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Up-regulation of tumour necrosis factor-alpha receptors on monocytes by desferrioxamine

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SUMMARY

The effect of endogenously generated reactive oxygen metabolites on the interaction of human blood monocytes with tumour necrosis factor-alpha (TNF-α) was investigated. Pre-exposure of unactivated human blood monocytes to dimethylthiourea, a scavenger of hydroxyl radical ($OH^{\cdot}$), or to desferrioxamine (DFX), an iron chelator preventing the synthesis of $OH^{\cdot}$, enhanced the specific binding of ^{125}I-TNF-α to its receptors. Scavengers of superoxide anion or hydrogen peroxide were without effect. DFX-induced up-regulation of ^{125}I-TNF-α binding depended on the concentration of the drug (1–5 mM) and on the duration of the treatment (1–18 h). It was not due to a reduction of receptor occupancy by endogenously generated TNF-α. Scatchard analysis of binding data revealed that DFX caused an approximately two-fold increase in the number of type II TNF-α receptors, with no change in their affinity. This up-regulation, that did not require synthesis of new proteins, was associated with a decrease in the internalization rate of TNF-α receptors, the half-life of which was doubled. Conversely, these findings suggest that $OH^{\cdot}$ generation by monocytes may have a physiological role in reducing the activity of membrane-associated TNF-α receptors.

Keywords tumour necrosis factor receptors hydroxyl radical human monocytes

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a monocyte-derived cytokine that exerts several effects on these cells. Its synthesis is induced by adherence of monocytes to extracellular matrix components [1], by cell–cell adhesion [2], and by monocyte exposure to various agents including viruses [3], bacterial lipopolysaccharide (LPS) [4], immune complexes [5], and inflammatory peptides (IL-1, TNF-α itself, substance P, and substance K) [6]. Among the effects of TNF-α on monocytes are its ability to enhance platelet activating factor synthesis [7], superoxide anion ($O_2^{\cdot-}$) and lysozyme release [8,9], migration [10], and expression of receptors for urokinase [11] and advanced glycosylation endproducts [12]. It is believed that these actions are initiated by TNF-α interaction with its specific receptors. Monocytes, as other myeloid cells, express the type A or type II TNF-α receptor which has a molecular weight of 73 kD and leads to a cross-linked complex of 98–100 kD [13–15]. It has been shown recently that the TNF-α binding capacity of human monocytes can be up-regulated by activators of protein kinase A [16]. This regulation appears to be due to an increase in the amount of type A TNF-α receptor [17]. In contrast, activation of protein kinase C by phorbol esters results in a rapid decrease of the number of transmembrane TNF-α receptors [18], and an increase of their proteolytic cleavage [19]. Soluble TNF-α binding proteins released in that way compete for TNF-α with the cell-associated receptors and hence inhibit the action of the ligand [20, 21]. Likewise, LPS considerably reduces TNF-α binding to the surface of cultured human monocytes and induces the appearance of soluble TNF-α binding proteins in the extracellular space [22, 23].

Because TNF-α generation is frequently associated with the release of $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), these reactive oxygen metabolites may be involved in a potentially important mechanism of the modulation of TNF-α receptor expression. We have recently shown that exposure of different cells including human blood monocytes to H_2O_2 can down-regulate the cellular response to TNF-α by reducing the TNF-α binding capacity of these cells [24]. The purpose of the present study was to determine whether monocyte TNF-α receptors are a target for regulation by endogenously generated reactive oxygen metabolites.

