SYNTHESIS OF TUMOR NECROSIS FACTOR α (TNF-α) BY HUMAN MONOCYTES IN VITRO

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Tumor necrosis factor α (TNF-α) is secreted by macrophages and is able to induce hemorrhagic necrosis of tumors and also to act on functions of different cells [4]. There is experimental and clinical evidence that TNF-α may act as the principal mediator of septic shock [4, 9, 11]. It was considered important to study the mechanisms regulating TNF-α synthesis by activated human blood monocytes and by tissue macrophages. It was shown previously that mouse peritoneal macrophages can contain presynthesized mRNA of TNF-α, whose translation begins after activation of the cells by endotoxin [3].

The aim of this investigation was to determine whether human blood monocytes contain mRNA of TNF-α or the preformed protein of TNF-α. To rule out activation of monocytes during isolation by methods of adhesion to plastic, monocytes were isolated on a Percoll density gradient. Considering the instability of the mRNA of certain cytokines [15], the method of isolation of RNA directly from donated blood was used in some experiments.

EXPERIMENTAL METHOD

Mononuclear leukocytes were isolated from freshly donated heparinized human blood on Ficoll-Verografin (d = 1.077 g/ml) at 4°C, and then fractionated on a continuous Percoll gradient ("Pharmacia") [8] at 4°C. The monocyte fraction contained about 80% of cells which reacted positively for α-naphthyl acetate esterase (kit from "Sigma"). The monocytes (2 ml/ml) were incubated in medium RPMI 1640 ("Gibco") with 5% fetal calf serum and antibiotics in plastic cultural Petri dishes ("Flow Laboratories").

To induce TNF-α *Staphylococcus aureus* strain Cowan 1 (SAC) was used. It was prepared by the method in [10], in a final concentration in the culture medium of 0.001% (by volume). In some experiments part of the donated blood was collected directly in flasks with actinomycin D (AcD) ("Serva," final concentration 10 μg/ml blood) or cycloheximide (CCH) ("Calbiochem," 10 μg/ml blood), after which the cell fractions were isolated as described above. To induce TNF-α synthesis in cells of unfractionated blood, a portion of blood stabilized with heparin was incubated in 50-ml polypropylene tubes ("Nunc"), in a waterbath at 37°C for 3 h in the presence of SAC. Part of the blood was used to isolate RNA (see below) and part was centrifuged for determination of TNF-α activity in the plasma. The cytotoxic activity of TNF-α in supernatants from cultures of monocytes and in blood plasma was determined in a test on L-929 cells in the presence of AcD (1 μg/ml) [7]. To identify cytotoxicity, dilutions of samples were incubated with neutralizing monoclonal 3C7N antibodies against human recombinant TNF-α for 30 min at 37°C [2]. TNF-α activity was determined on the basis of the dilution of supernatant at which 50% lysis of sensitive cells was observed [7]. Activities of the samples in cytotoxicity units were calculated relative to activity of a standard of the biological activity of TNF-α (1 U = 25 pg rTNF-α), provided by the National Institute of Biological Standards and Control (England). Intracellular TNF-α in lysates of monocytes was determined by enzyme immunoassay (EIA) on the basis of natural monoclonal antibodies against rTNF-α, using human rTNF-α as the

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TABLE 1. Content of TNF-α (intracellular and secreted) in Human Monocytes

Monocytes	Quantity of TNF- α, ng		Activity	
	intracellularly, per 2×10^7 cells	secreted, per 10 ml medium	TNF-α, standard	units/ml
Freshly isolated	0,02±0,08	n.t.	n.t.	n.t.
SAC-stimulated, 2 h	n.t.	36,52±0,98	41,4	<4*
SAC-stimulated, 18 h	1,14±0,22	433,47±35,7	874,7	<4
SAC-stimulated + CCH, 18 h	0,03±0,05	13,67±0,43	<4	<4

Legend. Asterisk indicates TNF-α activity in the presence of monoclonal antibodies against TNF-α; n.t.) not tested.

standard [12]. Total cellular RNA was isolated from cultured monocytes by the method in [5]. To detect mRNA of TNF-α actually in cells of unfractionated blood, 5 ml of blood from a healthy donor was collected in a test tube containing an equal volume of hot (60°C) lytic solution (8 M guanidine thiocyanate, 50 mM sodium citrate, 1% sarcosyl, 200 mM mercaptoethanol), after which total RNA was isolated from the lysate [5]. Analysis of the isolated RNA was carried out after electrophoresis on 1.4% formaldehyde agarose gel and transferred to a "Zeta-Probe" nylon membrane ("Bio-Rad") [1]. The mRNA of TNF-α was revealed by hybridization with a labeled cDNA-probe (Hae III — the Hae III fragment 4 of the exon of the human TNF-α gene, genomic sequence 2315-2458 [13], subcloned in phage M13mp18).

