removed and the cells were washed 2–4 times with ice-cold PBS(–) (34). Cells were solubilized in 1 ml of 0.3 *N* NaOH and radioactivity was determined by gamma counter. When the effect of PMA and LPS on $\text{rTNF}\alpha$ binding was examined, unless indicated otherwise, cells were incubated with LPS and PMA at 37°C before the binding assay.

Biosynthetic Labeling of TNF-R.

There are cysteine-rich domains in the $\text{TNF}\alpha$ receptor (12). Therefore, we decided to use [^{35}S]cysteine to label cells. THP-1 cells (3×10^7 cells) were washed by PBS(–), suspended in 10 ml of cysteine-free RPMI 1640 (Select-Amine Kit, GIBCO) containing 10% dialyzed FBS and [^{35}S]cysteine 50 $\mu\text{Ci/ml}$ (sp act 1113.6 Ci/mmol) (DuPont, Wilmington, DE), and then incubated with PMA and LPS for 24 hr. After the 24-hr incubation, supernatants were collected and used in cross-linking assays.

Cross-linking Assay

One-milliliter culture supernatants were co-incubated with 1 ng of ^{125}I -labeled $\text{TNF}\alpha$ for 2 hr at 37°C . Similarly, 1-ml culture supernatants from ^{35}S -labeled cells were co-incubated with 1 ng of cold $\text{TNF}\alpha$ for 2 hr at 37°C . BS3-nonreversible linker (34) and DTSSP-thiol reversible linker (35) (Pierce, Rockford, IL) were then added to a final concentration of 5 mM, respectively. After 20 min on ice, the reaction was stopped by adding 20 mM glycine (36). Five microliters of rabbit anti-h $\text{TNF}\alpha$ polyclonal anti-serum was then added and the mixture was incubated for 2 hr at room temperature. Finally, 10 μl of immunoprecipitin (BRL, Gaithersburg, MD) (37) was added and the mixture was incubated overnight at 4°C . The samples were centrifuged and the precipitates were washed in 50 mM Tris, pH 7.5, once containing 0.005% Tween-20 and were resuspended in SDS–PAGE buffer with 100 mM 2-mercaptoethanol (J. T. Baker, NJ).

SDS–PAGE

SDS–PAGE was performed under reducing conditions according to the technique of Laemmli (38). The gels were dried and exposed to X-Omat XAR-5 films (Kodak). The intensities of bands were determined by scanning with a fiber optic scanner Model 800 (Kontes, Vineyard, NJ).

RESULTS

Induction of TNF α Secretion by PMA and LPS Stimulation of THP-1 Cells in Vitro

PMA and LPS are activators of the cell lines THP-1 and HL-60 and can induce $\text{TNF}\alpha$ secretion by these cells *in vitro* (39–41). We first established the dose-response and time-course of activation by PMA and LPS on these cell lines *in vitro*. A dose of 10^{-8} *M* PMA induced the highest amount of $\text{TNF}\alpha$ production by THP-1 cells (Fig. 1A). $\text{TNF}\alpha$ cytolytic activity appeared at 4 hr and plateaued 8 hr after treatment with

FIG. 1. Production of TNF α by THP-1 and HL-60 cells after stimulation with PMA and LPS. Cells were stimulated with PMA and LPS as described under Materials and Methods, and the cytolytic activity of TNF α was assayed in supernatants after 4, 8, and 12 hr of culture. Concentrations of PMA were 10^{-7} M (Δ), 10^{-8} M ($\circ$), 10^{-9} M ($\square$) (A). The inset shows the result of HL-60. Concentrations of LPS were 0.5 μ g/ml (Δ), 5 μ g/ml ($\circ$), 50 μ g/ml ($\square$) (B). Ten milliliters of 10^7 cells in total was used in each assay.

PMA. Stimulation with 5 μ g/ml LPS resulted in TNF α production which peaked at 4 hr and then declined thereafter (Fig. 1B). HL-60 cells produced TNF α after treatment with an optimal dose of PMA (Fig. 1A, inset), but did not produce TNF α when stimulated with the levels of LPS we employed in these studies (data not shown). All the cytolytic activity could be neutralized by polyclonal rabbit anti-human TNF α antibody (data not shown).

The Effect of PMA and LPS Stimulation on the Expression of TNF α Cell Membrane Receptors

The binding of 125 I-labeled TNF α to unstimulated and PMA- and LPS-stimulated THP-1 cells was examined as described under Materials and Methods. Specific binding of 125 I-labeled TNF α was saturable, reaching the maximum at 30 ng of 125 I-labeled TNF α (Fig. 2A). Scatchard analysis of these data (Fig. 2A, inset) indicated that there was a single class of binding sites. There were approximate 1848/cell in THP-1 cells. Preexposure of 10^{-8} M PMA for 1 hr rapidly down-regulated the number of 125 I-labeled TNF α binding sites by 95–99% (Fig. 2B). After 12 hr, the number of TNF α membrane receptors was still only 13% of normal. LPS (5 μ g/ml) induced downregulation of 125 I-labeled TNF α binding sites at 1 hr by 70%, then the number of TNF α -binding sites increased back up to the level of untreated controls by 12 hr (Fig. 2B).