MATERIALS AND METHODS

Purification and stimulation of human monocytes

Heparinized venous blood (25–50 ml) drawn from healthy volunteers was diluted 1/1 (vol/vol) in calcium-free HBSS (Flow

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Laboratories, Irvine, UK) supplemented with 0·08% EDTA, and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphopaque (Nyegaard, Oslo, Norway) [25]. PBMC were resuspended in the same medium diluted 1/9 (vol/vol) in calcium-free HBSS and centrifuged (100 *g* for 10 min) to remove the platelets. Thereafter, PBMC were resuspended in culture medium consisting of RPMI 1640 (Flow Laboratories) buffered with 20 mM HEPES to pH 7·4, and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, counted after staining with acridine orange, and adjusted to a concentration of $2{\cdot}5 \times 10^6$ cells/ml. The cells were plated into 24-well tissue culture plates (Nunc, Roskilde, Denmark) and allowed to adhere for 4 h at 37°C. The wells were extensively rinsed with culture medium, leaving only adherent monocytes. The monocytes were further incubated for 1–18 h at 37°C in humidified 95% air, 5% CO_2, in culture medium with or without 10 μg/ml superoxide dismutase, 10 μg/ml catalase (Sigma Chemical Co., St Louis, MO), 1–10 mM dimethylthiourea (Merck, Darmstadt, Germany), 1–5 mM DFX (Ciba-Geigy, Rueil Malmaison, France). After the incubation, supernatants were removed, and the cells were washed once before binding studies were performed.

Binding studies

Monocytes in 24-well plates were overlaid with 0·2 ml per well of binding buffer (RPMI 1640 containing 10% FCS), and incubated with 0·5 nM human recombinant (r) ^{125}I-TNF (400–600 Ci/mmol; Radiochemical Centre, Amersham, UK), except where otherwise stated, and varying concentrations of unlabelled human rTNF-α (Boehringer, Mannheim, Germany). After 2 h at 4°C, the binding buffer was removed and monocytes were washed with ice-cold medium, incubated with 0·05 M glycine–HCl buffer (pH 3·0) containing 0·15 M NaCl for 10 min at 4°C, and finally solubilized in 1 M NaOH. The acid-dissociable radioactivity and the radioactivity present in solubilized cells represented cell surface-bound ^{125}I-TNF-α and internalized ^{125}I-TNF-α, respectively. Non-specific binding determined in the presence of a 100-fold excess of unlabelled TNf-α was 10–20% of total binding, and was subtracted to calculate specific binding. In some selected cases, monocytes were pre-exposed for 10 min to low-pH glycine buffer, to reveal possible occupation of TNF-α receptors by endogenously generated TNF-α or for 2 h to 1 μg/ml MoAb directed against TNF-α binding protein type I or II to identify the molecular species of TNF-α receptor. Both antibodies (number 20 and 13, respectively) were kindly provided by Dr D. Wallach from the Weizmann Institute of Science, Rehovot, Israel.

To determine the TNF-α receptor half-life, monocytes in 24-well plates were incubated at room temperature for periods of 0–120 min in the presence of 30 μg/ml cycloheximide with or without 5 mM DFX. Thereafter, the assay of ^{125}I-TNF-α binding to surface receptors was performed at 4°C, as indicated above.

For internalization studies, monocytes were incubated for 18 h with or without 5 mM DFX, washed, and exposed to ^{125}I-TNF-α at 4°C for 2 h. Thereafter, the medium was removed, and the cells were shifted to 37°C by adding pre-warmed binding buffer. They were incubated at 37°C for 0–240 min, before cell surface-bound ^{125}I-TNF-α and internalized ^{125}I-TNF-α were determined.

