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Membrane expression and shedding of tumour necrosis factor receptors during activation of human blood monocytes: regulation by desferrioxamine

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SUMMARY

Previous studies have shown that desferrioxamine (DFX), an iron chelator preventing the synthesis of hydroxyl radical (OH·), up-regulates the cell-surface expression of tumour necrosis factor-α (TNF-α) receptors on unactivated human blood monocytes. In the present study, we investigated the regulatory action of DFX on ^{125}I-TNF-α binding to monocytes upon exposure to bacterial lipopolysaccharide (LPS). Exposure to LPS (1 μg/ml) resulted in almost complete loss of ^{125}I-TNF-α binding to the surface of monocytes. This down-regulation was reversible and the recovery observed after 18 hr was enhanced by addition of DFX (5 mM). However, binding studies on monocytes pre-exposed to low pH suggested that the DFX-induced increase of ^{125}I-TNF-α binding was not due to differences in the number of receptors available but was probably due to a reduction of receptor occupancy by endogenously generated TNF-α. Time-course studies of TNF-α release from monocytes confirmed the ability of DFX to reduce the extracellular concentration of bioactive TNF-α through a decrease of its synthesis and an increase of its inactivation. The latter process was associated with an increased expression of the soluble form of TNF-α receptor type II. These results indicate that, in the presence of LPS, DFX increases the release of soluble TNF-α receptors from monocytes. Thus, conversely, OH· generated *in situ* could reduce the shedding of soluble TNF-α receptors and, hence, increase the widespread release of bioactive TNF-α.

INTRODUCTION

Tumour necrosis factor-α (TNF-α) is a monocyte-derived cytokine that exerts autocrine actions. Among its different functions on monocytes, TNF-α enhances platelet-activating factor synthesis,[1] superoxide anion (O_2^-) and lysozyme release,[2,3] migration[4] and expression of receptors for urokinase[5] and advanced glycosylation endproducts.[6] It is believed that these actions are initiated by the interaction of TNF-α with specific receptors. Monocytes, as other myeloid cells, express mainly type A or type II TNF-α receptor (MW of 73,000).[7–9] It has been shown recently that the TNF-α binding capacity of human monocytes can be up-regulated by activators of protein kinase A.[10] This regulation appears to be due to increased levels of type A TNF-α receptor.[11] In contrast, activation of protein kinase C by phorbol esters results in a rapid decrease of the number of transmembrane TNF-α receptors,[12] and an increase of their proteolytic cleavage.[13] 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.[14,15]

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Because TNF-α generation is frequently associated with the release of O_2^-, hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·), 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.[16] In addition, we have shown that endogenously generated OH· may also reduce the activity of membrane-associated TNF-α receptors on monocytes.[17] Indeed, both desferrioxamine (DFX), an iron chelator preventing the synthesis of OH· via the Haber–Weiss reaction, and dimethylthiourea, an OH· scavenger, enhance ^{125}I-TNF-α binding to the surface of unactivated monocytes. This up-regulation is due to an increase in the number of type II TNF-α receptors, which reflects an extension of their half-life.

Exposure of human monocytes to bacterial lipopolysaccharide (LPS) has been found to augment TNF-α release[18] and to reduce TNF-α binding to cell-surface receptors.[19] Because LPS simultaneously affects O_2^- and H_2O_2 generation,[20] the question arises as to whether endogenously generated reactive oxygen metabolites are capable of also regulating TNF-α receptor expression of LPS-activated human monocytes. In the present

study, we evaluated the effect of DFX on the ^{125}I-TNF-α binding to these cells. The results described here indicate that DFX weakly enhances ^{125}I-TNF-α binding to the surface of LPS-activated human monocytes. The difference is probably due to a decrease in receptor occupancy by endogenously generated TNF-α, which may be related to a rise of TNF-α interaction with soluble type II TNF-α receptors.

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 Hanks' balanced salt solution (HBSS; Flow Laboratories, Irvine, U.K.) supplemented with 0·08% EDTA, and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphopaque (Nyegaard, Oslo, Norway).[21] 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 10 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 hr at 37°. The wells were extensively rinsed with culture medium, leaving only adherent monocytes. The monocytes were further incubated for 1–18 hr at 37° in humidified 95% air, 5% CO_2, in culture medium with 1 μg/ml LPS from *Escherichia coli* 026B6 (Sigma Chemical Co., St Louis, MO) together with or without 5 mM DFX (Ciba-Geigy, Rueil Malmaison, France). After the incubation, supernatants were harvested, centrifuged (10,000 *g* for 3 min) to remove cell debris and stored at −70° until they were assayed for production of TNF-α and TNF-α-binding protein. The cells were washed once, before binding studies were performed.

