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Original Research Communication

Secretion of 10-kDa and 12-kDa Thioredoxin Species from Blood Monocytes and Transformed Leukocytes

BITA SAHAF and ANDERS ROSÉN

ABSTRACT

Thioredoxins (TRX) are ubiquitous, small redox-active proteins with multiple functions, including antioxidant, cytoprotective, and chemoattractant activities. In addition to a 12-kDa intracellular form, extracellular 10-kDa and 12-kDa TRX have been defined. The biological activities of the 10-kDa TRX were previously measured as eosinophil cytotoxicity enhancing activity or B-cell stimulatory activity. Cytotrophoblastic cell lines also release a 10-kDa TRX form. To study the biological role of 10-kDa TRX, we established two highly sensitive enzyme-linked immuno-spot assays (ELISPOT), which detect secreted truncated 10-kDa and full-length 12-kDa TRX at the single cell level. TRX secretion was investigated in several cell lines including the T-helper cell hybridoma MP6, the Jurkat T-cell leukemia, the U-937 myelomonocytic leukemia, and the 3B6, EBV-transformed, lymphoblastoid B-cell line. The highest number of secreting cells was found in 3B6 cultures, median = 34 (quartiles, 27–39) per well (10^5 cells). Peripheral blood monocytes isolated from healthy donors secreted significantly more TRX after stimulation with ionomycin, phorbol myristate acetate (PMA), fMLP, and lipopolysaccharide (LPS), compared to unstimulated cells. Oxidative stress induced by thioloxidant diamide also induced the secretion of both truncated and full-length TRX measured in ELISPOT ($p = 0.047$ and $p = 0.031$, respectively). The biological activity of the truncated and full-length forms was tested in a cell migration assay. Truncated TRX was devoid of protein disulfide reductase activity, but retained strong chemoattractant activity for human monocytes, in the same range as full-length TRX, as previously reported (Bertini *et al.*, 1999). Antiox. Redox Signal. 2, 717–726.

INTRODUCTION

THIOREDOXINS (TRX) constitute a family of small redox active proteins with a molecular weight of 12 kDa. Full-length TRX is a ubiquitous protein with a conserved CXXC active site (Holmgren, 1985, 1989) and was first defined as a proton donor for ribonucleotide reductase (Laurent *et al.*, 1964). TRX catalyzes dithiol-disulfide oxidoreduction, and oxidized TRX is reduced by thioredoxin reductase (TRXR) and NADPH (Holmgren and Björnstedt, 1995). TRX is secreted by monocytes, lymphocytes, and other normal and neoplastic cells through a leaderless pathway (Ericson *et al.*, 1992; Rubartelli *et al.*, 1992, 1995; Tagaya *et al.*, 1989; Wakasugi *et al.*, 1990). Mammalian cells have three types of TRX, one is located in the cytoplasm (Wollman *et al.*, 1988), one in the mitochondria (Spyrou *et al.*, 1997), and a third in the plasma membrane (Sahaf *et al.*, 1997). Furthermore, another member of the TRX family, a TRX-like protein, which is of 32.2 kDa and cannot serve as substrate for TRXR has been

Department of Biomedicine and Surgery, Division of Cell Biology, Linköping University, S-581 85 Linköping, Sweden.

defined (Miranda-Vizuete *et al.*, 1998). Silberstein and co-workers purified a 10-kDa eosinophil cytotoxicity enhancing factor (ECEF) from phorbol myristate acetate (PMA)-stimulated U-937 cells. ECEF shared identical amino-terminal amino acid sequence with human TRX (Balcewicz-Sablinska *et al.*, 1991). Based on the electrophoretic mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as a 10-kDa protein and its homology in the amino-terminal amino acid sequence to full-length 12-kDa TRX, it was suggested that the last 20 or 24 amino acids were missing from the ECEF compared to 12-kDa TRX. This truncation was shown to increase the ECEF activity 200-fold compared to the 12-kDa TRX. Opposing regulatory effects of full-length TRX and truncated TRX on development of human immunodeficiency virus-1 (HIV-1) was also shown (Balcewicz-Sablinska *et al.*, 1991; Newman *et al.*, 1994). Our previous results on the T-helper cell hybridoma MP6-derived TRX, which was a mixture of 10-kDa and 12-kDa TRX, revealed that its B-cell stimulatory activity was >500-fold higher than purified 12-kDa placental-derived TRX (Rosén *et al.*, 1995).

We have earlier shown that low quantities of truncated TRX were present in cells of monocyte/macrophage origin, using specific monoclonal antibodies (mAbs) (Sahaf *et al.*, 1997). Co-localization studies showed that full-length TRX was mainly in cytoplasm, whereas the truncated form was associated with the plasma membrane (Sahaf *et al.*, 1997). For detection of TRX released from single cells, we developed an enzyme-linked immuno-spot assay (ELISPOT). Using these newly developed methods, we studied the secretion of TRX and its physiological effects on cell migration of blood monocytes.