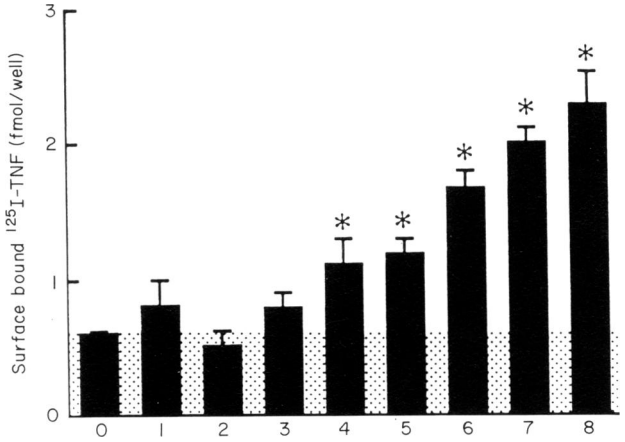

Fig. 1. Effect of oxygen metabolite scavengers or DFX on ^{125}I-TNF-α binding to unstimulated monocytes. Monocytes were incubated for 18 h under control conditions (0); or with 10 μg/ml superoxide dismutase (1); 10 μg/ml catalase (2); 1, 5, 10 mM dimethylthiourea (3–5); or 1, 2, 5 mM DFX (6–8), before specific ^{125}I-TNF-α binding was determined. Means and s.e.m. of values obtained in 4–6 experiments are given. $*P < 0{\cdot}05$ *versus* untreated monocytes.

Statistical analysis

Results are given as the mean ± s.e.m. The statistical significance of differences between groups was analysed by Student's *t*-test for unpaired samples. $P < 0{\cdot}05$ was considered significant.

RESULTS

Exposing unactivated monocytes to DFX or dimethylthiourea for 18 h to prevent ·OH-induced cytotoxicity dose dependently enhanced the subsequent binding of ^{125}I-TNF-α to their surface (Fig. 1). Maximum stimulation was obtained with 5 mM DFX. In contrast, superoxide dismutase, a scavenger of O_2^-, and catalase, which destroys H_2O_2, had no effect. In kinetic studies, ^{125}I-TNF-α binding increased after less than 4 h of culture in the presence of 5 mM DFX, reaching its highest value at 18 h (Fig. 2). Scatchard analysis of the binding data indicated that pre-exposure of monocytes to 5 mM DFX for 18 h resulted in a 103% increase of the average number of receptor sites per cell, without modification of their affinity (K_D value for control cells, 0·41 nM; K_D value for DFX-treated cells, 0·33 nM) (Fig. 3). This increase was due to enhanced expression of type II TNF-α receptors, since pre-exposure of monocytes to a MoAb directed against type II TNF-α binding protein completely suppressed specific ^{125}I-TNF-α binding, whereas a MoAb directed against type I TNF-α binding protein had no effect.

To examine whether DFX-induced up-regulation of ^{125}I-TNF-α binding was due to a decrease in receptor occupancy by endogenously generated TNF-α, bound TNF-α was first dissociated from the surface of monocytes by pre-exposure to low-pH glycine buffer. No significant increase in ^{125}I-TNF-α binding to both DFX-treated and untreated monocytes could be observed under these conditions, indicating that the level of TNF receptor occupancy by endogenously generated TNF-α was unimportant (Fig. 4). The half-life of TNF-α receptors was also examined by incubating monocytes in the presence of cycloheximide to inhibit protein synthesis. Figure 5 shows the progressive decay of ^{125}I-TNF-α binding to the surface of DFX-untreated mono-

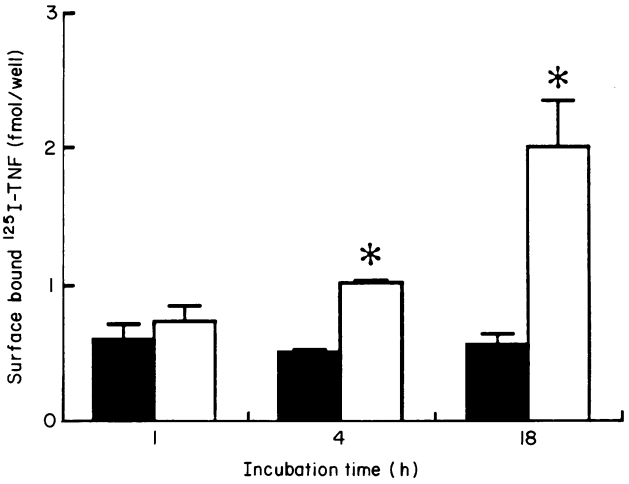

Fig. 2. Time course of DFX effect on ^{125}I-TNF-α binding to unstimulated monocytes. Monocytes were pre-incubated with 5 mM DFX (□) or kept untreated (■) for the indicated time periods, before specific ^{125}I-TNF-α binding was determined. Means and s.e.m. of values obtained in 3–7 experiments are given. *$P < 0.05$ *versus* untreated monocytes.