FIG. 2. The effects of PMA and LPS on the expression of TNF α membrane receptors on THP-1 cells *in vitro*. The indicated amounts of ^{125}I -labeled TNF α were added to 10^6 THP-1 cells at 4°C for 3 hr as described under Materials and Methods. Specific binding represents total bound radioactivity minus that bound in the presence of a 100-fold excess of unlabeled TNF α . (A) The results of the 0-hr control. (A, inset) Scatchard analysis of the equilibrium-binding data. B/F: bound/free. (B) The calculated numbers of receptors after treatment with PMA and LPS for various intervals.

The affinities ranged from $1.2 \times 10^{-10} \text{ M}$ to $7.7 \times 10^{-10} \text{ M}$ (0 hr, $5.0 \times 10^{-10} \text{ M}$; TPA 1 hr, $7.2 \times 10^{-10} \text{ M}$; 4 hr, $7.7 \times 10^{-10} \text{ M}$; 12 hr, $1.2 \times 10^{-10} \text{ M}$; LPS 1 hr, $6.8 \times 10^{-10} \text{ M}$; 4 hr, $4.2 \times 10^{-10} \text{ M}$; 12 hr, $1.2 \times 10^{-10} \text{ M}$). Similar results were observed with HL-60 cells as previously reported by Ding *et al.* (6).

Examination of the Level of 55 kDa TNF-R mRNA in THP-1 Cells after PMA and LPS Treatment

As described under Materials and Methods, we next examined whether the levels of mRNA transcript for 55-kDa TNF-R is affected when stimulated with PMA or LPS. We treated THP-1 cells with PMA or LPS for 30 min to 12 hr, then the total RNA was extracted and analyzed for the presence of TNF α and 55-kDa TNF-R mRNA by Northern blotting. As shown in Fig. 3A, the level of 55-kDa TNF-R mRNA of THP-1 cells stimulated with LPS remained relatively constant. When stimulated with PMA, the level of 55-kDa TNF-R mRNA of THP-1 cells showed some fluctuation during the first 12-hr period (Fig. 3B). The pattern of the mRNA expression did not correlate with the change of the TNF α -binding sites expression on cell membrane. The 55-kDa TNF-R mRNA level in HL-60 cells was unaffected by exposure to LPS during the same time period (Fig. 3E). The level of TNF α mRNA of THP-1 cells showed a slight increase after LPS and PMA activation (Figs. 3C and 3D).

FIG. 3. The level of 55 kDa TNF-R and TNF α mRNA expression in THP-1 and HL-60 after stimulation with PMA and LPS for various intervals. THP-1 cells were treated with LPS (5 μ g/ml) or PMA (10^{-8} M), and total RNA was extracted from cells collected at different time points. Ten micrograms of each total RNA was used in Northern blotting. The membranes were probed with 55 kDa TNF-R (A,B) and TNF α (C,D) oligonucleotide probes. (E) Level of TNF-R mRNA from HL-60 cells stimulated with LPS.

Release of TNF-Binding Factor (BF) by LPS and PMA Activated THP-1 Cells in Vitro

We next examined the 24-hr supernatants from resting and stimulated cells for the presence of TNF-BF. In the first study, the 24-hr supernatant was incubated with 125 I-labeled TNF α in the presence of reversible or nonreversible cross-linkers. The complex was immunoprecipitated by polyclonal rabbit anti-human TNF α antibody and then analyzed on reducing SDS-PAGE (Fig. 4A). A 55-kDa band of cross-linked rTNF α trimer was visible in each lane, and an extra band of 85 kDa was detected in lanes treated with PMA, LPS, and the 24-hr THP-1 cell control (Fig. 4A, a-c). The intensity of the 85-kDa bands which were treated with PMA and LPS showed a 129% (b) and 81% (c) increase compared to the control (a) (Fig. 4A, b, c). This 85-kDa extra band disappeared in lanes using reversible linkers. The 85-kDa band appeared to be due to the combination of the 55-kDa rTNF α trimer and a 30-kDa TNF α -binding factor. The soluble TNF α receptor we reported (23) and TNF-BF in general has a molecular weight (28 kDa) very close to this range (19-21). In a second set of experiments, we cultured THP-1 cells with 35 S-labeled cysteine. The supernatants were processed the same as the above except that the rTNF α was not radio-labeled (Fig. 4B). The same 85-kDa bands were observed in activated lanes (Fig. 4B, f, g).

