Protective effect of thioredoxin upon NO-mediated cell injury in THP1 monocytic human cells

P. J. FERRET, E. SOUM, O. NEGRE, E. E. WOLLMAN and D. FRADELIZI¹

Laboratoire INSERM Unité 477, Pavillon Hardy, Hôpital Cochin, 27 rue du Fbg St Jacques, 75674 Paris cedex 14, France

Although NO has been postulated to play important roles in host defences, it is potentially damaging for exposed cells, including for the macrophages producing the NO. Thus a network of radical acceptors and enzymes is thought to play an important redox-buffering role to protect cells against NO-mediated injury. We examined the properties of the redox systems superoxide dismutase (SOD)/catalase, glutathione (GSH) and thioredoxin (Trx), in regulating the viability of two human monocytic cell lines (THP1 and U937) exposed to the NO-generating compound diethylene triamine-nitric oxide (DETA-NO). We observed that NO-induced cytotoxic effects were time- and dose-dependent towards the two cell lines. After vitamin-induced differentiation *in vitro* with retinoic acid (RA) and 1,25-dihydroxy vitamin D₃ (VD), termed RA/VD, we observed that THP1 RA/VD cells became more resistant to NO-mediated cytotoxicity whereas the susceptibility of U937 cells was not modified. Using Western

blotting and reverse-transcriptase PCR methods, we observed that gene transcription and protein expression of Trx and thioredoxin reductase were significantly increased upon RA/VD treatment and differentiation in THP1 cells. By contrast, SOD/catalase and GSH redox state remained unmodified. Finally, a stable transfectant THP1 line overexpressing Trx was found to be more resistant than THP1 control cells that were untransfected or transfected with an empty plasmid, when exposed to DETA-NO *in vitro*. In conclusion, we observed an inverse correlation between cell susceptibility to NO damaging effects and Trx expression, suggesting that the Trx system may have important preventative capacities towards NO-mediated cellular injury in monocytic macrophage cells.

Key words: glutathione, macrophages, monocytes, nitric oxide, redox protection.

INTRODUCTION

NO [1–4], a short-lived radical molecule, is synthesized from the oxidation of the terminal guanido-nitrogen atom of L-arginine by an NADPH-dependent enzyme, termed NO synthase (NOS) [2,3]. It can be produced abundantly by macrophages following stimulation-differentiation signals [5,6]. Macrophage-derived NO is known for its anti-microbial and anti-tumoral effects *in vitro* and *in vivo* [7–9]. The mechanisms of NO-mediated cell injury largely depend upon subsequent reactions leading to reactive oxygen and/or nitrogen species such as hydrogen peroxide (H₂O₂), superoxide radicals (O₂^{•−}), hydroxyl radicals (OH[•]) and peroxynitrite (ONOO[−]) [10,11]. Their high reactivity results in damage to proteins, lipid membranes and DNA.

A network of radical acceptors and redox enzymes acting as buffering systems [4,12,13] maintains the redox status of cells. Among these intracellular mechanisms, the glutathione system [14,15] and the thioredoxin (Trx) system [16–19] are thought to play a major role; both are ubiquitous and found in many species such as bacteria, plants and mammals, including man. The Trx system, comprising the flavoprotein thioredoxin reductase (TR), Trx and NADPH, operates to transfer reducing equivalents from NADPH to the redox-active cysteine residues in Trx, using TR. Reduced Trx provides reducing equivalents to a wide variety of metabolic pathways and regulatory proteins such as ribonucleotide reductase, activator protein-1 (AP-1), nuclear factor κ B (NF- κ B) transcription factors, vitamin K epoxide reductase, thiolperoxidase and plasma glutathione peroxidase. Trx proteins present a dithiol-disulphide active site (Trp-Cys-Gly-Pro-Cys)

that is strongly conserved across all species investigated thus far. The rest of the molecule displays sequence variations in different species. The cDNA cloning of human Trx performed by our group [18], followed by the description of the human Trx genomic organization [20], indicated that human Trx has distinctive features, one being that human Trx displays five cysteines compared with only two (the reactive site) in *Escherichia coli* Trx. The Trx protein has no signal peptide. It is released in the culture supernatant of various cell lines through a non-classical pathway [21].

Since the Trx/TR system possesses disulphide reductase properties, it could have an important protecting function in cells exposed to NO-mediated cytotoxic attack [17,22]. The aim of the present study was to elucidate the relationship between the expression of Trx and the susceptibility to the damaging effects of NO of two monocytic cell lines, THP1 and U937, before and after monocytic differentiation [23–29].

MATERIALS AND METHODS

Chemicals

Retinoic acid (RA), 1,25-dihydroxy vitamin D₃ (VD), leupeptin, pepstatin, PMSF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dithiothreitol were purchased from Sigma (St. Louis, MO, U.S.A.). The NO donor diethylene triamine-nitric oxide (DETA-NO) was obtained from Research Biochemicals International (Natick, MA, U.S.A.). ECL[®] (enhanced chemiluminescence) reagents were from Amersham.

