[CANCER RESEARCH 60, 2281–2289, April 15, 2000]

Thioredoxin Reductase, a Redox-active Selenoprotein, Is Secreted by Normal and Neoplastic Cells: Presence in Human Plasma[1]

Anita Söderberg, Bita Sahaf, and Anders Rosén[2]

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

ABSTRACT

Thioredoxin (Trx) and Trx reductase (TrxR) are redox-active proteins that participate in multiple cellular events, including growth promotion, apoptosis, and cytoprotection. Studies on overexpression of Trx and TrxR in human cancers have indicated a role of these proteins in tumor development. In this study, we analyzed the expression of TrxR in peripheral blood cells, tumor-transformed leukemia, and melanoma cells and found, in addition to abundant plasma membrane localization, that TrxR was released from these cells. Secretory cells were observed at the single cell level using a sensitive enzyme-linked immunospot assay. The release was inducible, and physiological stimulation of human monocytes by IFN-γ, lipopolysaccharide, and interleukin 1α significantly increased the number of TrxR-secreting cells ($P = 0.004$). Secretion of TrxR followed the classical Golgi pathway, and it was confirmed by metabolic labeling using [^{35}S]methionine and [^{35}S]cysteine. TrxR was also detected for the first time in fresh healthy blood donor plasma ($n = 21$; median concentration, 18.0 ng/ml), with biological activity as determined by insulin reduction assay.

These results highlight the role of extracellular Trx and TrxR during inflammation and tumor progression. Released Trx, with its active site motif containing amino acids Cys-X-X-Cys, was recently shown to have chemoattractant properties beside its previously described antioxidant and cocytokine activities. Regeneration of oxidized Trx requires available TrxR outside the cell, the presence and induction of which is described in this paper for normal and transformed cells.

INTRODUCTION

The Trx[3] system, consisting of Trx, TrxR, and NADPH, plays a key role in maintaining a reducing cellular milieu (1, 2). Mammalian TrxR was first isolated and purified from rat liver as a dimer with two identical subunits of M_r 58,000 (3). Human placenta TrxR (TrxR1) has been purified and cloned (4). A selenocysteine is present in the penultimate COOH-terminus, and it is important for the enzyme activity (5). All mammalian TrxR are homologous to GSH reductase but with the catalytically active COOH-terminal elongation: -Gly-Cys-SeC-Gly- (6). Recently, novel TrxR family members (TrxR2, TrxR3, TrxRβ) were described (7, 8), of which TrxR3 was of mitochondrial origin. They were also described in parallel by Miranda-Vizuete *et al.* (9) and Lee *et al.* (10).

TrxR substrates, beside Trx, are protein disulfide isomerase and NK-lysin, which is a disulfide-containing effector peptide, derived from T lymphocytes and NK cells (1, 11). Low molecular weight substrates include selenite, selenodiglutathione, vitamin K, S-nitrosoglutathione, and lipoic acid (1).

Constitutive Trx and TrxR expression has been observed in several cell types of the mammalian body, including keratinocytes of the skin, placental cells, liver cells, secretory cells, and leukocytes (12, 13). Physiological stimuli, including UV light, hydrogen peroxide, and mitogens, can induce the expression of Trx and TrxR, pointing at an important role in protection against oxidative stress and in regulating cell growth and cell death (2), and recently, Trx, with its active site motif containing amino acids Cys-X-X-Cys, was shown to have chemoattractant properties (14). TrxR is required for redox regeneration of Trx. A number of human primary cancers, such as adult T-cell leukemia and lung, colon, cervical, and liver cancer, have been shown to overexpress Trx (15), and lung adenocarcinoma was shown to overexpress TrxR (16–18).

Many of the previous techniques for the detection of TrxR were based on polyclonal antibodies or enzyme activities, which were not exclusive for TrxR. Therefore, we have used highly specific novel mAbs against human TrxR (19) in this study to analyze the TrxR expression in normal and malignant human cells. The mAbs recognized TrxR1, but not TrxR2, as described recently (7), nor the mitochondrial TrxR (TrxR3; Refs. 7 and 9).

During the course of this study, we found that TrxR was secreted both from normal and transformed cells *in vitro.* TrxR release, studied at the single cell level, was inducible by physiological stimuli. *In vivo*, it was found in human plasma.

MATERIALS AND METHODS

Materials. Native human TrxR was prepared from human placenta according to previously described methods (3), and it was purchased from IMCO (Stockholm, Sweden). Insulin was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Two mAb clones of IgG1κ, designated anti-TrxR-cl.2 and anti-TrxR-cl.3, were specific for native placenta-derived human M_r 58,000 TrxR (TrxR1; SDS-PAGE and Western blot; Ref. 19), but they did not react with TrxR2 of M_r 65,000, as described by Sun *et al.* (7), nor mitochondrial TrxR, as described by Miranda-Vizuete (9).[4] The clones producing mAbs against human Trx were derived from mouse hybridomas recently established by our research group (A. S., B. S., and A. R.; Ref. 20). The anti-CD23 (EBVCS-2) hybridoma (21) was grown in our lab. The mAbs were purified from hybridoma culture medium by Sepharose-protein A (Pharmacia Biotech, Uppsala, Sweden) affinity chromatography. For stimulation, the following reagents were used: IL-1α, *Escherichia coli* LPS (Sigma Chemical Co., St. Louis, MO), PMA (Biomol Research Labs, Inc., Plymouth Meeting, PA), IFN-γ (Boehringer Ingelheim, Mannheim, Germany), ionomycin (Calbiochem, La Jolla, CA), sodium selenite (Sigma Chemical Co.), and FMLP (Sigma Chemical Co.).

