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Tumor Necrosis Factor–Alpha Gene Expression in Human Whole Blood

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Tumor necrosis factor-alpha (TNF) is recognized as a principal mediator of a variety of pathophysiologic and immunologic events. Lipopolysaccharide (LPS) challenge, either in vitro or in vivo, results in significant TNF production. In this study we present data demonstrating LPS-induced TNF mRNA expression and bioactivity using an in vitro tissue system of whole blood (WB). The kinetics of LPS-induced TNF production by WB was significantly accelerated as compared to isolated cultured peripheral blood monocytes (PBM). At post-LPS challenge, plasma from WB demonstrated a rapid rise in TNF bioactivity, peaking by 4 hr (1,021 units/ml/10^6 cells), plateauing between 4 and 8 hr, and then decreasing over the next 16 hr. In contrast, the highest measured TNF bioactivity from PBM did not occur until the 24-hr time-point (175 units/ml/10^6 cells). Whole blood buffy-coat TNF mRNA was assessed by Northern blot analysis, and demonstrated significant TNF mRNA accumulation at 1 hr and a peak 2 hr post-LPS challenge. By 8 hr TNF mRNA was undetectable. Concomitant administration of LPS with either prostaglandin E_2 (10^{-6}M) or Dexamethasone (10^{-6}M) resulted in significant suppression of LPS-induced TNF production. This data supports WB as a useful in vitro medium for the molecular and cellular analyis of TNF. As specialized connective tissue, WB may provide an important environment to study the pharmacologic manipulation of TNF mRNA and bioactivity.

Key words: monocytes, inflammation, cytokines

INTRODUCTION

Tumor necrosis factor-alpha (TNF) is a mononuclear phagocyte-derived cytokine that may orchestrate a variety of physiological and immunological events [1,2]. At the cellular level, TNF has diverse effects on a variety of cell types and serves as an effective signal for cell-to-cell communication [3–9]. At the systemic level, TNF plays a significant role in promotion of cachexia and mediation of septic shock [10,11]. Human-derived TNF has been studied both in vivo and in vitro post-lipopolysaccharide (LPS) challenge [12,13]. The production of human TNF has been documented in vivo both clinically, septic patients [13], and experimentally, after normal subjects received systemic LPS [14]. In vitro the majority of data regarding the synthesis and regulation of human TNF has been generated using primary cells in culture or tumor lines [12,15]. Although these systems have provided valuable approaches in studying the production and regulation of TNF, they both have inherent limitations. In order to bridge the in vivo and in vitro TNF studies, we have utilized whole blood as a system to assess the production and mRNA expression of human TNF. Since blood is defined as a specialized circulating connective tissue that communicates with other organs/tissues [16], we have used this medium to: 1) examine the production of biologically active TNF and compare this with TNF levels from monolayers of peripheral blood monocytes, 2) assess TNF mRNA expression from the cellular constituents of intact whole blood, and 3) evaluate pharmacologic regulation of TNF bioactivity.

MATERIALS AND METHODS

Recovery of Whole Blood and Isolation of Peripheral Blood Monocytes

Peripheral blood was obtained by venipuncture from healthy human volunteers (n = 9). Five ml of heparinized (50 USP units/ml) whole blood (WB) was aliquoted into 15 ml conical tubes. Peripheral blood monocytes (PBM) from WB diluted 1:1 with normal saline were

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separated by density centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Isolated mononuclear cells were resuspended in sterile RPMI-1640 (Whitaker Biomedical Products, Whitaker, CA), 1 mM glutamine, 25 mM HEPES, 100 units penicillin, 100 μg streptomycin/ml (Hazelton Research Products, Denver, PA) (complete media) and washed three times. Differential cell counts and viability analyses by trypan-blue exclusion were conducted for PBM. Peripheral blood monocytes (10^6 cells/ml) were plated in 10 ml volumes in 100 mm culture plates (Costar, Cambridge, MA) and incubated for 1 hr at 37°C in 95% air and 5% CO_2. Cells were washed twice with complete media for adherence enrichment. Total and differential cell counts for WB were performed. Whole blood in 15 ml conical tubes was stimulated in a dose or kinetic fashion with specific reagents, then placed on a rocker at 37°C in 95% air and 5% CO_2. At specified times, WB was centrifugated at 600*g* for 5 min. Plasma and buffy-coat (cells) were isolated for bioassay and nucleic-acid extraction, respectively. Cell-free supernatant from cultured PBM was removed for TNF assay and the adhered cells extracted for mRNA analysis.