MATERIALS AND METHODS

Materials

The mAb clone 4H9 (IgG_{2a}) and clone 7D11 (IgG_1) against truncated human TRX were both specific for the 1–80 (1–84) recombinant TRX, but did not recognize the conventional 12-kDa TRX. They were produced at the Department of Biomedicine and Surgery, Linköpings University, and are now commercially available from Pharmingen, a Beckton Dickinson Company, San Diego CA) (Sahaf *et al.*, 1997). Polyclonal goat anti-human TRX antibodies were a kind gift from Professor Arne Holmgren, Karolinska Institute, Stockholm, Sweden (sold by IMCO Ltd, Stockholm, Sweden). For stimulation of the monocytes, the following reagents were used: interleukin-1α (IL-1α) and *Escherichia coli* lipopolysaccharide (LPS) (Sigma Chemicals Co., St. Louis, MO); PMA (Biomol Research Labs Inc. Plymouth Meeting, PA): interferon-γ (IFN-γ) (Boehringer Ingelheim, Mannheim, Germany); ionomycin (Calbiochem, La Jolla, CA); and fMLP (Sigma Chemical Co). Recombinant human TRX was purchased from IMCO Ltd. (Stockholm, Sweden) and recombinant truncated TRX was produced as previously described (Sahaf *et al.*, 1997). Anti-hTNF-α mAbs were obtained from R&D systems (Minneapolis, MN).

Cell lines and culture conditions

The cell lines used in this study were: MP6-T cell hybridoma (Rosén *et al.*, 1986), Jurkat (Gillis and Watson, 1980), U-937 (Sundström and Nilsson, 1976), and 3B6 (Wakasugi *et al.*, 1987) were maintained in RPMI-1640 (Gibco BRL, Glasgow, UK) with 10% fetal calf serum (FCS) (Gibco BRL), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, and 2 m*M* glutamine. All cell lines were incubated at 37°C, in humidified, 5% CO_2-containing air.

Monocyte isolation

Human peripheral blood monocytes were isolated according to the method of Freunlich and Avdalovic (1983). Briefly, fresh buffy coats were derived from healthy blood donors. The peripheral blood mononuclear cells were prepared by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation according to manufacturer's recommendations. The cells were washed in media and suspended in AIM-V medium (Gibco BRL) without any FCS supplementation at a concentration of 5×10^6 cells per ml. A total of 10 ml of the suspension was applied to 2% gelatin (Microbiol-

ogy-grade, Merck, Darmstadt, Germany) and autologous serum-coated 75-cm^2 cell culture flasks. After an incubation time of 40 min, adhered cells were isolated using 5 m*M* EDTA in AIM-V medium Purified monocytes (>90% were monocytes according to CD14 marker) were then suspended in RPMI-1640 with 10% FCS.

ELISPOT for detection of 10-kDa and 12-kDa TRX secretion at the single cell level

ELISPOT assay was performed as described before (Czerkinsky *et al.*, 1984) with minor modifications. Plates (Multiscan HA, Millipore, Danvers, MA), were coated with 100 μl of anti-TRX goat polyclonal IgG (25 μg/ml) in sterile phosphate-buffered saline (PBS). Anti-hTNF-α mAbs (R&D Systems, Minneapolis, MN) were used as positive control in separate wells. The plates were incubated at 4°C overnight. Then the plates were washed in PBS and blocked with 200 μl of 0.5% bovine serum albumin (BSA; Fraction V, Boehringer Mannheim, GmbH, Germany) for 2–4 h at 37°C. The cell suspension (10^5 per well) was applied. Blood monocytes were tested as unstimulated and stimulated (1 μg/ml of LPS, 200 U/ml IFN-γ; 0.5 ng/ml IL-1α; 10^{-7} *M* fMLP; 0.5 μ*M* PMA) for 20 hr at 37°C, in six replicates. The effect of thioloxidant diamide (Sigma Chemical Co.) was tested on monocytes at 100 μ*M*, 50 μ*M*, and 25 μ*M* concentration over 20 hr. Cell lines were suspended in RPMI-1640 with 10% FCS, and incubated overnight at 37°C. Cells were then removed and the plate was washed two times in PBS and three times in PBS-T (PBS containing 0.05% Tween 20; Merck Darmstadt, Germany). Released, truncated TRX was detected by biotinylated mAb clone 7D11 (500 ng/ml) (Sahaf *et al.*, 1997) and tumor necrosis factor-α (TNF-α) released was detected by biotinylated anti-hTNF-α, 300 ng/ml (R&D). Full-length TRX was detected by 300 ng/ml of biotinylated IgG of clone 2G11 (Sahaf *et al.*, 1997). Finally, the plates were developed at room temperature by incubation in Vectastain ABC-AP kit, AP standard AK 5000 (Vector Laboratories Inc. Burlingame, CA), for 1 hr and AP-conjugate substrate kit (BioRad Laboratories, Hercules, CA), both according to manufacturer's recommendations. Developed plates were then rinsed thoroughly in deionized water, and allowed to dry at room temperature overnight before inspection in a Nikon stereo microscope. Two persons evaluated the plates blindly. Median values for the six replicates and quartiles were calculated and plotted in diagrams. No spots were detected in control wells lacking cells.