Cell Lines and Culture Conditions. The following cell lines were used in this study: U-937, a human histiocytic/monocytic leukemia cell line (22); $FM55_{M2}$, a human melanoma cell line (23); mouse NIH 3T3 fibroblasts (American Type Culture Collection); THP-1, a human monocytic cell line (24); and FLEB, a human pre-B-cell line (25). The cells were grown in humidified air with 5% CO_2 at 37°C in RPMI 1640 (Life Technologies, Inc., Glasgow, United Kingdom) with 10% FCS (Life Technologies, Inc.), supple-

Received 8/17/99; accepted 2/18/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grant 3171 from the Swedish Cancer Society.

[2] To whom requests for reprints should be addressed, at Department of Biomedicine and Surgery, Division of Cell Biology, Linköping University, S-581 85 Linköping, Sweden. Phone: 46-13-22-2793; Fax: 46-13-22-4314; E-mail: AndRo@mcb.liu.se.

[3] The abbreviations used are: Trx, thioredoxin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; AEBSF, amino-ethyl-benzenesulfonylfluoride; PBS-T, PBS containing 0.05% Tween 20; TNF, tumor necrosis factor; BSS-HEPES, balanced salt solution with 1% HEPES; BSS-HEPES-saponin, BSS-HEPES with 0.1% saponin; FACS, fluorescence-activated cell sorting; APAAP, alkaline phosphatase antialkaline phosphatase; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cell; B-CLL, B-type chronic lymphocytic leukemia; CL, chemiluminescence; ELISPOT, enzyme-linked immunospot assay; GSH, glutathione; HRP, horseradish peroxidase; PFA, paraformaldehyde; TrxR, Trx reductase; mAb, monoclonal antibody; IL, interleukin.

[4] A. Rosén and G. Spyrou, personal communications.

mented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Life Technologies, Inc.). B cells from B-CLL were isolated by Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation of heparinized peripheral blood from B-CLL patients, kindly provided by Kerstin Willander (Linköping, Sweden). Human foreskin keratinocytes were grown in tissue culture medium as previously described (26, 27) and were kindly provided by Dr. Margareta Lirvall (Linköping, Sweden). PBMCs were isolated from healthy blood donor buffy-coat by Ficoll-Hypaque density gradient centrifugation. Monocyte/macrophages were isolated from healthy blood donor buffy-coat according to Freundlich and Avdalovic (28). Briefly, 75-cm^2 tissue culture flasks were coated with 2% gelatin and 10 ml of autologous plasma. The mononuclear cell suspension was added to the flasks for 40 min at 37°C. Nonadherent cells were removed, and adherent monocytes were detached by 5 mM EDTA in PBS. The monocyte/macrophage cell purity was >90%. Monocyte/macrophage cells (20×10^6) were cultured in 20 ml of RPMI 1640 containing 5% FCS for 48 h. For stimulation, PMA was added at a concentration of 0.16 μM. At the end of the incubation, supernatants were collected and centrifuged at $400 \times g$ for 10 min at 4°C. Cell viability was determined by trypan blue dye exclusion, counting the cells in a Bürker chamber.

Blood Samples. Venous blood was collected from 21 healthy blood donor volunteers. The plasma samples were collected in EDTA-containing tubes. The tubes were precooled and immediately transferred to ice (0°C). No hemolysis was detected in plasma. These samples were tested in duplicates and in serial dilutions (1:8, 1:16, 1:32) in a sandwich ELISA for determinations of TrxR concentration and tested for TrxR activity in the insulin reduction assay, as described below.

Tissue Homogenates and Cell Extract Preparation for ELISA and Western Blot. Human placenta and rat liver tissue were collected and immediately frozen and stored at −70°C. Tissue homogenates were prepared on ice (0°C) in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.2 mM AEBSF as a protease inhibitor (Calbiochem, La Jolla, CA). Two ml per g fresh tissue was added. The tissue was homogenized with a Polytron tissue disrupter and centrifuged for 1 h at $150{,}000 \times g$ in a Beckman Optima TLX Ultracentrifuge equipped with a TLA100.2 rotor. The supernatants were kept frozen at −70°C in 1.0-ml aliquots. Cell detergent extracts were prepared from 20×10^6 cells. Cells were washed twice in PBS and lysed in 100 μl of lysing buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% NP40 (Merck, Darmstadt, Germany), 0.5% sodium deoxycholate (Merck), 0.1% SDS (BDH, Poole, United Kingdom), 0.4 mM AEBSF, 10 μg/ml aprotinin (Sigma Chemical Co.), and 1 mM sodium orthovanadate (Sigma Chemical Co.) for 30 min on ice. The cells were then disrupted by repeated aspiration through an 18-gauge syringe needle and further incubated on ice for 30 min.

ELISA for Human TrxR. Ninety-six-well ELISA plates (Costar, Cambridge, MA) were coated with 100 μl/well of anti-TrxR-cl.3 mAbs (5 μg/ml) in carbonate buffer (pH 9.6) for 16 h at 4°C. The plates were rinsed with PBS-T and blocked with 200 μl of PBS containing 3% BSA for 1 h. The wells were rinsed three times with PBS-T and incubated with 100-μl samples serially diluted in PBS containing 0.1% BSA, 0.05% Tween 20, and 0.5 mM AEBSF for 2 h at 4°C. The wells were rinsed three times with PBS-T and then incubated with 100 μl of biotinylated anti-TrxR-cl.2 mAbs (100 ng/ml) for 1 h at room temperature. This mAb has a different epitope specificity compared to the coating mAb. The wells were then rinsed three times with PBS-T and incubated with 100 μl of alkaline phosphatase-conjugated streptavidine (diluted 1:500; Amdex, Amersham Pharmacia Biotech, Rainham, United Kingdom) in PBS containing 0.1% BSA, 0.05% Tween 20. The plates were washed three times in PBS-T and incubated with p-nitrophenyl phosphate (Sigma Chemical Co.) in diethanolamine (pH 9.0) containing 0.5 mM $MgCl_2$ and 0.02% NaN_3 for 30 min. The absorbance was measured at 405 nm in a Multiskan RC photometer Labsystem (Stockholm, Sweden). The method showed linearity from 0.15–40 ng of TrxR/well.