Abbreviations used: Trx, thioredoxin; TR, thioredoxin reductase; SOD, superoxide dismutase; RA, retinoic acid; VD, 1,25-dihydroxy vitamin D₃; DETA-NO, diethylene triamine-nitric oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse-transcriptase PCR; AP-1, activator protein-1; NF- κ B, nuclear factor κ B; IL, interleukin; TNF- α , tumour necrosis factor α ; NOS, NO synthase; HPRT, hypoxanthine phosphoribosyl transferase; TBS, Tris-buffered saline.

¹ To whom correspondence should be addressed (e-mail fradeliz@cochin.inserm.fr).

Cell lines and differentiation procedures

The two pro-monocytic human cell lines used in this study, THP1 [30] and U937 [31], were maintained in culture medium (RPMI 1640/glutamax 1 from Gibco supplemented with 7% foetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin). The differentiation procedure consisted of adding to the culture medium 10^{-7} M RA and VD for 72 h at 37 °C in 5% CO₂ in a humid atmosphere. These treated cells were designated THP1 RA/VD and U937 RA/VD.

Susceptibility to NO-mediated cell injury: MTT assay

Susceptibility of cells to NO-mediated cell injury was evaluated by the MTT cytotoxicity assay using the following procedure: 5×10^4 cells/well were distributed in 96-well plates. Either DETA-NO or H₂O₂ (1 mM, a concentration established to be optimally toxic in preliminary testing) were added to the wells. Cell viability was assessed by the mitochondrion-dependent reduction of MTT to formazan. After the various incubation times indicated, cells in 96-well plates were exposed to 20 µl of MTT (5 mg/ml in 1 × PBS) and incubated at 37 °C for 4 h. Then, 150 µl of medium was removed from each well, and 100 µl of 0.5% HCl/propan-1-ol was added to lyse the cells. The absorbance at 550 nm in each well was recorded immediately using an ELISA microplate reader. The net absorbance in the wells containing cells cultured with control medium was taken as the 100% viability value. The percentage of viable cells exposed to DETA-NO was calculated using the formula $[(A_{550-630})_{\text{sample}} / (A_{550-630})_{\text{control}}] \times 100$.

Cytofluorimetric analysis

For cell-cycle analysis, 1×10^6 to 3×10^6 cells recovered from 72-h cultures (in differentiating conditions, or not) were fixed with 1 ml of ice-cold 70% ethanol for 18 h. After centrifugation, the pellet was resuspended in PBS containing 50 µg/ml propidium iodide (Sigma) and 100 units of RNase A (Sigma). The analysis was subsequently realized by cytofluorimetry using a Facscan machine (Beckton Dickinson, le Pont de Claix, France) at 488 nm.

ELISA detection of cytokine synthesis

Human-cytokine-detection ELISA kits were purchased from Medgenix Diagnostics (Rungis, France) and used according to the manufacturer's instructions. Interleukin (IL)-1β and tumour necrosis factor α (TNF-α) were measured in undiluted supernatants and each experiment was performed in triplicate.

Reverse-transcriptase PCR (RT-PCR)

Total RNAs were extracted from 1×10^6 cells in 500 µl of TRI reagent (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. Then cDNAs were synthesized in 40-µl reactions containing 2 µg of RNA, 8 µl of 5 × buffer (250 mM Tris/HCl, 250 mM KCl, 50 mM MgCl₂, 50 mM dithiothreitol and 2.5 mM spermidine), 2 µl of each dNTP (25 mM), 0.4 µl of 50 mM oligo-dT primer, 0.4 µl of RNasin at 22000 units/ml and 0.6 µl of avian myeloblastosis virus reverse transcriptase (Appligene, Strasbourg, France) at 42 °C for 2 h. PCR was performed from one-twentieth of the cDNA reaction in a 100-µl reaction volume containing 10 µl of 10 × buffer (10 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100 and 0.2 mg/ml gelatine), 0.5 µl of each dNTP (25 mM), 0.4 µl of *Taq* polymerase (5000 units/ml; Appligene) and 1 µl of each primer (20 µM). The RT-PCR products were subjected to electro-

phoresis on a 2% agarose gel and stained with ethidium bromide. The sequences of sense and antisense PCR primers used for amplification and the predicted sizes of products were as follows. Trx, upper, GAGCAAGACTGCTTTTCAGG, lower, GGCTCCAGAAAATTCACC; Mn superoxide dismutase (SOD), upper, GTCGACAAGCATGGCCACGAAGGC, lower, CCGGTCGACAGGGAATGTTTATTG; Cu/Zn SOD, upper, AAGGCCGTGTGCGTGCTGAA, lower, CAGGTCTCCAACATGCCTCT; catalase, upper, GCAGATACCTGTGAAGTGC, lower, GTAGAATGTCCGCACCTGAG; TR, upper, TCCTCTTTTCTACCCACTG, lower, GTATTCCTTGCTCTGTCATCCA; and HPRT (hypoxanthine phosphoribosyl transferase), upper, TATGGACAGGACTGAACGTCTTGC, lower, GACACAAACATGATTGAAATCCCTGA.