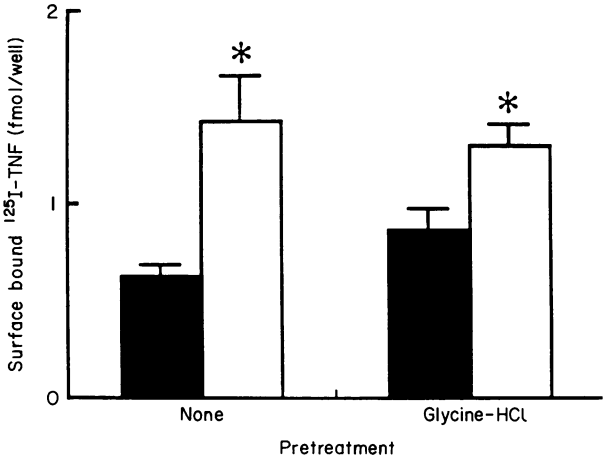

Fig. 4. Effect of low pH treatment of monocytes on DFX-induced TNF-α receptor up-regulation. Monocytes were treated with 5 mM DFX (□) or kept untreated (■) for 18 h, and then exposed to low-pH glycine buffer, before specific ^{125}I-TNF-α binding was determined. Means and s.e.m. of values obtained in four experiments are given. *$P < 0.05$ *versus* untreated monocytes.

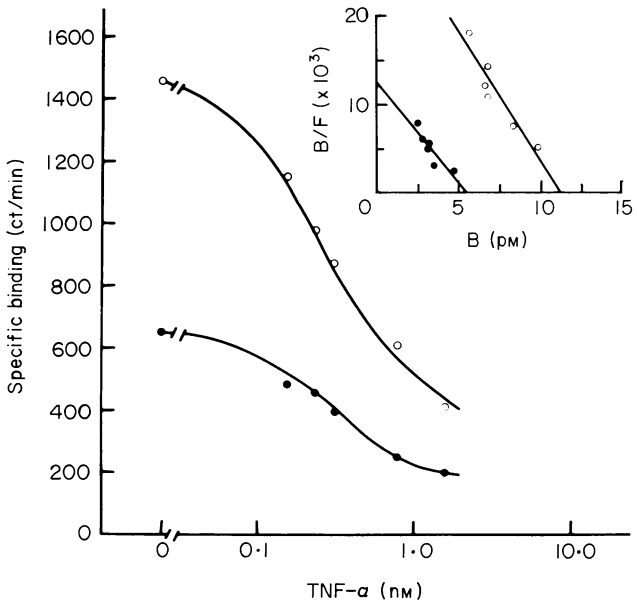

Fig. 3. Competitive displacement of ^{125}I-TNF-α bound in the presence or absence of DFX. Monocytes were treated with 5 mM DFX (○) or kept untreated (●) for 18 h, and then incubated for 2 h at 4°C with 0·3 nM ^{125}I-TNF-α and the amount of unlabelled TNF-α indicated in the abscissa. Scatchard analysis of the specific binding data is shown in the inset.