Insulin Reduction Assay for Analysis of TrxR Activity. Insulin reduction assay was performed as previously described (1). In short, a reaction mixture of 40 μl containing 200 μl 1.0 M HEPES (pH 7.6), 40 μl of 0.2 M EDTA, 40 μl of NADPH (40 mg/ml; Sigma Chemical Co.), and 500 μl of insulin (10 mg/ml; Novo Nordisk A/S, Bagsvaerd, Denmark) was mixed with 10 μl of 360 μM *E. coli* Trx (Promega, Madison, WI), and finally, 70 μl of plasma (diluted 1:5 in water) were added, and the volume was adjusted to 120 μl with water and incubated at 37°C for 20 min. The reaction was stopped by adding 500 μl of a stop solution containing 0.4 mg/ml of 5,5′-dithiobis-nitrobenzoic acid (Sigma Chemical Co.) in 6 M guanidine hydrochloride (GuHCl; Sigma Chemical Co.). Absorbance was recorded at $A_{412\,nm}$ in a Beckman DU-640 photometer.

ELISPOT for Detection of TrxR Secretion at the Single Cell Level. ELISPOT assay was performed essentially as previously described (29). Sterile nitrocellulose-bottomed 96-well microtiter plates (Multiscreen HA; Millipore, Danvers, MA) were coated with 100 μl of anti-TrxR-cl.3 (15 μg/ml) in PBS. Anti-human TNF-α mAbs (15 μg/ml; R&D Systems, Minneapolis, MN) were used as a positive control. The wells were then incubated overnight at 4°C. The plates were washed four times with 100 μl/well of sterile PBS. The nitrocellulose were blocked by incubation with 0.5% BSA in PBS for 2 h at 37°C. After emptying, 100 μl of cell suspension in RPMI 1640 with 5% FCS containing 10^5 cells were added to each well. Control cells or stimulated cells (1 μg/ml of LPS; 200 units/ml of IFN-γ; 0.5 ng/ml of IL-1α; 10^{-7} M of FMLP; 0.5 μM PMA; and 10 μg/ml of anti-CD23 mAb or control medium only) were tested, in six replicates, with 5% CO_2 at 37°C in humidified air. After 20 h of incubation, the cells were removed from microtiter plates by two washes in PBS and three washes in PBS-T. Thereafter, the microtiter plates were developed by the addition of 100 μl of biotinylated anti-TrxR-cl.2 or biotinylated anti-hTNF-α, respectively, diluted to 300 ng/ml in PBS-T with 1% BSA and incubated for 2 h at room temperature. After five washes with PBS-T, a final incubation at room temperature for 1 h with 100 μl of Vectastain ABC-AP kit, AP standard AK 5000 (Vector laboratories Inc., Burlingam, CA) prepared in PBS-T with 0.1% BSA was performed. The plates were then washed three times in PBS. Spots were developed using an AP-conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA). The reaction was stopped by rinsing the plates in tap water. The plates were then emptied and dried overnight at room temperature. The numbers of spots were counted blindly by two persons in a Nikon stereo microscope (35× magnification). Mean values of the six replicate wells and SE were calculated. Negative controls included wells without cells. No spots were found in these wells.

CL. CL was measured as previously described by Johansson *et al.* (30). Briefly, cells were incubated at 37°C in a six-channel Biolumat LB9505 (Berthold Co., Wildbad, Germany) instrument using disposable 4-ml polypropylene tubes. The reaction mixture contained 1 ml of cell suspension with 1×10^6 cells in PBS containing 5×10^{-5} M luminol and 4 units/ml HRP. The tubes were allowed to equilibrate for 5 min at 37°C. The cells were stimulated in the same way as the cells used for ELISPOT. The CL response is expressed as cpm and calculated from the integral surface area under each response peak.

Immunoprecipitation and Metabolic Labeling. $FM55_{M2}$ melanoma cells were precultivated in RPMI 1640 medium with 5% FCS for 24 h. Cells (5×10^6) were then resuspended in 5 ml of cysteine- and methionine-free RPMI 1640 medium (Amersham Life Science) with 5% PBS-dialyzed FCS added. Fifty μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine (>1000 Ci/mmol; Amersham Life Science) were added. The supernatants were collected after 24 h. Cells were harvested for extract preparation as described above. The supernatants and cell extracts were preabsorbed with Sepharose-protein A for 1 h and immunoprecipitated using 1 μg of anti-TrxR-cl.3 mixed into 50 μl of a 50% slurry of Sepharose-protein A for 18 h at 4°C. The precipitates were separated in 10–20% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Life Science). Autoradiography exposure was performed at −70°C for 10 days using Kodak X-Omatic intensifying screens and Hyperfilm enhanced CL (Amersham Life Science).