Binding studies

Washed monocytes in 24-well plates were overlaid with 0·2ml/well of binding buffer (RPMI-1640 containing 10% FCS), and incubated with 0·5 nM human ^{125}I-rTNF-α (400–600 Ci/mmol; Radiochemical Center, Amersham, U.K.). After 2 hr at 4°, 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°, 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 (Boehringer, Mannheim, Germany) 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.

TNF-α assays

TNF-α content of monocyte supernatants was assayed by both TNF-α immunoradiometric assay (TNF-α-IRMA; IRE-Medgenix, Fleurus, Belgium) and TNF-α bioassay, using the L-929 fibroblast lytic assay.[16] One TNF-α unit was defined as the reciprocal of the dilution which caused 50% cytotoxicity under the conditions of the present assay. An internal standard of human rTNF-α (2×10^7 U/mg; Genzyme, Boston, MA) was included as control. Increasing DFX concentrations up to 10 mM only minimally affected the TNF-α activity measured in the L-929 bioassay. Nevertheless, to exclude the possibility that modifications of TNF-α activity would be the consequence of the presence of DFX in the medium, TNF-α bioassays were performed after adding comparable amounts of DFX to each well of L-929 cells.

Soluble TNF-α receptor assays

The presence of TNF-α-binding material in the culture medium of monocytes was first assessed using the method described by J. G. Giri *et al.* for the identification of soluble interleukin-1 (IL-1)-binding protein.[22] The culture medium from monocytes was concentrated 10-fold by ultrafiltration (Centricon 10 with a 10,000 molecular weight cutoff; Amicon, Beverly, MA). Aliquots (0·125 ml) of the resulting medium were incubated at 4° for 180 min with 2·5 nM ^{125}I-TNF-α in the absence or presence of 50-fold excess unlabelled TNF-α, at a final volume of 200 μl. Free ^{125}I-TNF-α was separated from bound ^{125}I-TNF-α by the addition of 200 μl of 20% polyethylene glycol and 67 μl of 1% γ-globulin, and centrifugation at 10,000 *g* for 5 min. The pellet was washed twice with phosphate-buffered saline (PBS), and the radioactivity counted.

TNF-α-binding material was also identified in the culture medium of monocytes by a filter binding assay, according to the technique previously described by R. A. Heller *et al.*, with minor modifications.[23] Briefly, a 0·25 ml aliquot of concentrated culture medium from monocytes was spotted on a prewetted membrane of nitrocellulose (Schleicher and Schuell, Dusseldorf, Germany) held in a hybridot manifold (Bethesda Research Laboratories, Gaithersburg, MD). The membrane was stamped out to obtain a 8-mm patch which was dried and blocked by incubation at room temperature for 120 min in 5% defatted milk solution. The patch was then incubated at room temperature for 60 min in 0·2 ml PBS containing 10% FCS and 0·5 nM ^{125}I-TNF-α in the absence or presence of 100-fold excess unlabelled TNF-α. It was washed three times with PBS before bound radioactivity was determined.

Finally, soluble TNF-α receptors type I and type II (sTNF-RI and sTNF-RII, respectively) were assayed in the culture medium of monocytes by enzyme-linked immunological binding assay using monoclonal antibodies (mAb) to sTNF-RI and sTNF-RII and recombinant human sTNF-RI and sTNF-RII (kindly provided by F. Hoffmann-La Roche Ltd, Basel, Switzerland), according to the technique described previously.[24]

Statistical analysis

Results are given as the mean ± standard error of the mean (SEM). The statistical significance of differences between groups was analysed by the Student's *t*-test for paired or unpaired samples and by the Wilcoxon signed rank test. A P-value $< 0{\cdot}05$ was considered significant.