Reagents

Prostaglandin E_2 (PGE_2) was the generous gift of the Upjohn Company. Stock PGE_2 was prepared at a concentration of 10^{-2}M in ethanol. Dexamethasone (Dex; Sigma Chemical Co., St. Louis, MO) stock was prepared at a concentration of 10^{-2}M in dimethyl sulfoxide (DMSO). Serial dilutions of PGE_2 and Dex were prepared in complete media. Lipopolysaccharide (*Escherichia coli* 0111:B4; Sigma Chemical Co.) stock was prepared at a concentration of 200 μg/ml in complete media.

Tumor Necrosis Factor Bioassay

Tumor necrosis factor bioactivity was measured in both the cell-free supernatants and plasma. Bioactivity of TNF was monitored using a semi-automated WEHI 164 subclone 13 fibrosarcoma lytic assay. WEHI cells were suspended 5×10^4 in 100 μl of RPMI-1640, 10% fetal calf serum, 1 mM glutamine, 0.5 μg/ml actinomycin D, and plated in 96-well culture plates. Samples were serially diluted and 100 μl added to each well. Plates were allowed to incubate for 20 hr at 37°C in 95% air and 5% CO_2. This was followed by the addition of 20 μl of MTT tetrazolium (5 mg/ml; Sigma Chemical Co.) for an additional 4 hr. One hundred fifty microliters of supernatant fluid was removed from each well and replaced with 100 μl of isopropanol–0.04 N HCL to dissolve the tetrazolium crystals. Culture plates were then read in a microELISA reader at 540 nm. Units of TNF were defined using an internal standard of human recombinant TNF with a specific activity of 22 units/ng protein (Cetus Corp., Emeryville, CA). To allow comparison of TNF bioactivity between different experiments or between WB and PBM, TNF was further standardized to either the number of adherent monocytes (PBM) or absolute quantity of monocytes derived from the total cell and differential counts from WB. Rabbit anti-human TNF neutralizing antibody was used to establish specific TNF-induced cell lysis.

Northern Blot Analysis of TNF mRNA

Total RNA from PBM and WB buffy-coats was isolated as previously described [17]. Briefly, at specific time-points PBM and WB buffy-coats were placed into a solution of 25 mM Tris, pH 8.0, containing 4.2 M guanidine isothiocyanate, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, an equal volume of 100 mM Tris, pH 8.0, containing 10 mM EDTA and 1.0% SDS was added and the mixture was extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in 10 mM Tris/0.1 mM EDTA (TE) buffer with 0.1% sarkosyl. RNA (10 μg/lane) was separated using formaldehyde/1% agarose gels and transblotted to nitrocellulose. The blot was baked, prehybridized, and hybridized with ^{32}P-5′ end-labeled synthetic oligonucleotide probe for human TNF. The nucleotide sequence for TNF was the 25-mer 5′-GGT-CAC-CCT-TCT-CCA-GCT-GGA-AGA-C-3′ [18]. Blots were washed and autoradiographed with intensifying screens. Relative sensitivity of autoradiographs were quantitated using laser densitometry (Ultrascan XL, LXB Instruments, Inc., Houston, TX). Equivalent amounts of total RNA/gel were assessed by monitoring 28S and 18S rRNA.

RESULTS

LPS-Induced TNF Bioactivity From WB and PBM

The classical stimulus for induction of TNF at both the cellular and molecular level is LPS [1]. This stimulus was utilized as a primary challenge in our experiments. We first examined the dose-response of LPS-induced TNF bioactivity from both WB and PBM. Whole blood and PBM monolayers were incubated for 3 hr post-LPS (10 pg/ml to 10 μg/ml) challenge. Concentrations of LPS above 10 ng/ml resulted in significant increases in TNF bioactivity from both WB and PBMs. TNF bioactivity was noted to be maximal with 10 μg/ml of LPS (data not shown). We next examined the kinetics of TNF production from WB and PBM after LPS stimulation. Both WB and PBM were challenged with 10 μg/ml LPS and either plasma or cell-free supernatant was removed at the designated times. As depicted in Figure 1A, kinetic analysis of LPS-induced TNF bioactivity from WB was deter-