Intracellular immunofluorescence staining

Freshly isolated monocytes from healthy individuals or cultured cells were washed in balanced salt solution (BSS) containing 1% HEPES (Sigma Chemical Co.) (BSS-HEPES) and adjusted to 2×10^6 cells per ml of buffer. BioRad adhesion slides (180-7001; BioRad Clin. Div., München, Hercules, CA) were washed in deionized water and BSS-HEPES. A 20-μl amount of the cell suspension was applied to each field, and the cells were allowed to adhere for 15 min. The cells were then fixed in 4% paraformaldehyde for 5 min. After rinsing in BSS-HEPES with 0.1% saponin (Kebo) (BSS-HEPES-saponin), cells were incubated with primary mAb 2G11, 7D11, or IgG_1 isotype control (Dako Co., Glostrup, Denmark) at 5 μg/ml, for 30 min. After one wash in BSS-HEPES-saponin, cells were incubated in a R-phycoerythrin-conjugated $F(ab)_2$ goat anti-mouse IgG (H+L), preadsorbed anti-human IgG, A, M (CalTag Laboratories, Burlingame, CA), 1:80 diluted in BSS-HEPES-saponin, for 30 min. Slides were washed twice in BSS-HEPES-saponin and three times in BSS-HEPES. Then the slides were kept in the dark at 4°C in a 50% glycerol (Merck), 50% BSS solution with 20 mg/ml 1,4 diazobicyclo-2,2-octane (Sigma Chemical Co.), until analyzed under a Nikon fluorescence microscope.

Migration assay

Migration assay in a micro-Boyden chamber was performed as described previously (Falk *et al.*, 1980). Briefly, monocytes were isolated as described above. Cells were then suspended in RPMI-1640 with 25 m*M* HEPES and 1% BSA at a concentration of 4×10^6 cells per ml. Then, 10-kDa TRX (10 ng/ml) and LPS (1 μg/ml) were used as chemoattractants. Cells were ap-

plied into a chamber with a PVP membrane with 5-μm pore size (Costar, Nucleopore, Cambridge, MA). Cells were incubated for 2 hr at 37°C. The membrane was then stained using a Diff-quick kit (Labex, Svenska Labex AB, Helsingborg, Sweden). Cells that migrated through the pores in the membrane were counted at 40× magnifications. 9 fields-of-vision were evaluated. Mean ± SE was calculated for each stimulatory factor. Numbers of monocytes migrating in medium alone were regarded as nonspecific background migrations.

Statistical analysis

Median for six replicates and quartiles were calculated and noted. Mean ± SE (standard error of mean) is calculated for migrating cells. Difference in response between stimulated and nonstimulated cells were evaluated statistically by the nonparametric Mann-Whitney *U* test. All statistical evaluations were performed with JMP version 3.2.5 (SAS Institute Inc., Cary, NC) software and a Dell Latitude, PC.

RESULTS

ELISPOT measurement of secreted 10-kDa and 12-kDa TRX

First we investigated the spontaneous release of 12-kDa TRX in unstimulated monocytes isolated from healthy blood donors. Figure 1A shows that the median = 11 (quartiles, 9–15) per 10^5 yielded detectable spots. fMLP stimulation did not yield more secretory cells, whereas physiological stimulation by PMA/ionomycin, LPS/IFN-γ/IL-1α or LPS resulted in increase of number of secretory cells to median values 22 (quartiles, 18–27), 14 (quartiles, 11–23), or