RESULTS

Previous binding studies showed a high specific binding of ^{125}I-TNF-α (0·5–0·6 fmol/well) to monocytes that were incubated in

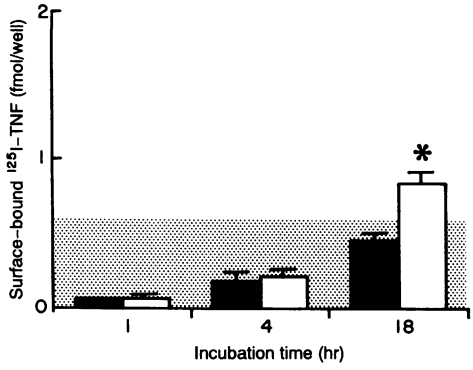

Figure 1. Time-course of DFX effect on ^{125}I-TNF-α binding to LPS-activated monocytes. Monocytes were preincubated for the indicated time periods with 1 μg/ml LPS without (■) or with (□) 5 mM DFX, before specific ^{125}I-TNF-α binding was determined. Means and SEM of values obtained in four to eight experiments are given. $*P < 0.05$ versus untreated monocytes. The hatched zone represents the magnitude of ^{125}I-TNF-α binding to unactivated monocytes.

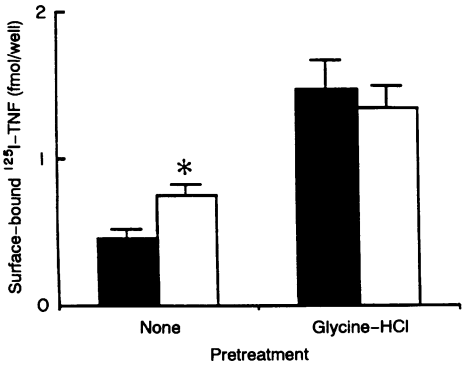

Figure 2. Effect of low pH treatment of LPS-activated monocytes on TNF-α receptor up-regulation by DFX. Monocytes were treated for 18 hr with 1 μg/ml LPS without (■) or with (□) 5 mM DFX, and then exposed to low pH glycine buffer, before specific ^{125}I-TNF-α binding was determined. Means and SEM of values obtained in six experiments are given. $*P < 0.05$ versus untreated monocytes.

the absence of LPS.[17] A decrease of ^{125}I-TNF-α binding to the surface of monocytes was induced by a short (1 hr and 4 hr) pre-exposure to LPS, and incomplete recovery was observed after 18 hr (Fig. 1). DFX treatment, which prevents the synthesis of OH·, significantly enhanced the binding of ^{125}I-TNF-α solely at 18 hr. Low pH treatment of LPS-activated monocytes significantly increased the subsequent binding of ^{125}I-TNF-α (Fig. 2). This increase was weaker in DFX-treated monocytes (0.60 ± 0.20 fmol/well) than in untreated monocytes (0.99 ± 0.22 fmol/well). Moreover, low pH treatment increased the amount of TNF-α bound to the same level and completely suppressed the DFX-induced increase of ^{125}I-TNF-α binding. The most likely explanation of this increase in the number of available receptors is a DFX-induced reduction of receptor occupancy by unlabelled TNF-α related to a corresponding decrease of TNF-α synthesis and release. This was confirmed by the analysis of TNF-α release from LPS-activated monocytes. The kinetics of

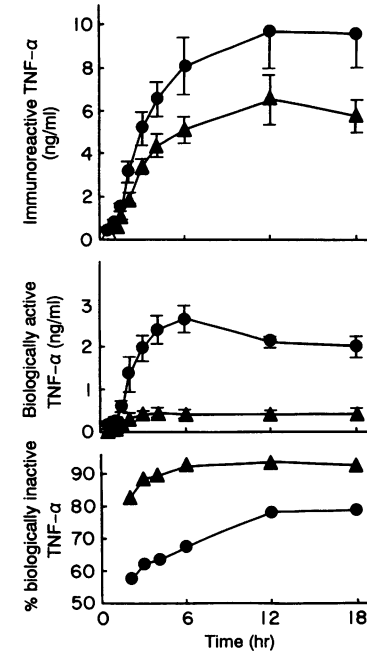

Figure 3. Time-course of TNF-α production by LPS-activated monocytes. Monocytes were incubated for the indicated time periods with 1 μg/ml LPS without (●) or with (▲) 5 mM DFX. Extracellular TNF-α levels were measured by immunoradiometric assay and bioassay. Differences between the two curves represented the level of TNF-α inactivation. Means and SEM of values obtained in four experiments are given.