Western Blot. All samples were electrophoresed under reducing conditions in 10–20% SDS-PAGE. After electrophoresis, proteins were transferred to 0.2-μm nitrocellulose membranes (Pharmacia). The membranes were blocked with 5% skim-milk powder (Semper, Stockholm, Sweden) and incubated with anti-TrxR mAbs (50 ng/ml). The bound antibodies were visualized with HRP-labeled antimouse IgG (1:10,000) and an enhanced chemiluminescence detection kit purchased from Amersham Life Science.

Flow Cytometry. Cells were harvested and washed in BSS-HEPES and fixed in a 4% PFA solution for 10 min. After another rinse with BSS-HEPES-saponin, 1×10^6 cells were incubated with 100 μl of unlabeled anti-TrxR-cl.3 (10 μg/ml) diluted in BSS-HEPES-saponin for 30 min according to the method of Sander *et al.* (31). Cells were washed in BSS-HEPES-saponin and incubated with 100 μl of FITC-conjugated $F(ab')_2$ fragment of goat antimouse immunoglobulin (Dako Co., Glostrup, Denmark; F0479; 25 μg/ml) for 30 min. After another rinse with BSS-HEPES-saponin, cells were blocked with 10 μl of

normal mouse serum (Dako, X0910) for 10 min and incubated with 100 μl of biotinylated anti-Trx (10 μg/ml; Ref. 20) for 30 min, followed by two washes and a final incubation with 100 μl of streptavidine-conjugated RPE-Cy5 (Dako, C0050; 10 μg/ml). Cells were analyzed on a FACS Calibur flow cytometer with a secured voltage supply of 220–230 V and further analyzed with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Immunohistological Staining. Immunohistological staining of cells with APAAP. After cytospin preparations, the cells were air-dried for 2–18 h and fixed in 4% PFA in PBS for 10 min. After three washes in Tris-buffered saline [0.05 M Tris-HCl (pH 7.6), 0.15 M NaCl], the cells were incubated for 30 min in a moist chamber at room temperature with 10 μl of anti-TrxR-cl.3 (10 μg/ml). After one wash, the cells were incubated for another 30 min with PBS-dialyzed rabbit antimouse immunoglobulin at 1:50 dilution (Dako, Z259). The cells were washed again and incubated for 30 min in APAAP. Mayer's hematoxylin stain was used as a counterstain for 3 min. The slides were then mounted in aqueous medium for microscopic examination under a Nikon Microphot FXA microscope.

Confocal Three-Dimensional Fluorescence Microscopy. $FM55_{M2}$ melanoma cells were cultured in RPMI 1640 supplemented with 10% FCS on 16-mm diameter coverglasses in a 12-well tissue culture plate (Costar) for 2 days. Cells were prepared for immunofluorescence according to the method of Sander *et al.* (31). Briefly, cells were rinsed in BSS-HEPES and then fixed in a 4% PFA, BSS-HEPES solution for 10 min. After being rinsed with BSS-HEPES-saponin three times, the cells were incubated with 100 μl of anti-TrxR-cl.3 mAb (10 μg/ml) in BSS-HEPES-saponin for 30 min. The cells were washed three times in BSS-HEPES-saponin, incubated with 100 μl of goat antimouse (FITC-conjugated $F(ab')_2$-fragment), 1:20 diluted in BSS-HEPES-saponin for 30 min, and then finally washed in BSS-HEPES three times. For the two-color analysis of TrxR and Trx, each incubation of 30 min at room temperature was followed by two washes in BSS-HEPES-saponin in the following sequence: anti-TrxR-cl.3 mAb (10 μg/ml); FITC-conjugated $F(ab')_2$ fragment of goat antimouse immunoglobulin (12 μg/ml); normal mouse serum (Dako, X0910); biotinylated anti-Trx mAb (10 μg/ml); and streptavidine-RPE-Cy5 conjugate (Dako, C0050; 10 μg/ml). The coverslip glasses were mounted upside down on microscope slides, with 10 μl of antifading solution (glycerol and carbonate/bicarbonate buffer at 1:1 ratio (v/v) containing 2% (w/v) 1.4-diazobicyclo 2.2.2-octane; Sigma Chemical Co.) to reduce the fading of FITC. The cells were examined under a confocal laser-scanning microscope (Phoibos 1000, Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis. Statistical differences between stimulated and non-stimulated control cultures of monocytes or PBMC were evaluated by using 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 Gateway G6–300 PC microcomputer.

RESULTS

Constitutive Cellular TrxR Expression. Trx and TrxR are ubiquitous redox-active, cytoprotective proteins that act in synergy. Trx-expression is rapidly up-regulated, and Trx is secreted by normal and tumor transformed upon oxidative stress, mitogen, cytokine, UV light, and virus exposure (32–34). However, TrxR and Trx protein patterns of expression are not congruent, and little is known how TrxR expression is regulated and whether it is actively released.

Table 1 *TrxR and Trx expression in cells determined by flow cytometry*[a]

Cell type	TrxR (MFI)[c]	Trx (MFI)[c]
Normal monocytes[b]	104	153
Normal lymphocyte[b]	38	35
U-937	681	765
FLEB pre-B cell	353	1268

[a] This experiment represents one of five separate flow cytometry experiments in which different blood donors were used together with U-937 and FLEB, staining for TrxR and Trx. Twenty-five thousand double-stained cells were analyzed by FACS.
[b] Ficoll-Hypaque-separated blood cells were derived from a healthy donor. Monocytes and lymphocytes were gated on their forward scatter and side scatter characteristics.
[c] Background isotype controls were subtracted.