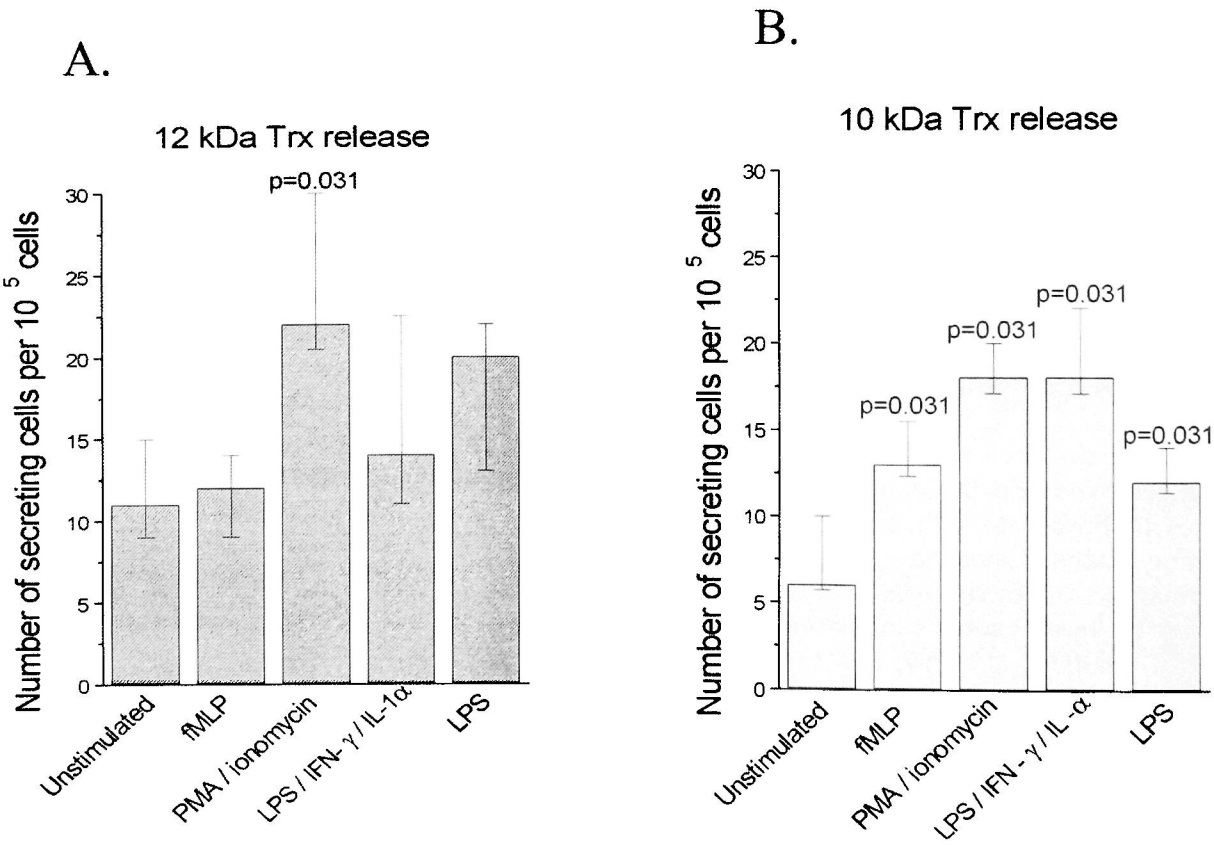

FIG. 1. (A) Secretion of full-length TRX from peripheral blood monocytes detected by ELISPOT. Isolated monocytes from healthy blood donors were stimulated by fMLP (10^{-7} *M*), PMA/ionomycin (0.5 μ*M*/300 ng/ml); LPS/IFN-γ/IL-1α (1 μg/ml, 200 U/ml, 0.5 ng/ml); and LPS (1 μg/ml). Numbers of secreting cells were evaluated by two persons in a double blind manner. Median and quartiles (n = 6) are shown. Significant p values from comparison by the Mann-Whitney *U* test are given. This ELISPOT is representative from three separate experiments. **(B)** Secretion of truncated TRX from peripheral blood monocytes. Cells were stimulated as in (**A**). This is a representative ELISPOT from three separate experiments.

20 (quartiles, 13–22), respectively. The PMA/ionomycin stimulation showed a significant increase ($p = 0.03$, Mann-Whitney U test). Second, we investigated the release of truncated TRX from the same monocyte population as described above (Fig. 1B). Interestingly, the stimulation profile was different compared to 12-kDa TRX. LPS/IFN-γ/IL-1α and PMA/ionomycin stimulation yielded the highest number of secretory cells median = 18 (quartiles, 14–22) and median = 18 (quartiles, 14–20), respectively. Here the background, unstimulated level was lower (median = 6, quartiles, 4–10).

Truncated TRX was secreted from transformed leukocytes

ELISPOT analysis of the T-cell hybridoma cell line MP6, the T-cell leukemia cell line Jurkat, the histiocytic/monocytic cell line, U-937, and EBV-transformed lymphoblastoid cell line, 3B6, showed different levels of secretion. The frequency of 10-kDa TRX-secreting cells was by far the highest in 3B6 cell line (median = 34, quartiles, 27–39) (Fig. 2A). MP6 and U-937 cells also secreted truncated TRX but in lower frequency (median = 10, quartiles 9–13, and median = 9, quartiles, 8–12, respectively). The same profile was seen for 12-kDa TRX release (Fig. 2B).

Oxidative stress induced secretion of truncated and full-length TRX

We also investigated whether oxidative stress induced by the thioloxidant, diamide, tested at sub-apoptotic levels 25–100 μM could induce the secretion of TRX. Secretion of truncated TRX was increased after incubation with diamide in a dose-dependent manner (Fig. 3A). Diamide (100 μM) induced significantly higher number of monocytes to secrete truncated TRX ($p = 0.047$, Mann-Whitney U test). Full-length TRX secretion (Fig. 3B) was also increased in a dose-dependent manner ($p = 0.031$, 0.063, and 0.031; Mann-Whitney U test) for 25 μM, 50 μM, and 100 μM diamide concentrations, respectively.