Immunoblot analysis

Cell pellets (1×10^6 cells) were lysed in lysis buffer (25 mM Hepes, 0.5% Nonidet P40, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 0.1 mM sodium deoxycholate, 1 mM PMSF and 0.1 mg/ml leupeptin and pepstatin, pH 7.8) at 4 °C for 30 min. Nuclei and membranes were removed by centrifugation at 12000 g for 15 min, and the amount of protein in each lysate was measured using the BSA microburet assay from Pierce. Loading buffer (42 mM Tris/HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol and 0.002% Bromophenol Blue) was added to each lysate, which was subsequently boiled for 3 min and electrophoresed on an SDS/polyacrylamide (15%) gel. Proteins were transferred on to a nitrocellulose membrane. The membrane was saturated overnight with Tris-buffered saline (TBS; 0.9% NaCl/3 M Tris/HCl, pH 7.5) containing 10% skimmed milk, washed twice for 15 min with 0.1% TTBS (TBS containing 0.1% Tween-20) and incubated with rabbit anti-(human Trx) polyclonal antibody (1/2000) in 0.1% TTBS/3% skimmed milk for 1 h. The membrane was washed twice for 15 min each with 0.1% TTBS, and incubated for 45 min with the secondary antibody, peroxidase-conjugated goat anti-rabbit (1/5000). Bound antibodies were visualized by chemiluminescence using an ECL Western Immunoblotting Kit (Amersham) and quantification of the bands was performed using the Bio-profil system (Vilbert Lourmat, Torcy, France).

cDNA construct and cell-line transfection

The cDNA encoding human Trx isolated by Wollman et al. [18] was cloned as a *EcoRI*–*EcoRI* fragment into the eukaryotic expression vector pcDNA 3.1 (Invitrogen NV, Leek, The Netherlands). The correct sequence of the construct was confirmed by DNA sequencing. For transfection, cells were seeded in six-well plates at 2.5×10^6 cells/well in 0.8 ml of serum-free growth medium and incubated for 6 h with a solution containing 4 µg of DNA of the coding (or empty) vector, and 12 µl of lipofectamine (Gibco-BRL) diluted into 200 µl of serum-free medium Opti-MEM. Following incubation, 4 ml of the appropriate complete growth medium was added to each well. Following transfection (72 h), cells were selected with G418 (400 µg/ml; Gibco-BRL).

Statistics

Student's *t*-test, with $P < 0.05$, was used to determine the statistical significance of the data.

RESULTS

RA and VD treatment *in vitro* induces the differentiation of the human macrophage cell line THP1

THP1 cells cultured for 72 h *in vitro* in the presence of optimum doses (10^{-7} M) of the differentiating agents RA and VD (designated RA/VD treatment) tend to acquire a more differentiated

Table 1 Effect of RA/VD treatment on THP1 and U937 cell differentiation: cell-cycle progression and cytokine production

Cells were cultured for 72 h *in vitro* in the presence of optimum doses (10^{-7} M) of the differentiating agents RA and VD (RA/VD treatment), or incubated in culture medium alone. DNA content and cytokine production were determined as detailed in the Materials and methods section. Results are expressed as the percentage of cells in each cell-cycle phase for DNA content, or as pg/ml for cytokine production. Each value is the mean $\pm$ S.D. from three independent experiments. Statistical analysis was performed using Student's *t* test in comparison with untreated cells: **P* < 0.05.

Cell-cycle phase			Cytokine production (pg/ml)		
Sample type	G ₀ /G ₁	S	G ₂ /M	IL-1 β	TNF- α
THP1	49.5 $\pm$ 2.5	38.5 $\pm$ 0.5	12 $\pm$ 2	5 $\pm$ 3	62 $\pm$ 37
THP1 RA/VD	65.5 $\pm$ 0.5*	25.5 $\pm$ 0.5*	9 $\pm$ 1	68 $\pm$ 27*	778 $\pm$ 219*
U937	71.5 $\pm$ 3.5	24.5 $\pm$ 1.5	4 $\pm$ 2	15 $\pm$ 12	5 $\pm$ 1
U937 RA/VD	78 $\pm$ 2.5	17 $\pm$ 1.5	2 $\pm$ 2	48 $\pm$ 11*	415 $\pm$ 135*

state in the macrophage lineage. For instance, RA/VD treatment induced the secretion of the pro-inflammatory cytokines IL-1 β and TNF- α , which could be titrated by ELISA in the culture supernatant of *in vitro*-differentiated THP1 cells (THP1 RA/VD; Table 1). This treatment also increased the proportion of cells arresting their progression in the cell cycle and accumulating in the G₀/G₁ phase (Table 1).