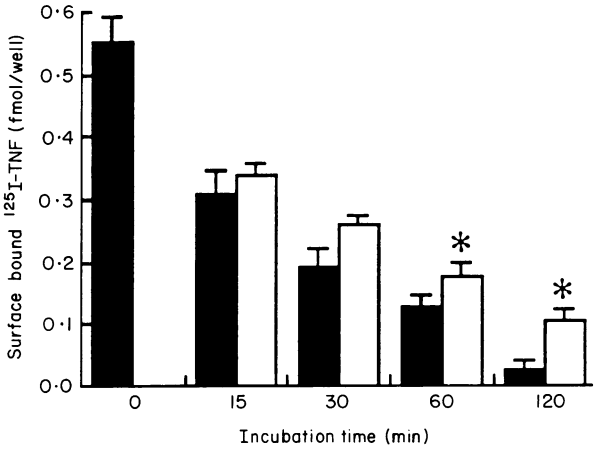

Fig. 5. Effect of DFX treatment on the half-life of cell surface TNF-α receptors. Monocytes were first incubated for the indicated time periods in the presence of 30 μg/ml cycloheximide with (□) or without (■) 5 mM DFX, and then examined for specific binding of ^{125}I-TNF-α. Means and s.e.m. of values obtained in 3–4 experiments are given. * $P < 0.05$ *versus* untreated monocytes.

cytes, with its value reaching one-third of the initial value in approximately 30 min. In the presence of DFX, this decline was reduced, and ^{125}I-TNF-α binding reached one-third of its initial value after 60 min of incubation. This extension of the half-life of TNF-α receptors could be related to a decrease in their internalization rate. As shown in Fig. 6, ^{125}I-TNF-α bound at 4°C to the surface of monocytes was rapidly internalized at 37°C. The increase of intracellular radioactivity was then followed by a small decay. These results are in agreement with those obtained previously [26]. Treatment of monocytes with DFX resulted in a time-dependent decrease of the internalization of ^{125}I-TNF-α bound to the surface. The rate of internalization was similar in DFX-treated and untreated monocytes at time 0, but was significantly reduced in DFX-treated monocytes when these cells were incubated for 2–4 h at 37°C.

DISCUSSION

Several important functions of monocytes are regulated by TNF-α through its binding to cell membrane receptors. The

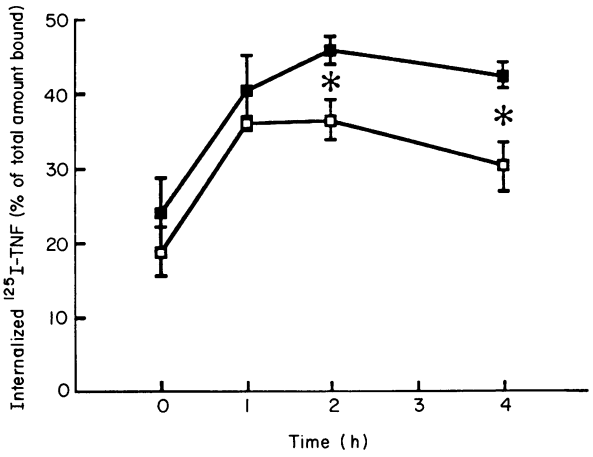

Fig. 6. Effect of DFX treatment on the internalization of ^{125}I-TNF-α bound to the surface of monocytes. Monocytes were treated with 5 mM DFX (□) or kept untreated (■) for 18 h, and then incubated for 2 h at 4°C with 0·5 nM ^{125}I-TNF-α. Thereafter, they were washed and incubated for the indicated time periods at 37°C. Surface bound and internalized ^{125}I-TNF-α were determined. Means and s.e.m. of values obtained in three experiments are given. $*P<0·05$ *versus* untreated monocytes.