Table 2 *Immunohistological staining of TrxR in human blood cells by the APAAP technique*[a]

Cell type	TrxR expression[b]
Monocytes	+++
Lymphocytes	++
Neutrophils	+
Eosinophils	++
Erythrocytes	+
Platelets	–
U-937 (positive control)	+++++
THP-1 (positive control)	+++++

[a] Isotype IgG control was negative. The evaluation was performed separately by two persons. The immunohistological staining represents one of three separate experiments.
[b] –, negative; +, low intensity staining; ++ and +++, moderate intensity staining; +++++, very high intensity staining.

Table 3 *TrxR content in cell detergent lysates determined by sandwich ELISA*[a]

Cell type	TrxR (ng/mg total protein)
Human skin keratinocytes	31.1 ± 6.0
Human skin melanocytes	57.8 ± 0.1
Human placenta extract	41.3 ± 0.1
U-937 monoblastic cell line	95.2 ± 13.7
B-CLL cells	44.0 ± 0.7
$FM55_{M2}$ melanoma cell line	62.6 ± 11.6
Mouse fibroblast	<2
Rat liver extract	<2

[a] The results represent mean values ± SE of two or three separate experiments each performed in serial dilutions of cell extracts in duplicates.

Therefore, we first analyzed constitutive TrxR expression in comparison with Trx expression normal and transformed cells by two-color flow cytometry (Table 1). The transformed cell lines, U-937, a monocytic leukemia-derived cell line, and FLEB, an EBV-transformed pre-B-cell line, showed much higher TrxR expression with MFI values of 681 and 353, respectively, compared to their normal cellular counterparts, peripheral blood monocytes, and lymphocytes, which had MFI values of 104 and 38, respectively. Thus, U-937 monocytes had >6-fold higher TrxR expression, compared to normal monocytes. In these transformed cells, Trx levels were also higher compared to their normal cellular counterparts (Table 1), but no strict covariation between Trx and TrxR expression was found. Microscopic inspections (Table 2) using immunohistological staining of peripheral blood cells confirmed that monocytes were the most intensely stained cells. Platelets had no detectable TrxR. U-937 and THP-1 showed much higher TrxR expression than healthy blood donor peripheral blood cells did (Table 2).

Quantitative determinations of human TrxR (Table 3) by ELISA in cell detergent extracts and tissue homogenates of various origins revealed that the U-937 monocytes contained the highest amount of TrxR: 95.2 ± 13.7 (SE) ng/mg of total protein. TrxR concentrations varied from 31.1 to 95.2 ng/mg of total protein. Intracellular TrxR content was higher in malignant melanoma cells compared to normal human skin keratinocytes but similar to that of normal melanocytes (Table 3). In separate experiments, comparisons of normal monocytes and lymphocytes to the transformed monoblastic U-937 and pre-B-cell lines, FLEB, confirmed FACS and immunohistochemical findings (data not shown). Rat liver extracts and a mouse fibroblast cell line were used as negative controls. The anti-TrxR-cl.2 and anti-TrxR-cl.3 mAbs used in this study did not cross-react with other mammalian TrxR.

The distribution of TrxR in the cells was investigated by three-dimensional confocal fluorescence microscopy. In melanoma cells, we found a high intensity of TrxR staining in the cytoplasm and in the cell membrane, localized to the cell-matrix adhesion sites/focal adhesion sites. Images from 16 different focal planes were collected, and two of them are represented in Fig. 1, *A* and *B*. The first micrograph