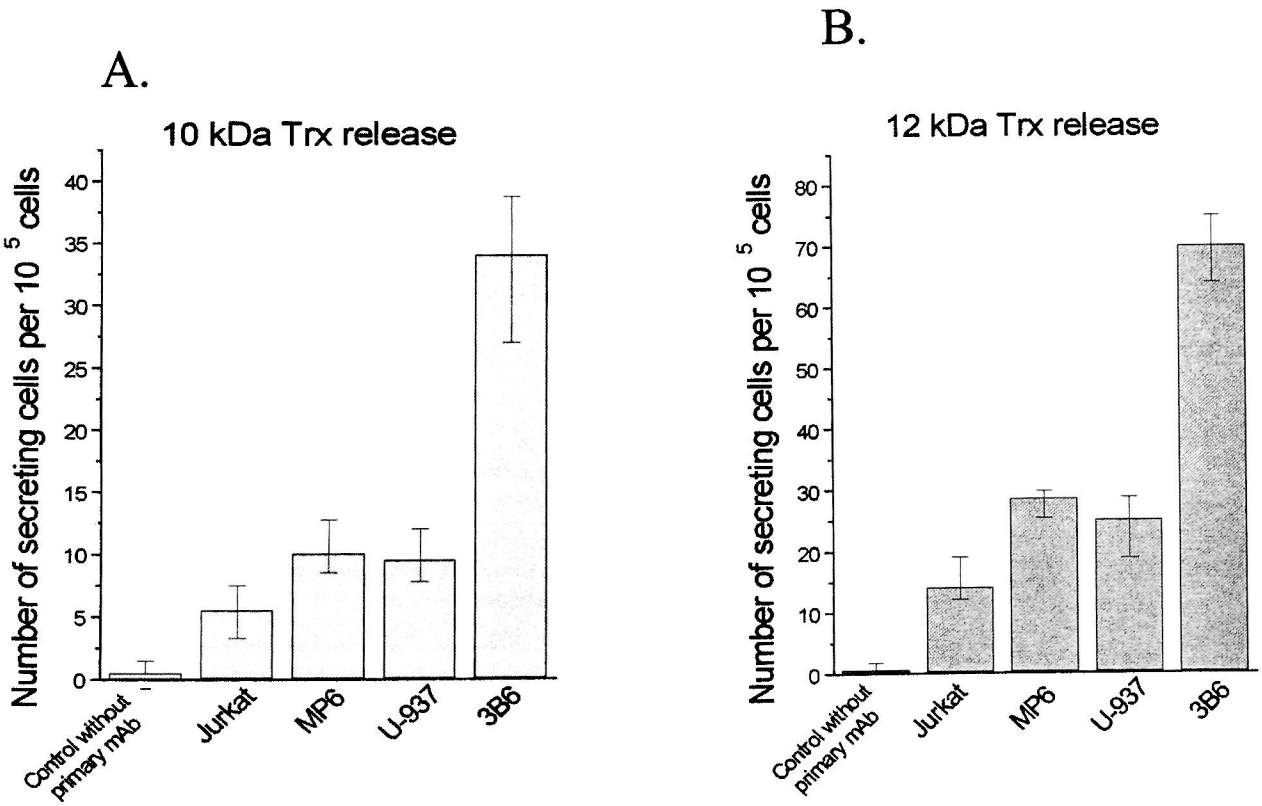

FIG. 2. Secretion of TRX from transformed leukocytes evaluated by ELISPOT. Jurkat, MP-6, U-937, and 3B6 cells were analyzed for the truncated, 10-kDa form by mAb 7D11 (**A**) and for full-length 12-kDa TRX by mAb 2G11 (**B**). The p value for comparison with well with cells without primary antibody is calculated to 0.016 for each cell line, using the Mann-Whitney U test.