Treatment of the human promyelocytic line U937 with RA and VD (U937 RA/VD) also induced secretion of the pro-inflammatory cytokines IL-1 β and TNF- α . However, using the U937 cell line, no change in the proportion of cells in the different phases of the cycle could be detected (Table 1).

RA-and-VD-differentiated THP1 cells were more resistant to NO- or H₂O₂-induced cell death *in vitro*

THP1 cells were first differentiated *in vitro* by 72-h cultures in the presence of optimum doses (10^{-7} M) of the differentiating agents RA and VD (THP1 RA/VD), or left in medium alone (THP1 cells). The cells were subsequently exposed *in vitro* to a graded concentration of either the NO donor DETA-NO, or H₂O₂. The global number of cells surviving exposure to the donors of radical species was evaluated using Trypan Blue dye exclusion (results not shown) and the mitochondrial reduction of MTT, at *T*₀ (just before exposure) and at various times for the next 48 h.

Figure 1 Effect of RA/VD treatment on susceptibility of THP1 and U937 cells to NO or H₂O₂ cytotoxicity properties

Cells were cultured for 72 h *in vitro* in the presence of optimum doses (10^{-7} M) of differentiating agents RA and VD (RA/VD treatment), or incubated in culture medium alone. Then, cells seeded at 5×10^4 cells/well in 96-well plates were exposed to 1 mM DETA-NO (A and B) or 1 mM H₂O₂ (C and D) for the indicated times. The percentage of living cells was assessed 48 h post-treatment using the MTT assay. Percentage cell survival was calculated as the recovery of viable cells exposed to DETA-NO or H₂O₂ compared with the recovery of cells cultured in medium alone, not exposed to the oxidizing agents. The experiments were performed in triplicate and the results presented are representative of four separate experiments. Statistical analysis was performed using Student's *t* test in comparison with untreated cells: **P* < 0.05.

Figure 2 Effect of RA/VD treatment on NO-induced apoptosis in THP1 and U937 cell lines

Cells were cultured for 72 h *in vitro* in the presence of optimum doses (10^{-7} M) of the differentiating agents RA and VD (RA/VD treatment) or incubated in culture medium alone. After 6 h of incubation in the presence (or not) of 1 mM DETA-NO, cells were washed with PBS. Early apoptotic changes were identified by cell staining with propidium iodide and FITC-conjugated annexin V. The percentage of apoptotic cells was defined as the percentage of green fluorescent positive and propidium iodide negative cells measured by flow cytometry. Results are means $\pm$ S.D. from three separate experiments. Statistical analysis was performed using Student's *t* test in comparison with untreated cells: **P* < 0.05.

The results indicate that about 70 % of THP1 RA/VD cells could be recovered as functional surviving cells after 48 h in cultures supplemented with 1 mM DETA-NO (the highest dose tested), compared with less than 20 % of control undifferentiated THP1 cells, cultured under the same conditions (Figure 1A). Similar results were obtained when the cells were exposed to H_2O_2 : 80 % of THP1 RA/VD cells survived 48 h in cultures supplemented with 1 mM H_2O_2 , compared with less than 20 % for control THP1 cells (Figure 1C).

Experiments were performed with the monocytic line U937 using the same experimental protocol. They led to very different results. As can be seen in Figure 1, U937 cells were as sensitive to NO- (Figure 1B) or H_2O_2 - (Figure 1D) induced cell death as THP1 cells. However, in contrast to the results with THP1, no increased resistance to radical species, either NO or H_2O_2 , was observed when the U937 cells were first differentiated by RA/VD treatment.

RA/VD-differentiated THP1 cells are more resistant to NO-induced apoptosis *in vitro*

We next examined in more detail the mechanisms of cellular death of THP1 cells (differentiated or not), exposed to exogenous NO in culture. As previously, THP1 cells were first differentiated by 72 h of culture in the presence of the differentiating agents RA and VD, or left in medium alone. These cells were subsequently

Figure 3 Effect of RA/VD treatment (A) on redox acceptor/enzyme molecules and (B) on Trx expression in THP1 and U937 cell lines

Cells were cultured for 72 h *in vitro* in the presence of optimum doses (10^{-7} M) of the differentiating agents RA and VD (designated RA/VD treatment) or incubated in culture medium alone. (A) Total cellular RNA was extracted and used for semi-quantitative RT-PCR evaluation of mRNA of the redox acceptor/enzyme molecules indicated. HPRT mRNA was used as internal standard. The vertical axes indicate arbitrary mRNA units. Each value is the mean $\pm$ S.D. from five independent experiments. Statistical analysis was performed using Student's *t* test in comparison with untreated cells: **P* < 0.05. (B) Cell lysates (1×10^6 cells) were separated by SDS/PAGE, blotted on to nitrocellulose, and Trx was immunodetected by a rabbit polyclonal antibody. Quantification of the bands was performed using Bio-profil system (Vilbert Lourmat). The Western blot shown is representative of results reproduced in three independent experiments.