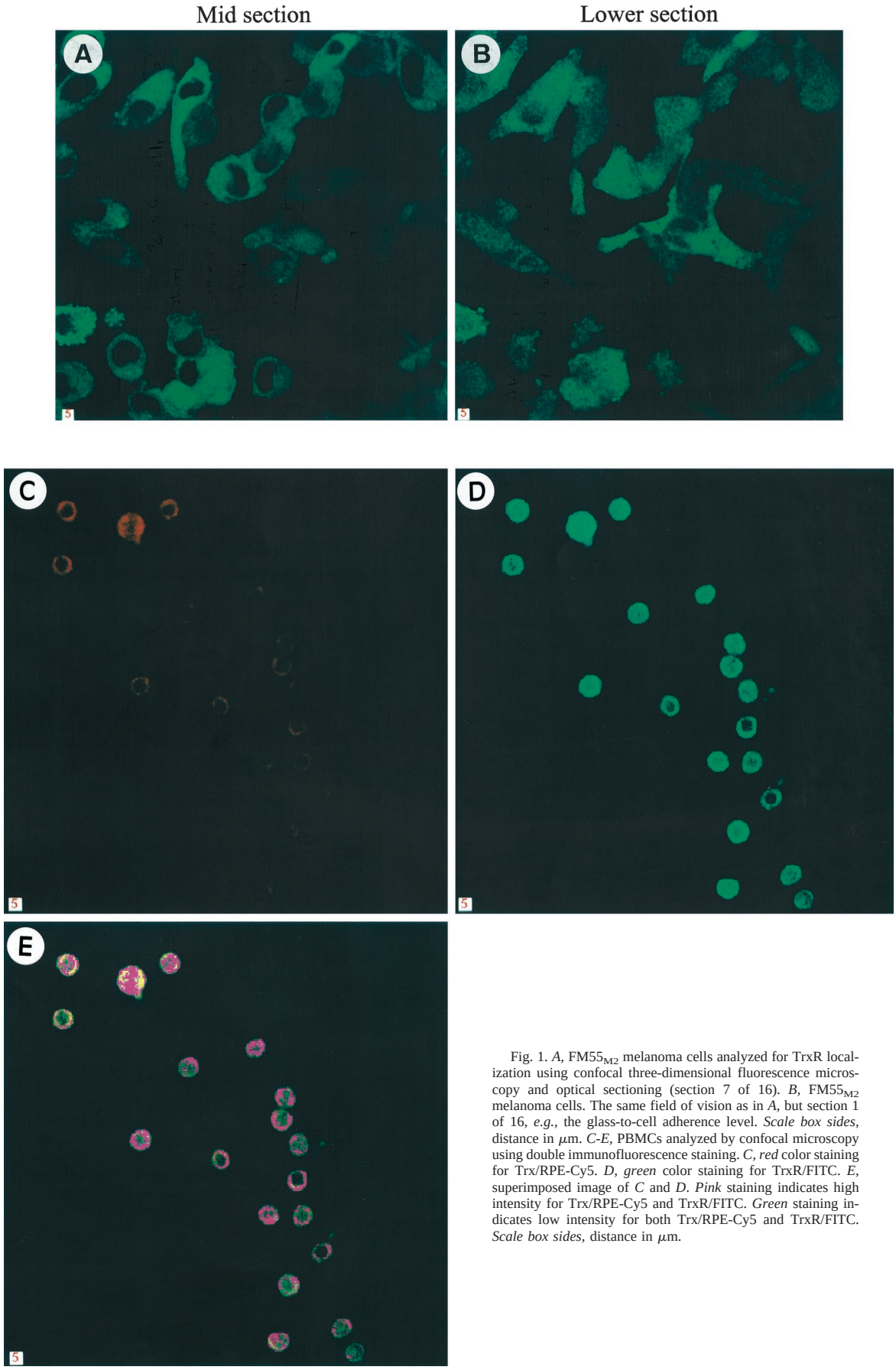

Fig. 1. *A*, $FM55_{M2}$ melanoma cells analyzed for TrxR localization using confocal three-dimensional fluorescence microscopy and optical sectioning (section 7 of 16). *B*, $FM55_{M2}$ melanoma cells. The same field of vision as in *A*, but section 1 of 16, *e.g.*, the glass-to-cell adherence level. *Scale box sides*, distance in μm. *C-E*, PBMCs analyzed by confocal microscopy using double immunofluorescence staining. *C*, *red* color staining for Trx/RPE-Cy5. *D*, *green* color staining for TrxR/FITC. *E*, superimposed image of *C* and *D*. *Pink* staining indicates high intensity for Trx/RPE-Cy5 and TrxR/FITC. *Green* staining indicates low intensity for both Trx/RPE-Cy5 and TrxR/FITC. *Scale box sides*, distance in μm.

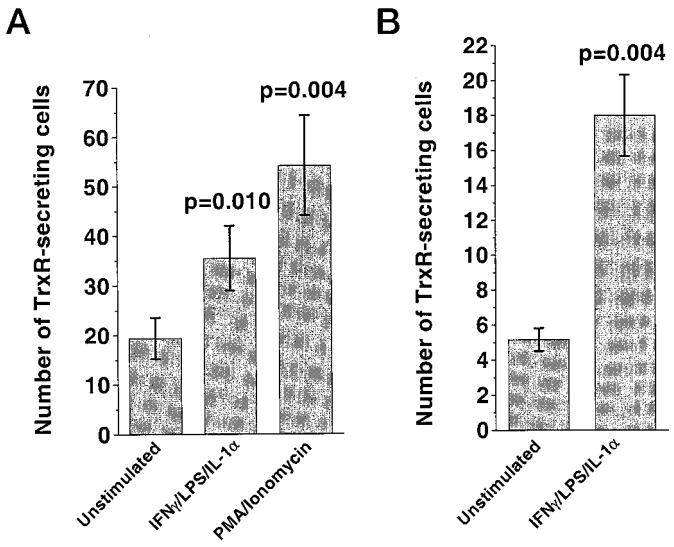

Fig. 2. *A*, number of TrxR-secreting cells generated per 10^5 PBMCs during 20-h incubation by ELISPOT. Nonstimulated (5% FCS in RPMI 1640) or upon stimulation with IFN-γ (200 units/ml), LPS (1 μg/ml), and IL-1α (0.5 ng/ml) or with PMA (0.5 μM) and ionomycin (300 ng/ml). *P*s show statistically significant differences between nonstimulated and stimulated peripheral blood lymphocytes (Mann-Whitney *U* test). *Error bars*, SE. *B*, number of TrxR-secreting cells generated per 10^5 peripheral monocytes during 20-h incubation by ELISPOT. Nonstimulated (5% FCS in RPMI) or upon stimulation with IFN-γ (200 units/ml), LPS (1 μg/ml), and IL-1α (0.5 ng/ml). *P*s show statistically significant differences between nonstimulated and stimulated peripheral blood lymphocytes (Mann-Whitney *U* test). *Error bars*, SE.

shows an image derived from the $FM55_{M2}$ melanoma cell line at a mid transsection level (Fig. 1*A*, section 7 of 16). Fig. 1*B* visualizes TrxR expression at the cell surface located at adhesion sites at the glass contact. In this cell line, grown to near confluence, TrxR was localized at the surface and in the cytoplasm. TrxR was highly concentrated in the lower level image. Comparing PBMC to U-937 and $FM55_{M2}$ cells, we found that PBMC expressed low constitutive levels of TrxR only and that TrxR was more diffusely localized throughout the cells. In U-937 cells, TrxR was overexpressed and distributed both in the cytoplasm and nucleus, although some nuclei were devoid of TrxR (data not shown). Detection of Trx and TrxR by two-color immunofluorescence using an anti-Trx mAb-biotin-conjugate stained by streptavidine-R-phycoerythrin-Cy5 (Fig. 1*C*, red fluorochrome) and anti-TrxR-cl.3 mAb detected by FITC-labeled goat antimouse immunoglobulin (Fig. 1*D*, green fluorochrome) was performed as detailed in the "Materials and Methods" section. Results revealed that maximum expression of TrxR and Trx was found in the plasma membrane (Fig. 1*E*, overlay image).