present study analyses the effect of monocyte-derived reactive oxygen metabolites on ^{125}I-TNF-α binding to human monocytes. DFX and dimethylthiourea caused an up-regulation of ^{125}I-TNF-α binding to human monocytes. Both agents belong to two structurally distinct classes. The only property they are known to share is the ability to suppress $OH^{\cdot}$ toxicity by interfering with the mechanism of its generation or by promoting its scavenging. In particular, DFX reduces $OH^{\cdot}$ generation by leucocytes without affecting the production of other reactive oxygen metabolites [27]. It is therefore suggested that DFX up-regulates TNF-α binding by blocking a reactive oxygen metabolite-mediated process on which TNF-α receptor degradation depends. In a previous study, the role of exogenously added H_2O_2 on the cellular response to TNF-α has been investigated [24]. This study concluded that H_2O_2 itself reduces the TNF-α binding capacity of several human cells including blood monocytes. By contrast, in this study, catalase, which destroys H_2O_2, did not have any significant effect on ^{125}I-TNF-α binding (Fig. 1). This does not conclusively indicate that endogenously generated H_2O_2 is not involved in the regulation of ^{125}I-TNF-α binding. Because dimethylthiourea and DFX can rapidly cross cell membrane and act intracellularly [28] whereas catalase and superoxide dismutase cannot, these data rather suggest a prevailing role for intracellularly generated reactive oxygen metabolites.

The Scatchard analysis of the binding data reveals that only the number of type II TNF-α receptors was increased, with no change in their affinity (Fig. 3). In this respect, the present investigation confirms that reactive oxygen metabolites affect the expression of TNF-α cell surface receptors but do not modify their affinity for the ligand [24]. DFX-induced increase of ^{125}I-TNF-α binding is not due to a decreased receptor occupancy by endogenously generated TNF-α since it persisted when endogenously generated TNF-α was first dissociated from monocytes. In all instances, cell exposure to low-pH glycine buffer uncovered few binding sites, indicating that the binding of endogenously generated TNF-α was unimportant. These results agree with other studies which have shown that fresh human monocytes secrete very low levels of TNF-α when not stimulated by LPS [4].

In investigations bearing on various cells, binding assays have demonstrated that TNF-α receptors undergo continuous internalization so that their half-life at the cell surface does not exceed 2 h in the absence of TNF-α and 30 min in its presence [29]. Results shown here indicate that the half-life of TNF-α receptors at the surface of monocytes was below 30 min and was significantly increased upon cell exposure to DFX (Fig. 5). Because these experiments were performed on cells whose protein synthesis was inhibited by cycloheximide, DFX induced up-regulation of ^{125}I-TNF-α binding cannot be explained by *de novo* TNF-α receptor synthesis. A more reasonable possibility might be that the drug prevents $OH^{\cdot}$ formation and hence the oxidative damage of the cystein-rich subdomain which has been described in the extracellular part of the type II TNF-α receptor molecule [21]. Another possibility could be that DFX prevents the proteolytic cleavage of the transmembrane type II TNF-α receptor. This might be due to a reduced toxicity of reactive oxygen metabolites that, by activating monocyte-derived metalloproteinases and by inactivating proteinase inhibitors, stimulate proteolytic processes [30,31]. A third possible mechanism is a DFX-induced decrease of the rate of TNF-α receptor internalization. Such a modification of the internalization process was observed at 37°C (Fig. 6). These results agree with our previous finding that exposure of L-929 cells to reactive oxygen metabolites slightly increases the internalization of prebound ^{125}I-TNF-α [24]. Such a mechanism has also been evoked to explain the up-regulation of TNF-α binding to the surface of human cervical carcinoma cell line ME-180 upon exposure to various lectines [32]. Finally, it might be expected that cell treatment by DFX similarly affects surface receptors for other ligands. In fact, Chaudhri *et al.* [33] reported that in contrast to its effect on monocyte TNF-α receptors, DFX inhibited the expression of IL-2 receptors on stimulated T cells. Thus, DFX-induced up-regulation of ^{125}I-TNF-α binding does not seem to reflect a generalized overexpression of plasma membrane receptors on mononuclear cells.

Our results demonstrate that DFX increases the number of cell-associated TNF-α receptors on monocytes. Conversely, one can speculate that reactive oxygen metabolites released from monocytes at the site of inflammation would limit autocrine and paracrine activities of TNF-α by decreasing the interaction of the ligand with these specific receptors.

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