Figure 4 Susceptibility of Trx-transfected THP1 cells to NO damaging effects

THP1 cell lines transfected with Trx cDNA or empty cDNA, and subsequently selected in culture with G418, were established. (A) Thioredoxin expression in THP1 transfected cell lines was assessed. Cell lysates (1×10^6 cells) were separated by SDS/PAGE, blotted on to nitrocellulose, and Trx was immunodetected by a rabbit polyclonal antibody. Quantification of the bands was performed using the Bio-profil system (Vilbert Lourmat). The Western blot shown is representative of results reproduced in three independent experiments. (B) Susceptibility of both THP1 transfected cell lines to exogenous NO cytotoxicity was evaluated using the MTT assay. Percentage cell survival was calculated as the recovery of viable cells exposed to 1 mM DETA-NO compared with the recovery of cells cultured in medium alone, not exposed to the oxidizing agents. The experiments were performed in triplicate and the results presented are representative of four separate experiments. Statistical analysis was performed using Student's *t* test in comparison with untreated cells: **P* < 0.05.

exposed *in vitro* to 1 mM DETA-NO. Early apoptotic changes were monitored after 6 h by flow-cytometry analysis of the number of living cells (staining negative with propidium iodine) expressing phosphatidylserine at their surface and binding annexin V.

The results indicate that RA/VD-differentiated THP1 cells were more resistant to NO-induced early apoptotic events than untreated THP1 cells (following NO exposure, 25% of THP1 RA/VD cells bound annexin V versus 45% of untreated THP1 cells; Figure 2).

In contrast to the results with the THP1 cell line, no increased resistance to apoptotic death induced by NO was observed with the U937 line, differentiated or not. Following NO exposure, 22% of the U937 cells bound annexin V, whether differentiated or not (Figure 2). The spontaneous apoptotic changes in cells cultured under the same conditions but not exposed to NO were always low: fewer than 12% of cells bound annexin V in these control cultures.

Increased resistance of differentiated THP1 cells to NO-induced cell death is associated with up-regulation of redox enzymes

We examined the effects of RA/VD differentiating treatment on THP1 cells with respect to the expression of several redox enzymes involved in protection against radical oxygen species. Using a semi-quantitative RT-PCR, we compared the level of expression of the mRNA of redox enzymes in THP1 cells, either differentiated (THP1 RA/VD) or not (THP1) (Figure 3A). Significantly increased expression of both Cu/Zn SOD and Mn SOD mRNA (30 and 40% increases respectively) was observed in THP1 RA/VD cells compared with untreated THP1 cells. The

highest increases in mRNA expression in THP1 RA/VD cells compared with untreated THP1 cells were those of the enzymes Trx and TR, which increased 70 and 120% respectively. No increase of catalase mRNA expression was observed when comparing THP1 RA/VD and THP1 cells.

Following these results obtained at the level of gene transcription, we examined whether Trx protein was also up-regulated in THP1 cells upon differentiation. Total protein extracts from THP1 RA/VD and THP1 cells were submitted to SDS/PAGE and studied by immunoblotting using a specific polyclonal rabbit anti-human Trx). As can be seen on Figure 3(B), Trx protein content was increased approx. 2-fold (2.15 ± 0.33 , *P* < 0.05) in THP1 RA/VD cells compared with undifferentiated THP1 cells.

U937 cells were also examined, which, at variance with THP1 cells, did not acquire resistance to NO damaging effects upon RA/VD differentiation. The same semi-quantitative mRNA evaluations of redox enzymes in U937 cells led to very different results compared with THP1 cells: no significantly increased expression of any of the enzymes examined was observed comparing U937 RA/VD and U937 cells. Rather, we observed a decreased expression of the two SOD enzymes in U937 RA/VD cells, no changes in catalase and Trx, and finally a low, non-significant, modified expression of TR (Figure 3A). Immunoblotting of Trx in U937 cells, differentiated or not, did not reveal any Trx protein increase following differentiation (Figure 3B).

Glutathione (GSH) levels of these various cells were also examined. No significant changes were observed between the THP1 cells, differentiated by RA/VD and not. In U937 cells there was also no significant variation in glutathione, although a lower level of expression was observed in these cells, whether differentiated or not (results not shown).

THP1 cells transfected with the *Trx* gene are more resistant to NO cell-damaging effects

In order to establish a causal relationship between the resistance to NO cytotoxicity and the up-regulation of oxidative stress protective enzymes in differentiated THP1 cells, we performed transfection experiments. Trx was selected because the Trx/TR redox enzymes displayed the highest up-regulation in differentiated THP1 cells. A stable THP1 transfected line overexpressing Trx was established by transfection with a plasmid construct (pcDNA 3.1-Trx or empty control plasmid). Following 1-month selection with G418, we examined whether Trx protein was increased in THP1 transfected cells. Total protein extracts were submitted to SDS/PAGE and studied by immunoblotting using a specific polyclonal rabbit anti-(human Trx). As can be seen on Figure 4(A), Trx protein content was increased approx. 2-fold (1.78 ± 0.21 , $P < 0.05$) in THP1 transfected cells (THP1 pcDNA 3.1-Trx) compared with untransfected THP1 cells or those transfected with empty plasmid (THP1 pcDNA 3.1).