EXPERIMENTAL RESULTS

To study the possibility of depositing preformed TNF-α protein in circulating blood monocytes, lysates of intact and stimulated monocytes and supernatants of monocytic cultures were tested by the EIA method with monoclonal antibodies against human TNF-α. As Table 1 shows, monocytes isolated from freshly donated blood under "nonactivating" conditions, did not contain TNF-α protein (sensitivity of the test system 20 pg). Similar results were obtained by the use of polyclonal serum against TNF-α in EIA (data not given), making the presence of preformed TNF-α or its precursor in monocytes unlikely. Stimulation of monocytes by SAC led to an increase in intracellular TNF-α, but no significant accumulation of a protein comparable with the level of secretion into the culture medium could be observed in monocytes (Table 1). Monocytes activated by SAC were secreted into the culture medium in excess of 400 ng TNF-α to $2\cdot 10^7$ cells (Table 1). In the presence of CCH, TNF-α production (intracellular and secreted) was suppressed in a culture of monocytes activated by SAC. It can be concluded from these data that TNF-α production by activated monocytes was the result of de novo protein synthesis.

The results of analysis of mRNA isolated from monocytes from the blood of healthy donors are shown in Fig. 1. No mRNA of TNF-α was found in freshly isolated monocytes. Monocytes isolated from blood in the presence of AcD or CCH (to prevent synthesis of new RNA during isolation of the cells or degradation of existing RNA), likewise did not contain transcripts of TNF-α. This may be the result of activation caused by adhesion of monocytes to the plastic [6]. Treatment of monocytes with SAC for 3 h at 37°C led to accumulation of mRNA of TNF-α (Fig. 1). Under these circumstances, considerable cytotoxic activity, fully neutralized by monoclonal antibodies against TNF-α, was determined in the conditioned medium of SAC-stimulated monocytes.

Data obtained previously showed that monocytes isolated from blood by adhesion to plastic do not contain mRNA of TNF-α [14]. However, the possibility of degradation of hypothetical preformed mRNA of TNF-α in the process of isolation of the monocytes cannot be ruled out, for some degree of instability of transcripts of cytokines in which the neutralized 3′-regions of the genes are rich in AU-sequences [15], is known to take place. Accordingly, we measured the level of mRNA of TNF-α in lysates of freshly isolated human donated blood, which had not been subjected to fractionation or other procedures. No mRNA of TNF-α was present in RNA samples isolated directly from blood (Fig. 2). A very small quantity of TNF-α mRNA was found in blood incubated for 3 h at 37°C without the addition of the activator. Treatment

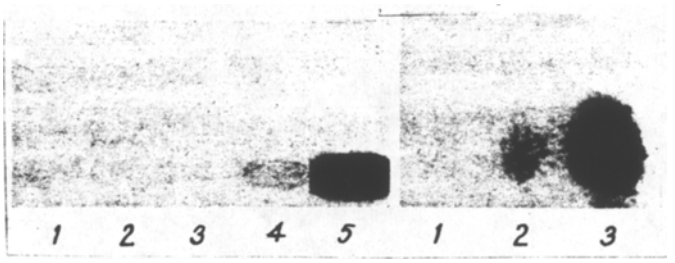

Fig. 1 Fig. 2

Fig. 1. Northern blot analysis of TNF-α mRNA in isolated monocytes in the presence of SAC and (or) CCH and AcD. Total cellular RNA (10 μg per point) was isolated and treated as described in the section "Experimental Method." 1) Freshly isolated monocytes, 0 h; 2) monocytes isolated in the presence of AcD, 0 h; 3) monocytes isolated in the presence of CCH, 0 h; 4) monocytes cultured for 3 h; 5) monocytes stimulated by SAC, 3 h.

Fig. 2. Northern blot analysis of TNF-α mRNA in preparations of whole blood. Total cellular RNA (20 μg per point) was isolated from samples of whole blood (see the section "Experimental Method"). 1) Fresh blood; 2) cultured blood, 3 h; 3) blood stimulated by SAC, 3 h.

of the blood with SAC for 3 h led to accumulation of TNF-α mRNA (Fig. 2). Blood plasma treated with SAC for 3 h contained biologically active TNF-α (about 300 standard units), which was completely neutralized by monoclonal antibodies against TNF-α.

The experimental results showed that TNF-α mRNA is not present in monocytes isolated from human blood on a Percoll density gradient or in samples of whole blood. TNF-α protein is not detected in lysates of freshly isolated monocytes by the EIA method using monoclonal and polyclonal antibodies against TNF-α. It can accordingly be concluded that induction of TNF-α in monocytes is the result of mRNA and protein synthesis de novo.

In view of earlier data [3, 14] it can be postulated that the appearance of TNF-α mRNA in blood monocytes may be the result of their differentiation into macrophages.

The method we used to isolate total RNA from unfractionated blood, like the method of induction of TNF-α synthesis with the aid of SAC in whole blood, may be of practical interest due to its relative simplicity and the small volume of blood required for analysis (5 ml). The method may be useful for the study of regulation of genes of cytokines under normal and pathological conditions, including in clinical laboratory practice.

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