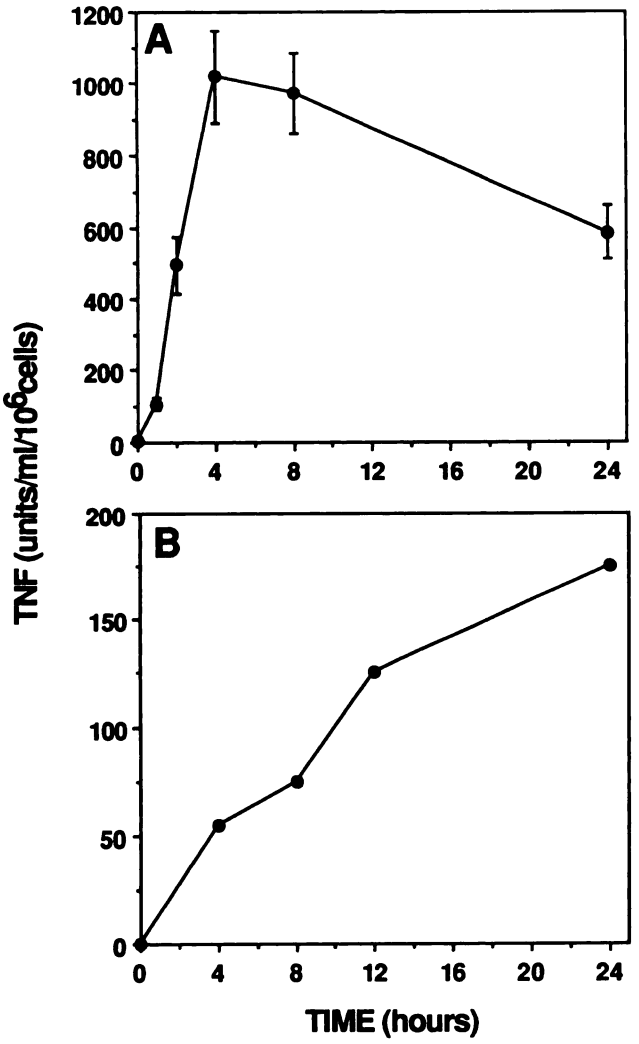

Fig. 1. The kinetic analysis of LPS-induced TNF bioactivity from WB and PBM. Whole blood and PBM were both challenged with LPS (10 μg/ml) and either plasma or cell-free supernatants were isolated and analyzed for TNF production at designated times. A: Whole blood TNF bioactivity from 9 subjects was significant at 1 hr (107 ± 17 units/ml/10^6 cells), peak bioactivity at 4 hr (1,021 ± 128 units/ml/10^6 cells), with a plateau from 4 to 8 hr followed by a gradual decline (43%) over the next 16 hr to a TNF level of 585 ± 73 units/ml/10^6 cells at 24 hr. Each time-point was statistically different from each other except 4 and 8 hr ($P < 0.05$). In contrast, B is a representative kinetic analysis of TNF bioactivity from PBM (n = 4). TNF bioactivity was found to rise gradually throughout the study period with maximal TNF activity achieved at 24 hr post-LPS challenge (175 units/ml/10^6 cells).

mined from nine subjects. TNF levels were significant at 1 hr (107 ± 17 units/ml/10^6 cells), peaking at 4 hr (1,021 ± 128 units/ml/10^6 cells) with a plateau in bioactivity between 4 and 8 hr. This plateau in bioactivity was followed by a gradual decline (43%) over the next 16 hr to a TNF level of 585 ± 73 units/ml/10^6 cells at 24 hr. Cellular viability after 24 hr was established by exclusion of trypan-blue from buffy-coat cells (>90%). To exclude a plasma inhibitor of TNF, plasma from time-points after 8 hr were treated with exogenous recombinant human TNF. Plasma from these time-points failed to inhibit exogenous TNF. In contrast, Figure 1B is a representative kinetic analysis of TNF bioactivity from PBM monolayer (n = 4). TNF bioactivity was found to rise gradually throughout the study period with maximal TNF activity being achieved at 24 hr post-LPS challenge (175 units/ml/10^6 cells). The 8 hr time-point from PBM monolayer represented <50% of the maximal TNF response at 24 hr.