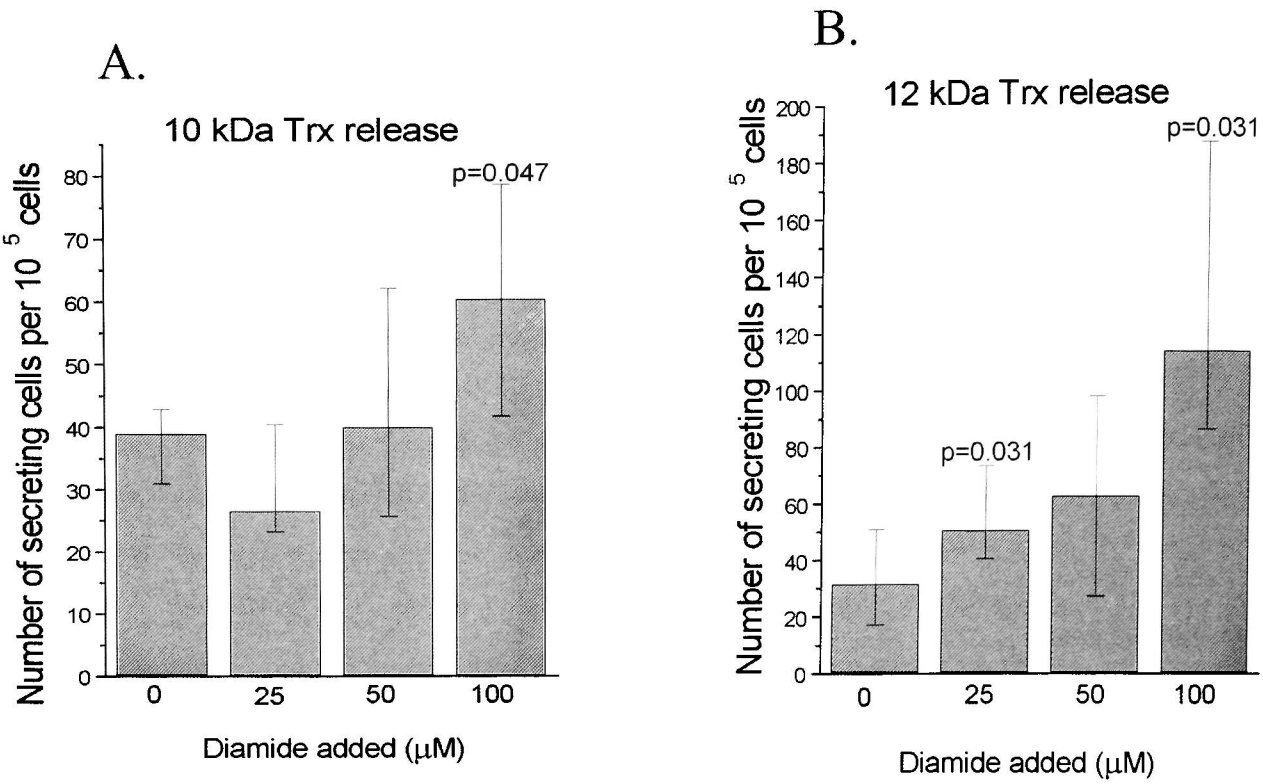

FIG. 3. (A) Oxidative stress release of induced truncated TRX in normal blood monocytes. ELISPOT results are shown as median ± quartiles (n = 6) for each diamide concentration added (0 μM, 25 μM, 50 μM, and 100 μM). Significant p value calculated by the Mann-Whitney U test is given. **(B)** Oxidative stress induced release of full-length TRX in normal blood monocytes. ELISPOT results are shown as median ± quartiles (n = 6) for each diamide concentration added (0 μM, 25 μM, 50 μM, and 100 μM). Significant p values calculated by the Mann-Whitney U test are noted. This is a representative ELISPOT from three separate experiments.

Intracellular TRX staining

To detect the truncated TRX in the secretory cell population, we stained the cell using mAb 7D11. Figure 4 shows that truncated TRX was stained at low intensity.

Cell migration assay

Figure 5 shows that 10-kDa TRX induced directed migration of monocytes: 230 ± 25 (mean ± SE) cells compared to 50 ± 6 for medium control. LPS induced 160 ± 10 cells to migrate.

DISCUSSION

The most important finding of this study is that human TRX was released in a truncated 10-kDa form, as well as a 12-kDa full-length TRX from normal monocytes and transformed leukocytes. A novel ELISPOT method detected single cells secreting TRX. The secretion was inducible by physiological stimuli and oxidative stress.

Early findings by our group and others (Silberstein *et al.*, 1993; Rosén *et al.*, 1995) detected a 10-kDa TRX form. We isolated a mixture of full-length 12-kDa and truncated 10-kDa TRX that synergized with several cytokines (IL-2, IL-4, IL-1, CD40 ligand, TNF-α) in activating B-cell chronic lymphocytic leukemia (B-CLL) (Rosén *et al.*, 1995). Di Trapani, and co-workers (1998) have also recently detected secreted TRX from transformed human cytotrophoblast cells as a 10- and 12-kDa bands on gels.

The sensitive ELISPOT assay, used in this study, allowed detection at the single cell level. Secretion was highly augmented by PMA and ionomycin, compared to unstimulated cells (p = 0.03; Mann-Whitney U test) (Fig. 1A,B). Physiological stimuli (LPS/IFNγ/IL-1α) also