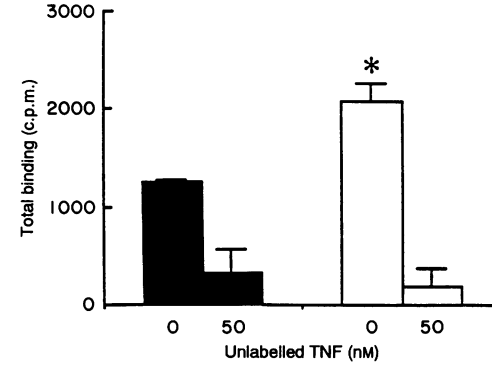

Figure 4. Effect of DFX treatment on the release of TNF-α binding material from LPS-activated monocytes. Monocytes were treated for 18 hr with 1 μg/ml LPS without (■) or with (□) 5 mM DFX. To immobilize proteins, 10-fold concentrated supernatant was dot-blotted to nitrocellulose membrane. This was then incubated with 0·5 nM ^{125}I-TNF-α in the absence or presence of a 100-fold excess unlabelled TNF-α, washed, and analysed for bound radioactivity. Means and SEM of values obtained in three experiments are given. $*P < 0.05$ versus untreated monocytes.

TNF-α release were studied by both immunoradiometric assay and bioassay (Fig. 3). The immunoreactive TNF-α concentration in the supernatant of monocytes increased after a 2-hr exposure to 1 μg/ml LPS, and levelled out after 12 hr. These results are in agreement with those obtained previously.[25] By comparison, TNF-α bioactivity reached a lower level which was

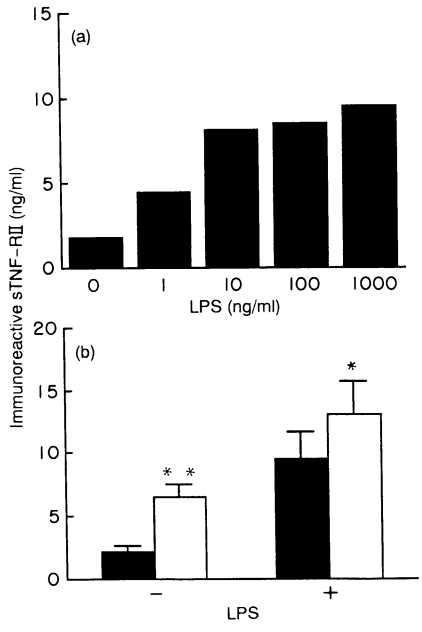

Figure 5. Effect of DFX treatment on the release of sTNF-RII from unactivated and LPS-activated monocytes. Monocytes were treated for 18 hr with the indicated amounts of LPS (a), and with or without 1 μg/ml LPS without (■) or with (□) 5 mM DFX (b). Concentrations of sTNF-RII were determined in the supernatants by enzyme-linked immunobiological binding assay (ELISA). Means of values obtained in two experiments (a) and means and SEM of values obtained in seven experiments (b) are given. $*P<0.05$ and $**P<0.005$ versus DFX-untreated monocytes (paired *t*-test).

maximal at 6 hr, after which it started to decline. The difference between the two time-course curves represents the degree of TNF-α inactivation. Its value increased progressively as a function of time from 60% at 2 hr to 80% at 18 hr. Treatment of monocytes with DFX, which prevents the synthesis of OH·, markedly reduced TNF-α production. Changes in biologically active TNF-α production were greater than changes in immunoreactive TNF-α production, indicating that the drug both decreased TNF-α release and increased TNF-α inactivation.

Binding of TNF-α to soluble TNF-α-binding proteins could be involved in the inactivation process. In order to demonstrate the presence of such TNF-α-binding material in the cell-free supernatants of LPS-activated monocytes, a soluble receptor binding assay was used first. Specific ^{125}I-TNF-α binding to soluble material was detected in 10-fold concentrated supernatants. Non-specific binding reached 40–50% of total binding in this assay. The activities in counts per min (c.p.m.) of ^{125}I-TNF-α specifically bound per 0·125 ml of medium were 951 ± 20 for untreated, and 2023 ± 228 for DFX-treated monocytes ($n=3$; $P<0.05$). Similar experiments were repeated using a filter binding assay (Fig. 4). Under these conditions, the non-specific binding did not exceed 25% of the total binding. As described above, the cell-free supernatants from monocytes treated with DFX and thus exposed to low levels of OH· demonstrated high levels of specific ^{125}I-TNF-α binding (1891 ± 212 c.p.m.) compared to that from untreated monocytes (986 ± 180 c.p.m.) ($n=4$; $P<0.05$).

To characterize further these soluble TNF-α-binding molecules, levels of sTNF-RI and sTNF-RII were determined in the culture medium of monocytes by enzyme-linked immunological binding assay. sTNF-RI was undetectable in all the conditions tested. sTNF-RII was detected in the 18-hr culture medium of unactivated monocytes (Fig. 5). Its release was increased in response to LPS addition in a dose-dependent manner. A significant increase in sTNF-RII concentration was also observed after DFX addition in the culture medium of both LPS-treated and untreated monocytes.