The THP1 cells, either overexpressing Trx or not, were exposed to a toxic dose (1 mM) of the NO donor DETA-NO or H_2O_2 . The number of cells surviving exposure to NO or H_2O_2 radicals was evaluated using the mitochondrial reduction of MTT, at T_0 (just before exposure) and at various times for the next 48 h. As can be seen in Figure 4(B), THP1 transfected cells overexpressing Trx had a better survival *in vitro* when exposed to the NO donor (70 % of cells surviving at 48 h) and H_2O_2 (90 % of cells surviving at 48 h) compared with THP1 cells transfected with the empty plasmid or that were left untransfected (18 and 20 %, or 18 and 25 % of cells surviving respectively for NO donor and H_2O_2 treatment at 48 h).

DISCUSSION

NO is a ubiquitous molecule produced continuously at low levels from L-arginine in many cells with vascular and neuronal functions [1]. Murine macrophages, when they are activated, express the inducible NOS (NOS2) and produce very large quantities of NO. Macrophage-derived NO is a very potent effector molecule with several effects resulting from the production of peroxides and oxygen radicals [9–11]. The macrophage-derived active radical species have two main functions. They destroy or inactivate a wide variety of micro-organisms, protecting the host from environmental infections [5,6]. Secondly, the radical species are cytotoxic for cells exposed to their action [6]. This later effect is an important regulatory process with ambivalent consequences, since death of tumour cells mediated by macrophage-derived NO has potent anti-tumour effects [7,8]; however, macrophage-derived active radicals are also toxic for bystander cells, including macrophages themselves [32].

A tightly regulated cellular redox state is necessary for proper cell functioning. Such equilibrated redox state is maintained by several mechanisms: metal- or thiol-containing proteins in the serum and/or extracellular spaces (albumin and haemoglobin) play the roles of acceptors of reactive radicals. Intracellularly, there are sets of acceptor metal- or thiol-proteins coupled to reductase enzymes that capture and neutralize toxic radicals produced when an oxidative or nitrosative stress occurs [33]. The acceptor/enzyme molecular partners belong to four main systems: the SOD/catalase system [34], the glutathione (GSH) system (γ -glutamylcysteine synthetase/GSH/GSH reductase/glutathione S-transferases) [35,36], the Trx/TR system [16] and the glutaredoxin/glutaredoxin reductase system. These four systems are considered to be the most important molecules in charge of neutralizing (detoxifying) active oxygen radicals [4].

Their respective importances vary from one tissue or cell type to another, and their roles in macrophages are not clearly defined.

The goal of our study was to identify the redox-buffering and enzymic systems protecting human macrophages against NO stress, and their regulation upon macrophage activation and differentiation. Our experiments were performed using two human leukaemic cell lines, THP1 [30] and U937 [31], which belong to the myelo-monocytoid lineage. They can be differentiated *in vitro* towards macrophages by cell treatment with active metabolites of RA and VD. Following treatment with RA and VD, these cells reduce their growth capacity and acquire monocyte/macrophage markers and functions [37–44], including secretion of pro-inflammatory cytokines [45] and gene transcription and functional expression of inducible NOS2 [46].

Our experiments indicated that RA/VD-differentiated THP1 cells were more resistant to exogenous NO-induced cell death *in vitro*. This resistance to NO correlated with an increased expression of Trx mRNA and overexpression of Trx protein in RA/VD-differentiated THP1 cells. A causal relationship between the acquisition of NO resistance by activated macrophage THP1 cells and the gene transcription and overexpression of Trx was suggested by our observation that the *Trx* gene transfected into THP1 cells induced the expression of a phenotype resistant to NO. Different results were obtained with U937 cells: RA/VD treatment of U937 cells neither modified their susceptibility to exogenous NO nor induced gene transcription and overexpression of Trx, in sharp contrast with the RA/VD-induced protection of THP1 cells reported above.

The different results obtained with both cell lines can be interpreted as follows: in THP1 cells, the cell-death protection induced upon RA/VD differentiation would result from the endogenous induction of Trx synthesis; on the other hand, U937 cells were not converted, upon exposure to RA/VD, to a NO-resistant phenotype. Other authors have reported that exogenously added human Trx protected U937 cells against apoptosis induced by TNF- α [47]. Our own failure to induce a NO-resistant phenotype in U937 cells upon RA/VD differentiation might result from the lack of response of these cells for unknown reasons to the differentiating agents and the subsequent failure to induce endogenous Trx in these cells.