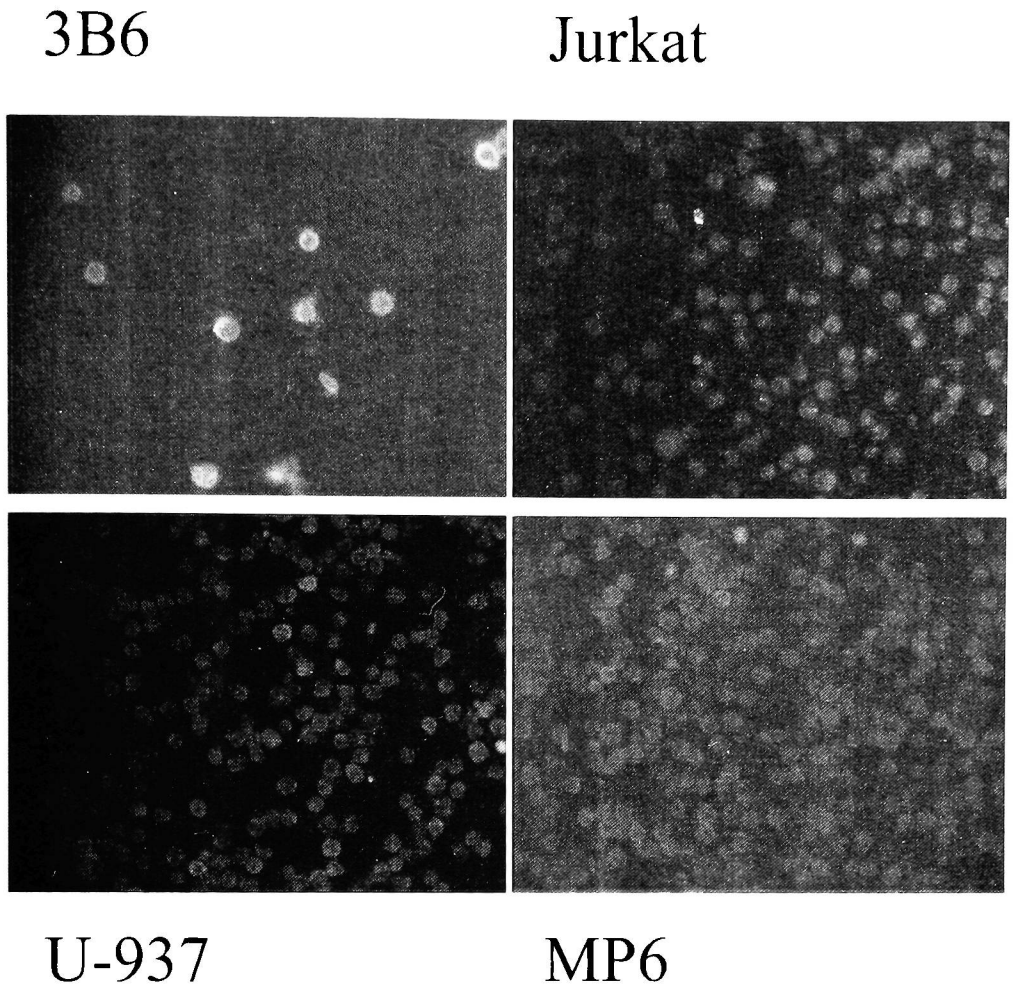

FIG. 4. Intracellular localization of truncated TRX. Jurkat, MP-6, U-937, and 3B6 cells were fixed with 4% paraformaldehyde, stained with 7D11 mAb, and detected with RPE-conjugated goat anti-mouse IgG.

increased the secretion significantly compared to the unstimulated monocytes ($p = 0.03$). It is intriguing that stimulated monocytes/macrophages, besides the full-length TRX, will also secrete a truncated form.

Four cell lines representing different cell types were analyzed with respect to release of 10-kDa TRX (Fig. 2A). All four secreted 10-kDa TRX at various levels (6–34 cells/10^5 cells). Freshly isolated human monocytes (Fig. 3A) responded at a higher level (39 cells/10^5 cells). ELISPOT offers a highly sensitive method for detection and enumeration of single secretory cells (Czerkinsky *et al.*, 1984). In comparison with the ELISA technique, ELISPOT quantifies frequencies of secretory cells in a cell population, whereas ELISA quantifies the concentration of an antigen secreted by a whole population. This concentration represents a mean value of the whole population. In experimental designs, such as used in this study, when nonstimulated cells are being compared with stimulated cells, ELISPOT offers great advantage. For example, a sandwich enzyme-linked immunosorbent assay (ELISA) optimized for the very same Abs (7D11 and goat anti-Trx) had a sensitivity of 4 ng/ml (background + 2 × SD) and failed to detect TRX in 24-hr conditioned media from the four cell lines (data not shown). Intraassay and interassay variations for the ELISPOT method were determined in parallel studies in our laboratory to be 28% and 31%, respectively (Ekerfelt, 1999).

A third immunological assay was used. Intracelullar immunofluorescence results of TRX expression (Fig. 4) obtained on the same four