The band intensities increased 90%(f) in the PMA-treated sample and 120%(g) in the LPS-treated sample. These results confirmed the presence of a 30-kDa TNF α binding factor induced by PMA and LPS. These same data had been observed three times in different experiments. Similar results were obtained from HL-60 (data not shown).

DISCUSSION

Recently, a soluble form of the extracellular domain of the 55-kDa TNF membrane receptor has been isolated and identified in cancer patient serum (23) and in urine

FIG. 4. Detection of TNF α binding factor(s) in supernatants of PMA- and LPS-stimulated THP-1 cells *in vitro*. (A) THP-1 cells were treated by PMA and LPS for 24 hr. The supernatants were incubated with 125 I-labeled TNF α and different cross-linkers (nonreversible and reversible) for 2 hr. The proteins were immunoprecipitated with polyclonal rabbit anti-TNF α antibody and analyzed on reducing SDS-PAGE. a, cells without stimulation; b, PMA (10^{-8} M); c, LPS ($5 \mu\text{g/ml}$); d, medium only (without cells). nonreversible linker (+), reversible linker (-). The arrow indicates the 85-kDa complex. (B) THP-1 cells were incubated with PMA, LPS, and medium containing 35 S-labeled cysteine for 24 hr. The supernatants were incubated with TNF α and different cross-linkers (nonreversible and reversible) for 2 hr. The proteins were then immunoprecipitated with the same antibody used above and run on reducing SDS-PAGE. e, Cells without stimulation; f, PMA (10^{-8} M); g, LPS ($5 \mu\text{g/ml}$). (+) and (-) are as described for A.

samples collected from patients with chronic inflammatory diseases (18). The soluble receptor: (A) inhibited the binding of human TNF α to its cellular receptor (18–21), (B) blocked the cytolytic activity of human TNF α and LT on cell lines *in vitro* (22), and (C) inhibited the necrotizing activity of human TNF α on murine Meth-A tumor *in vivo* (23). These data suggested that these soluble TNF-R may have an important role in controlling the action of TNF α and LT activities. However, the cellular origin and the mechanism(s) which regulate the source of this soluble receptor remained unclear.

Both LPS and PMA can induce TNF secretion by the human monocytic cell lines THP-1 and the promyelocytic leukemia cell line HL-60 *in vitro* (38–40). We also found TNF α secretion after the cells were stimulated with optimal levels of PMA and LPS. Northern blotting showed the mRNA for the 55-kDa receptor increased during a 1- to 12-hr period. But, the cytolytic activity of supernatant TNF α began to decline near the end of this time period. One possible explanation for the loss of secreted TNF activity is the appearance of an inhibitory factor in the supernatants. While we could not distinguish between 55 and 68-kDa TNF membrane receptors, we found that the level of 55-kDa TNF-R mRNA transcripts did not correlate with the significant drop in the expression of membrane TNF α -binding sites after PMA and LPS stimulation.

Studies were conducted to determine if TNF-BFs were released into the supernatants by resting cells and cells stimulated with PMA and LPS. These studies revealed that these supernatants contained a 30-kDa material which specifically bound to radio-labeled TNF α . We also demonstrated [35 S]cysteine-labeled 30-kDa material binding to unlabeled TNF α . The molecular weight of this material is close to the MW of soluble TNF α receptors found in urine (18, 19) and in cancer patient's serum (23). This BF may be responsible for the drop in supernatant TNF α activities observed in these cultures. The present study indicated that HL-60 and THP-1 cell lines, which resemble human macrophages, may release TNF-BF as well as TNF.

Previous investigators have shown TNF α and TNF-R complexes are rapidly internalized when formed on the cell surface (7). The downregulation observed for TNF α receptors in our studies might be due to internalization. However, downregulation of TNF α membrane receptors may also be due to the release of TNF α receptor(s). The mechanism of how these receptors are released is not yet apparent; however, there are several possibilities: (A) "shedding" directly from the membrane due to enzymatic cleavage, or (B) "secreted" after they have been endocytosed and cleaved by certain enzymes.

Shedding or release of membrane receptors for cytokines may represent a new mechanism of control of these highly active proteins. Release of IL-2 and IL-6 receptors has been reported by Rubin *et al.* (24), and Novick *et al.* (25), respectively. Weisman *et al.* (42) recently reported a soluble human complement membrane receptor type 1 which is biologically functional *in vivo*. These authors have suggested that this may represent a mechanism(s) to control secreted cytokines. The present study shows that TNF α -secreting human cell lines can release soluble TNF-BF when TNF α secretion was stimulated. Whether this release was caused by PMA and LPS, TNF α itself, or some other mechanism(s) is not yet clear. Further studies must be done in this system to: (A) specifically identify whether these BF are derived from 55- or 68-kDa receptors and (B) investigate the molecular mechanism leading to the release of the soluble TNF-R and TNF-BF. Studies of the mechanism(s) resulting in TNF-R release may help us understand the physiological and pathological roles of soluble TNF-R in immunological and cell-mediated reactions.

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