WB-Derived TNF mRNA

To examine TNF gene expression from WB, we next assessed LPS-induced TNF mRNA accumulation in a kinetic fashion from WB (buffy-coat cells). Whole blood (5 ml) was stimulated with LPS (10 μg/ml) at time zero, and total RNA from the buffy-coat was extracted at specified times. Figure 2 demonstrates the Northern blot analysis of WB-derived TNF mRNA accumulation at specific times post-LPS addition. Each lane represents 10 μg of total RNA loaded per well. TNF mRNA accumulation was significant at 1 hr, with a peak in TNF mRNA occurring at 2 hr post-LPS challenge. TNF mRNA rapidly decayed after 2 hr, with essentially baseline levels achieved by 8 hr (n = 4).

Pharmacologic Suppression of TNF Bioactivity From WB

To establish the efficacy of WB as a tool for monitoring pharmacologic manipulation of TNF production, both PGE_2 and Dex were evaluated for their ability to suppress WB-derived TNF bioactivity. PGE_2 and Dex at a concentration of 10^{-6}M have been shown to inhibit mononuclear phagocytic cell TNF synthesis in vitro [17,19]. Whole blood (5 ml) was incubated for 3 hr in the presence of either LPS (10 μg/ml) alone, PGE_2 (10^{-6}M) concomitant with LPS, Dex (10^{-6}M) concomitant with LPS, or media alone. As shown in Figure 3, concomitant administration of either PGE_2 or Dex resulted in a 91% and 80% suppression of LPS-stimulated WB-derived TNF bioactivity (n = 3), respectively. Neither PGE_2 nor Dex in concentrations utilized in these experiments was found to inhibit the activity of exogenous recombinant TNF in our WEHI bioassay.

DISCUSSION

Tumor necrosis factor-alpha (TNF) is increasingly recognized for its pleiotropic effects in immune and pathophysiologic events. It appears to be an essential cytokine at the cellular level, representing a pivotal signal in or-

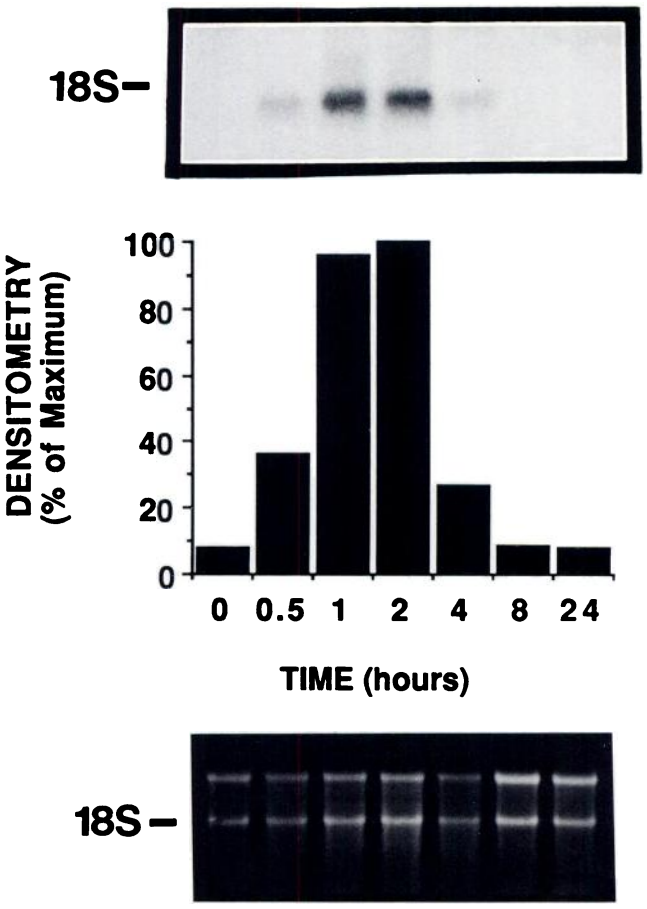

Fig. 2. Northern blot analysis of WB-derived TNF mRNA accumulation. Whole blood was stimulated with LPS (10 μg/ml) and buffy-coat nucleic-acid was extracted in a kinetic fashion. TNF mRNA accumulation was significant at 1 hr, peaking at 2 hr post-LPS challenge, and rapidly declined to baseline levels by 8 hr. Ten μg total RNA was load per lane.