Inducible TrxR Secretion. Secondly, based on previous observations that several cell types such as lymphocytes and hepatocytes secreted Trx, and the fact that Trx requires TrxR for maintenance of its reduced active state, we wanted to investigate whether TrxR was secreted from cells. TrxR secretion was analyzed in cultures from PBMCs and monocytes after 20-h incubation at 37°C in an ELISPOT assay detecting secretory cells at the single cell level. PBMCs and monocytes were stimulated by IFN-γ, LPS, and IL-1α. We found a significant increase in number of TrxR-secreting cells, compared to cultures without stimuli. Mean number of TrxR secretory PBMCs after stimulation was 41 $\pm$ 4 (SE)/10^5 cells compared to control cultures [23 $\pm$ 3 (SE); $P = 0.010$; Fig. 2*A*]. For purified monocytes, corresponding values were 18 $\pm$ 2 (control, 5 $\pm$ 0.7; $P = 0.001$; Fig. 2*B*). An increased TrxR release was also found with PMA/ionomycin (Fig. 2*A*). We also explored the effect of alternative stimuli, previously reported to activate human monocytes (Table 4). Reactive oxygen species released were determined directly after stimulation in a CL assay. Parallel identically stimulated cultures were analyzed for the number of TrxR-secreting cells in ELISPOT after 20 h. There was a dose-dependent response observed: increased CL response induced more TrxR secretory cells (correlation coefficient $r = 0.85$).

The quality of the ELISPOT assay was determined by including several positive and negative controls: (*a*) The secretory capacity of the cells were determined by analyzing TNF-α secretion in parallel identically stimulated cell cultures. A doubling of TNF-α-specific spots was found after IFN-γ/LPS/IL-1α stimulation compared to nonstimulated cells (data not shown). (*b*) Wells containing catcher and indicator mAbs with substrate did not give any spots in the absence of cells. (*c*) Only viable cells were secreting TrxR or TNF. Necrotic cells did not give any spots at all as determined in detergent-lysed cells (0.1% Tween 20). (*d*) The specificity of the two anti-TrxR mAbs were carefully controlled in separate immunoprecipitation experiments showing intracellular TrxR from cell extracts (C) and secreted TrxR (S) in monocytes and PMA-stimulated monocytes appearing as a single M_r 58,000 protein band with identical molecular weight as compared to purified human placenta TrxR (TrxR1; Fig. 3).

To analyze the TrxR secretion in detail, we performed metabolic labeling of $FM55_{M2}$ cells followed by immunoprecipitation. Results revealed that TrxR was actively secreted into the medium during 24 h (Fig. 4). The autoradiograph showed that intracellular TrxR had a slightly higher molecular weight, compared to secreted TrxR in the $FM55_{M2}$ melanoma cell line (Fig. 4, *left side*). The very same sample of $FM55_{M2}$ 24-h supernatant was also separated and analyzed by Western blot (Fig. 4, *right side*), showing identical molecular weights of TrxR compared to metabolic labeling. Purified placenta TrxR was used as positive control. In separate stimulation experiments, we titrated sodium selenite and checked the effect of these additions to

Table 4 *Reactive oxygen species determined by CL followed by analysis of number of TrxR-secreting cells*[a]

Stimuli	CL intensity[b] (cpm $\times$ 10^{-7})	No. of TrxR-secreting cells[c] (per 10^5 cells)
Unstimulated control	2.201	3 $\pm$ 0.5
FMLP[d]	10.71	13 $\pm$ 3
PMA/ionophore[e]	320.6	63 $\pm$ 13
LPS/IFN-γ/IL-1α[f]	1.878	16 $\pm$ 2
CD23-ligation[g]	13.96	53 $\pm$ 9

[a] A statistical evaluation by linear regression gives: $r = 0.85$, and $0.01 < P < 0.05$.
[b] CL intensity was calculated from the integral surface area under the response peak.
[c] The numbers represent mean value of six replicates $\pm$ SE.
[d] Concentration was 10^{-7} M.
[e] Concentrations were 0.5 μM and 300 ng/ml, respectively.
[f] Concentrations were 1 μg/ml, 200 units/ml, and 0.5 ng/ml, respectively.
[g] Concentration was 10 μg/ml of anti-CD23 mAb.

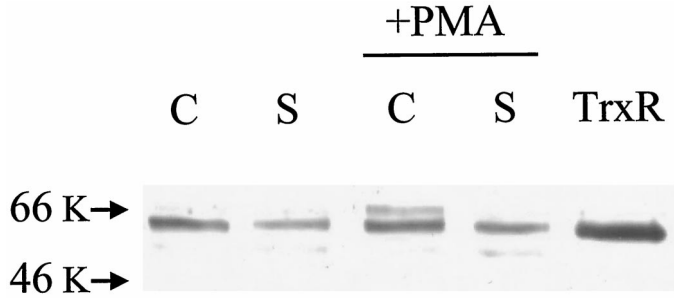

Fig. 3. Secretion of TrxR by peripheral blood monocytes in RPMI 1640 medium with 5% FCS. Human monocytes (40×10^6) were cultured for 4 days with or without PMA (0.16 μM). Cells (*C*) and supernatants (*S*) were harvested, immunoprecipitated, and analyzed by SDS-PAGE and Western blot. The Western blot was developed with biotin-anti-TrxR-cl.3 plus streptavidine-HRP and CL. TrxR denotes purified human placenta TrxR.