Our results suggesting that Trx has a central protective role against cell-death-inducing signals are in line with previous reports showing that Trx is protective for cells exposed to various aggressions. For instance, Trx-transfected WEHI7.2 cells have been shown to be protected against various apoptosis-inducing agents, such as dexamethasone, staurosporine, etoposide or *N*-acetyl-sphingosine [22]. The same authors observed that these apoptosis-resistant Trx-transfected WEHI7.2 cells formed fast-growing tumours in SCID mice. Trx was found to improve the stimulation of monocyte, fibroblast and endothelial cell lines, acting as a potent co-stimulus [48].

The protective effects of the Trx/TR system in activated macrophages against nitrosative and oxidative stress might have several explanations. First, they may result from its well-documented enzymic di-thiol reductase activity with NADPH as the source of protons and TR for reducing oxidized Trx S-S into Trx (SH)₂ [16,18]. The thiol-buffering activity of the system has no known restriction. It can regenerate SH on a broad spectrum of thiol-containing proteins possessing radical buffering activities and protecting macrophages from nitrosative and oxidative stress [19,49]. Furthermore, Trx was shown to selectively induce Mn SOD (mitochondrial), which, like Cu/Zn SOD, has been shown to protect cells against nitric oxide injury in many cell types [50–52]. Thus it added to its own functions the action of a second essential antioxidant enzyme that dismutates toxic superoxide

radicals into H₂O₂ and dioxygen. Another possible action of Trx is at the level of gene-transcription control. It has been reported that endogenous overexpressed Trx acts in the nucleus to directly reduce and activate nuclear transcription factors such as AP-1 (Fos/Jun heterodimer), which mediates transcriptional control of glutathione S-transferase [53]. On the other hand, reducing agents were shown to inhibit NF- κ B activation, possibly by inhibiting the process of I κ B (inhibitory κ B) phosphorylation [54,55].

The identification of regulated redox-buffering systems protecting differentiated macrophages against NO and active radical derivatives may have important implications. NO and oxygen radicals are the macrophage effector molecules. However, they have ambivalent opposite consequences, efficiently protecting the organism from infectious agents and growth of tumour cells. They are also potentially toxic for bystander cells, including macrophages themselves. Elucidating the self-protecting redox systems induced in activated and differentiated macrophages could help to define new strategies of therapeutic intervention aimed at boosting the efficiency of macrophage defences against infectious agents and malignant tumours.