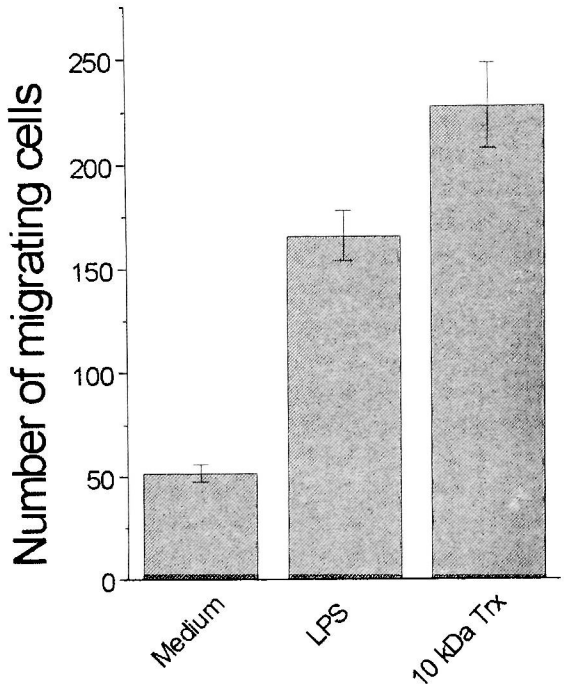

FIG. 5. Migration of peripheral blood monocytes. Five-micrometer PVP membranes were used. Cells were incubated for 2.5 hr at 37°C. Mean ± SE of nine fields of vision are shown.

cell lines indicate that the majority of cells expressed intracellular TRX, of which only a minority released TRX (Fig. 2A,B).

Previously, the 10-kDa, TRX was found to have potent biological activity: >200-fold increase of ECEF activity (Silberstein *et al.*, 1993) and >500-fold increased B-cell stimulatory activity (Rosén *et al.*, 1995) compared to 12-kDa TRX. It is noteworthy that the truncated TRX is devoid of protein disulfide reducing activity (Sahaf *et al.*, 1997), but, as we have shown in this study, retains chemoattractant properties (Fig. 5). Bertini *et al.* (1999) have recently shown chemotactic activity of full-length TRX for granulocytes, monocytes, and T cells. In this study we found that 10-kDa TRX has chemotactic activity for monocytes (Fig. 5). The 10-kDa and 12-kDa secretory forms of TRX may thus attract different cell types, although the 10-kDa TRX must be tested on several cell types before conclusions can be drawn. We have previously shown that truncated TRX does not retain reductase activity, measured by insulin reduction assay. This would implicate a new role for truncated TRX, pointing at an elegant regulatory pathway for reduced TRX in which a carboxy-terminal truncation generates a protein-disulfide reductase negative but biologically active novel TRX molecule. We have also shown that the truncated form not only is present and released from normal monocyte, but also released by transformed leukocyte MP6 and U-937. The EBV-transformed lymphoblastoid cell line 3B6 produced and secreted full-length and truncated TRX at the highest frequency (Fig. 2A,B). The delicate balance inside and outside the cells in our body between oxidants and antioxidants is maintained by well-controlled up- and down-regulation of cellular antioxidants, such as glutathione, superoxide dismutase, catalase, glutathione peroxidase, TRX/TRXR system (Halliwell, 1999). The TRX system seems to play a key role in sensing the redox cellular environment, and it has potent capacity for an extremely rapid and prompt response to these changes. Reduced TRX (TRX-SH_2) can, for example, reduce a protein disulfide bond 10^5 times faster than dithiothreitol (DTT) (Holmgren, 1985), and thus regulate the structure and function of several proteins including transcription factors NF-κB, AP-1, glucocorticoid receptor, surface receptors for IFN-γ (for review, see Nakamura *et al.*, 1997). TRX is also involved in apoptosis regulation (Nilsson *et al.*, 2000). The response to oxidative stress can be effectuated at several levels including mRNA stability, protein transcription, protein stability, *etc.* Oxidative stress induces TRX and nuclear transport of NFκ-B p50 subunit where TRX regulate the DNA binding efficiency through Cys-62 in p50. NFκ-B regulates transcription of several cytokine genes. A common feature of these cytokines is that their mRNA is rapidly turned over, which is regulated by dispersed AUUUA sequence in 3′-untranslated gene region. The presence of these segments correlates to rapid mRNA turnover (Koishi *et al.*, 1997). Human catalase and TRXR contain these regulatory AUUUA sequences (Koishi *et al.*, 1997). In a parallel study, we found that TRXR was rapidly turned on by identical stimuli used in this study and that TRXR was actively secreted extracellularly and could be found in human plasma (Söderberg *et al.*, 2000). There is no evidence for existence of AUUUA sequences in the TRX gene or any alternative

spliced shorter form of mRNA, although Hariharan *et al.* (1996) described the presence of nonproductive species of truncated TRX mRNA. We favor an interpretation that TRX regulation is controlled by protease cleavage at the plasma membrane. This was supported by preferential membrane localization of truncated TRX (Sahaf *et al.*, 1997).

The development of a method for direct analysis of the truncated TRX form opens a way to study new pathways of TRX regulation and also new strategies to find out more about the function(s) of the truncated form of the protein.

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ABBREVIATIONS

ELISPOT, enzyme-linked immune-spot assay; mAb, monoclonal antibodies; Redox, reduction/oxidation; Trx, thioredoxin; TrxR, thioredoxin reductase.

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Address reprint requests to:
Professor Anders Rosén
Department of Biomedicine and Surgery
Division of Cell Biology
Faculty of Health Sciences
Linköping University, S-581 85, Sweden

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