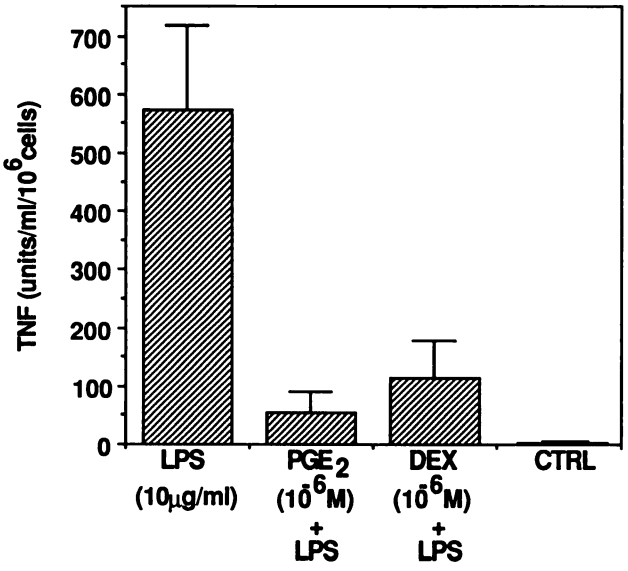

Fig. 3. PGE_2 and Dex suppression of LPS-induced TNF bioactivity from WB (n = 3). Whole blood was stimulated with either LPS (10 μg/ml), PGE_2 (10^{-6}M), and LPS (10 μg/ml), or Dex (10^{-6}M) and LPS (10 μg/ml) and the plasma analyzed for TNF bioactivity at 3 hr. Both PGE_2 and Dex demonstrated significant suppression of LPS-induced TNF production ($P < 0.05$).

chestrating a variety of local acute and chronic inflammatory processes [1]. The antithesis of local TNF production is its presence systemically, representing an essential mediator of septic shock or chronic cachexia [1]. TNF has been extensively investigated as an important inflammatory protein, yet relatively little is known regarding its synthesis and regulation in human cells. The production of human TNF in vivo has been measured in the serum of septic patients or normal subjects who have received intravenous LPS [13,14]. In vitro, both human TNF production and regulation have been evaluated only in cell systems, such as monolayers of blood monocytes or alveolar macrophages [12,15]. A tissue system, simulating the complexity of an in vivo system, would prove useful to assess the dynamics of TNF production and gene expression.

In this present study we demonstrate that intact whole blood can be utilized as a tissue system to examine the molecular and cellular expression of TNF. Our findings show that LPS in either a dose-dependent or kinetic fashion results in TNF production from WB. The peak in TNF production from LPS-treated WB was several magnitudes greater than PBM, standardized to equivalent numbers of monocytes. This discrepancy in TNF production between WB and PBM was not explained by lymphocyte-derived TNF, as LPS does not induce the expression of lymphocyte-derived TNF [15]. The peak in WB kinetic expression of TNF was shifted markedly toward the left as compared to PBM. TNF peaked at 4 hr in WB followed by a plateau in activity from 4 to 8 hr and then declined over the next 16 hr.

In contrast, the peak in TNF from PBM was delayed until 24 hr post-LPS. The difference in TNF kinetics between WB and PBM was not explained by altered cellular viability, as WB buffy-coat cells and PBM were found to have similar viability at 24 hr. The decrease in WB TNF after 8 hr was not due to a WB-derived TNF plasma inhibitor/protease. Plasma isolated from time-points after 8 hr failed to inhibit exogenous TNF in our bioassay. A plausible explanation for the decline in WB TNF production from 8 to 24 hr may be explained by the internalization of TNF after interaction with its cell-associated receptor [20–22]. In additional studies we demonstrate that both PGE_2 and Dex inhibited LPS-induced TNF from WB. This system provides a novel model to determine the efficacy of pharmacologic alteration of cytokines in WB. This aspect is important, since many

pharmacologic agents are less active in vivo yet retain activity in a less complex system such as cell culture. The use of whole blood as a model system, with the complexity of tissue, has enormous potential for the study of cytokine regulation and synthesis. The cellular and molecular level of cytokine regulation can be studied by monitoring specific mRNA and protein bioactivity in the buffy-coat and plasma, respectively. Furthermore, blood recovered from patients receiving a specific pharmacologic agent can be assessed to determine alterations in both the production of inflammatory cytokines and expression of cytokine mRNA. Regulation of cytokines in whole blood by various substances is relevant and may have significant implication for the use of this system prior to the analysis of drug effects in vivo.

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