DISCUSSION

The down-regulation of TNF-α receptors by LPS has been shown to occur temporarily via a rise in their internalization.[19] Likewise, the LPS-induced down-regulation of the TNF-α receptors observed here was reversible, and specific ^{125}I-TNF-α binding to LPS-activated monocytes reached 80% of the control levels after 18 hr of incubation (Fig. 1). At that time, DFX addition weakly enhanced ^{125}I-TNF-α binding, indicating that, conversely, endogenously generated OH· reduced the ^{125}I-TNF-α-binding capacity of monocytes. By comparison, DFX enhanced ^{125}I-TNF-α-binding to LPS-untreated monocytes from 0.57 ± 0.07 to 2.01 ± 0.32 fmol/well.[17] Moreover, while DFX-induced increase of ^{125}I-TNF-α binding persisted after low-pH treatment of LPS-untreated monocytes, it was stunted after low-pH treatment of LPS-treated monocytes (Fig. 2). These results indicate that, added to LPS, DFX causes only a reduction of receptor occupancy by endogenously generated TNF-α. This probably reflects the decrease in extracellular TNF-α concentration, which was demonstrated by both immunoradiometric assay and bioassay (Fig. 3). Recent studies also demonstrate that reactive oxygen metabolites may play a role in the release of TNF-α, and, conversely, that DFX added to the culture medium of elicited peritoneal macrophages[26] or mesangial cells[27] entails a dose-dependent reduction in the TNF-α production. There are different ways in which DFX might reduce the release of TNF-α from LPS-activated monocytes. DFX might impair TNF-α synthesis at the level of gene transcription, as well as at a post-transcriptional level. By preventing the formation of reactive oxygen metabolites and, hence, the activation of metalloproteinases and/or the inactivation of proteinase inhibitors, the drug might also interfere with the cleavage of membrane-associated TNF-α (26,000 MW molecule), the precursor of the secretory component of TNF-α (17,000 MW molecule).[27] The comparison of data obtained by immunoradiometric assay and bioassay reveals that DFX also enhanced the inactivation of TNF-α. The inactivation process could result from the action of proteolytic enzymes[28] either in the medium or in endosomes following internalization of ligand–receptor complexes. However, analysis by SDS-PAGE of ^{125}I-TNF-α degradation following 18 hr incubation with LPS-activated monocytes indicated that DFX treatment did not modify TNF-α proteolysis (data not shown). The finding of higher amounts of TNF-α binding proteins in the supernatant of DFX-treated monocytes (Fig. 4) is rather consistent with the notion that DFX treatment, by preventing the synthesis of OH·; increases the release or prevents the damage of soluble TNF-α-binding proteins, so that they can bind TNF-α with a higher affinity and/or a higher capacity.

As demonstrated by enzyme-linked immunological binding assay (Fig. 5), at least one part of these proteins represent a soluble form of the membrane TNF-α receptor type II found on

human monocytes. sTNF-RII release was increased by LPS. These findings are in line with recent observations that LPS administration to healthy adult volunteers promotes the appearance of circulating sTNF-RI and sTNF-RII, with peak levels occurring immediately after that of TNF-α.[29,30] Additionally, sTNF-RII release was increased by DFX added either alone or together with LPS. One possible explanation is that DFX prevents OH· formation and, thus, decreases the rate of internalization of TNF-α receptor type II. This mechanism is consistent with our previous finding that exposure of LPS-untreated monocytes to DFX decreases the internalization of TNF-α receptors, the half-life of which is doubled.[17] Consequently, proteolysis could affect long-lived membrane receptors and lead to the increased release of sTNF-RII. Alternatively DFX treatment could limit prostaglandin synthesis, since, conversely, reactive oxygen metabolites greatly enhance it.[31] In turn, a reduction in prostaglandin level may represent a mechanism for increasing sTNF-RII release, as recently demonstrated *in vivo*.[32]

In conclusion, our results demonstrate that DFX increases the release of soluble TNF-α receptors from LPS-activated monocytes. Conversely, one can speculate that reactive oxygen metabolites released at the site of inflammation would simultaneously increase the widespread release of TNF-α into a circulation by increasing the release of soluble TNF-α[26,27] and by reducing the level of soluble TNF-α-binding proteins. However, these speculations need further evaluation.

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