REFERENCES

- Schmidt, H. H. and Walter, U. (1994) *Cell* **78**, 919–925
- Nathan, C. and Xie, Q. (1994) *Cell* **78**, 915–918
- Marletta, M. (1994) *Cell* **78**, 927–930
- Stamler, J. (1994) *Cell* **78**, 931–936
- MacMicking, J., Xie, Q. and Nathan, C. (1997) *Annu. Rev. Immunol.* **15**, 323–350
- Stuehr, D. J. and Marletta, M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7738–7742
- Farias-Eisner, R., Sherman, M. P., Aeberhard, E. and Chaudhuri, G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9407–9411
- Lavnikova, N., Drapier, J. C. and Laskin, D. L. (1993) *J. Leukoc. Biol.* **54**, 322–328
- Evans, T. J., Buttery, L. D., Carpenter, A., Springall, D. R., Polak, J. M. and Cohen, J. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9553–9558
- Brunelli, L., Crow, J. P. and Beckman, J. S. (1995) *Arch. Biochem. Biophys.* **316**, 327–334
- Zingarelli, B., O'Connor, M., Wong, H., Salzman, A. L. and Szabo, C. (1996) *J. Immunol.* **156**, 350–358
- Foresti, R., Clark, J. E., Green, C. J. and Motterlini, R. (1997) *J. Biol. Chem.* **272**, 18411–18417
- Bouton, C., Hirling, H. and Drapier, J. C. (1997) *J. Biol. Chem.* **272**, 19969–19975
- Baez, S., Segura-Aguilar, J., Widersten, M., Johansson, A. S. and Mannervik, B. (1997) *Biochem. J.* **324**, 25–28
- Smyth, M. (1991) *J. Immunol.* **146**, 1921–1927
- Holmgren, A. (1979) *J. Biol. Chem.* **254**, 9627–9632
- Iwata, S., Hori, T., Sato, N., Hirota, K., Sasada, T., Mitsui, A., Hirakawa, T. and Yodoi, J. (1997) *J. Immunol.* **157**, 3108–3117
- Wollman, E. E., d'Auriol, L., Rimsky, L., Shaw, A., Jacquot, J. P., Wingfield, P., Graber, P., Dessarps, F., Robin, P., Galibert, F. et al. (1988) *J. Biol. Chem.* **263**, 15506–15512
- Spector, A., Yan, G. Z., Huang, R. R., McDermott, M. J., Gascoyne, P. R. and Pigiet, V. (1988) *J. Biol. Chem.* **263**, 4984–4990
- Kaghad, M., Dessarps, F., Jacquemin-Sablon, H., Caput, D., Fradelizi, D. and Wollman, E. E. (1994) *Gene* **140**, 273–278
- Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. E. and Sitia, R. (1992) *J. Biol. Chem.* **267**, 24161–24164
- Baker, A., Payne, C. M., Briehl, M. M. and Powis, G. (1997) *Cancer Res.* **57**, 5162–5167
- Botling, J., Oberg, F., Torma, H., Tuohimaa, P., Blauer, M. and Nilsson, K. (1996) *Cell Growth Differ.* **7**, 1239–1249
- Jurutka, P. W., Hsieh, J. C., Nakajima, S., Haussler, C. A., Whitfield, G. K. and Haussler, M. R. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3519–3524
- Zhang, D. E., Hetherington, C. J., Gonzalez, D. A., Chen, H. M. and Tenen, D. G. (1994) *J. Immunol.* **153**, 3276–3284
- Mossalayi, M. D., Paul-Eugene, N., Ouaz, F., Arock, M., Kolb, J. P., Kilchherr, E., Debre, P. and Dugas, B. (1994) *Int. Immunol.* **6**, 931–934
- Yamamoto, N. and Naraparaju, V. R. (1996) *Cell Immunol.* **170**, 161–167
- Oberg, F., Botling, J. and Nilsson, K. (1993) *J. Immunol.* **150**, 3487–3495
- Willheim, M., Gessl, A., Berger, R., Schedle, A., Luger, T., Foster, O. and Boltz-Nitulescu, G. (1991) *J. Immunol.* **147**, 1837–1842
- Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) *Int. J. Cancer* **26**, 171–176
- Sundstrom, C. and Nilsson, K. (1976) *Int. J. Cancer* **17**, 565–577
- Laubach, V. E., Shesely, E. G., Smithies, O. and Sherman, P. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10688–10692
- Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J. and Loscalzo, J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 444–448
- Sandstrom, P. A. and Butke, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4708–4712
- Buchmuller-Rouiller, Y., Corrandin, S. B., Smith, J., Schneider, P., Ransijn, A., Jongeneel, C. V. and Mauel, J. (1995) *Cell Immunol.* **164**, 73–80
- Hamilos, D. L. and Wedner, H. J. (1985) *J. Immunol.* **135**, 2740–2747
- Dodd, R. C., Cohen, M. S., Newman, S. L. and Gray, T. K. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7538–7541
- McCarthy, D. M., San Miguel, J. F., Freake, H. C., Green, P. M., Zola, H., Catovsky, D. and Goldman, J. M. (1983) *Leuk. Res.* **7**, 51–55
- Olsson, I., Gullberg, U., Ivhed, I. and Nilsson, K. (1983) *Cancer Res.* **43**, 5862–5867
- Koeffler, H. P., Amatruda, T., Ikekawa, N., Kobayashi, Y. and DeLuca, H. F. (1984) *Cancer Res.* **44**, 5624–5628
- Taimi, M., Chateau, M. T., Cabane, S. and Marti, J. (1991) *Leuk. Res.* **15**, 1145–1152
- Vey, E., Zhang, J. H. and Dayer, J. M. (1992) *J. Immunol.* **149**, 2040–2046
- Kim, Y. R., Abraham, N. G. and Lutton, J. D. (1991) *Leuk. Res.* **15**, 409–418
- Lubbert, M., Herrmann, F. and Koeffler, H. P. (1991) *Blood* **77**, 909–924
- Bhalla, A. K., Paavonen, T., Williams, M. M., Delves, P. J. and Lydyard, P. M. (1991) *Immunology* **72**, 61–64
- Dugas, N., Mossalayi, M. D., Calenda, A., Leotard, A., Becherel, P., Mentz, F., Ouaz, F., Arock, M., Debre, P., Dornand, J. and Dugas, B. (1996) *Blood* **88**, 3528–3534
- Matsuda, M., Masutani, H., Nakamura, H., Miyajima, S., Yamauchi, A., Yonehara, S., Uchida, A., Irimajiri, K., Horiuchi, A. and Yodoi, J. (1991) *J. Immunol.* **147**, 3837–3841
- Schenk, H., Vogt, M., Droge, W. and Schulze-Osthoff, K. (1996) *J. Immunol.* **156**, 765–771
- Lundstrom, J., Krause, G. and Holmgren, A. (1992) *J. Biol. Chem.* **267**, 9047–9052
- Das, K. C., Lewis-Molock, Y. and White, C. W. (1997) *Am. J. Respir. Cell. Mol. Biol.* **17**, 713–726
- De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinuer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y. and Fang, F. C. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13997–14001
- Brockhaus, F. and Brune, B. (1999) *Biochem. J.* **338**, 295–303
- Peunova, N. and Enikolopov, G. (1993) *Nature (London)* **364**, 450–453
- Schenk, H., Klein, M., Erdbrugger, W., Droge, W. and Schulze-Osthoff, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1672–1676
- Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) *EMBO J.* **12**, 2005–2015