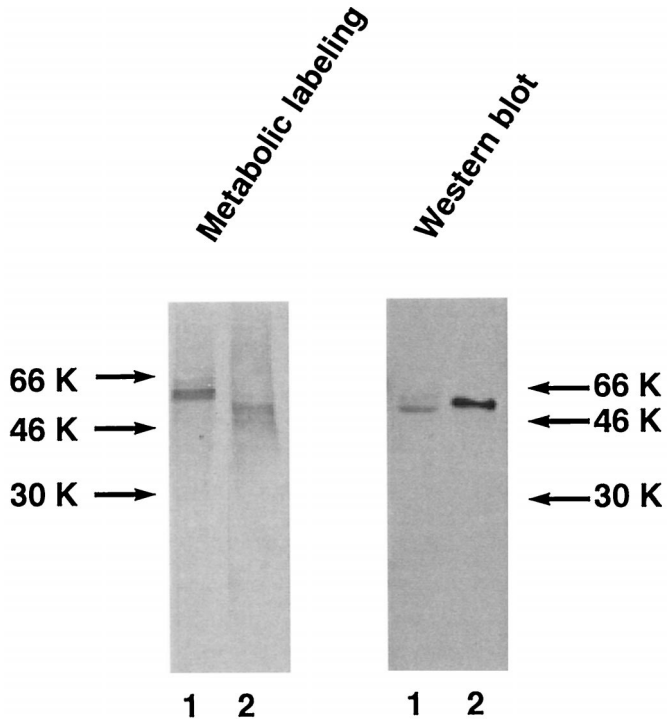

Fig. 4. Secretion of TrxR studied in the melanoma cell line $FM55_{M2}$. *Left side* (*metabolic labeling*), immunoprecipitation of L-[^{35}S]methionine and L-[^{35}S]cysteine metabolically labeled TrxR using anti-TrxR-cl.3 mAb. *Lane 1*, cell lysate/extract. *Lane 2*, 24-h supernatant from melanoma cell line $FM55_{M2}$. *Right side* (*Western blot*), immunoprecipitation followed by Western blot analysis. *Lane 1*, 96-h supernatant. *Lane 2*, purified control TrxR. Western blot was developed by biotin-anti-TrxR-cl.3 plus streptavidine-HRP and CL.

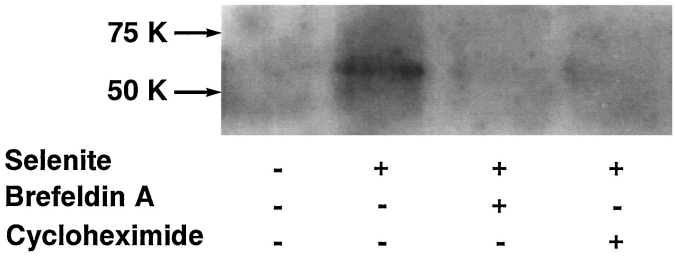

Fig. 5. Secretion of TrxR from peripheral blood monocytes stimulated by 10 μM selenite. Monocytes (13×10^6) were cultured for 20 h with and without selenite in the presence or absence of the Golgi and endoplasmic reticulum vesicular transport inhibitor, Brefeldin A (5 μg/ml), or the protein synthesis inhibitor, cycloheximide (10 μg/ml). Conditioned media were harvested, immunoprecipitated by anti-TrxR-cl.3 mAb, and analyzed by Western blot after SDS-PAGE using biotin-anti-TrxR-cl.2 plus streptavidine-HRP. Detection was enhanced by enhanced CL.

the various cell cultures using low FCS concentrations (2.5%). Monocytes were the most resistant cells and tolerated up to 10 μM of selenite for 20 h without negative effects on cell viability. But >10 μM, we found an apoptotic effect. Other cells did not tolerate >5 μM (data not shown). Twenty-h exposure of human monocytes to 10 μM selenite induced a TrxR secretion (Fig. 5). The secretion of TrxR follows the classical Golgi pathway, as indicated by a secretory block induced by the Golgi pathway inhibitor, Brefeldin A (5 μg/ml). This concentration of Brefeldin A was pretested to ensure that it would not affect the viability of the cells. As a positive control for blocked protein synthesis, we used the inhibitor cycloheximide (10 μg/ml), which clearly stopped TrxR synthesis. TrxR release contrasts to Trx-release, which previously was shown to follow an alternative, non-Golgi pathway (33).

TrxR Present in Human Blood. The finding that TrxR could be actively secreted upon physiological stimulation *in vitro* prompted us to explore whether TrxR could be detected in the circulation of normal healthy blood donors. We analyzed, in the specific TrxR ELISA, EDTA-plasma samples from 21 healthy blood donors. Serial dilutions of the plasma samples were analyzed in duplicates. The results obtained from these samples showed that median plasma TrxR was 18.0 ng/ml (Table 5). Biological activity in the plasma samples were tested in insulin reduction assay (1). Trx-dependent reducing activity was found in three separate experiments in the plasma of donor 18, whereas the other normal blood donor plasma were below or at the detection limit. Sample 18 had the highest TrxR ELISA concentration value of 578.5 ng/ml. The reducing capacity in this plasma corresponded to 18.8 nmol of oxidized NADPH/ml plasma.

DISCUSSION

The most important finding of this study is that normal and transformed cells can be induced to secrete TrxR, and we have described for the first time the presence of TrxR in human blood donor plasma. Secretion was found in cells that overexpressed TrxR, including normal monocytes, PBMCs, tumor-transformed monocytic leukemia, and melanoma cell lines (Table 3). It was not until we explored a sensitive single cell assay originally designed for secretory cytokines that we found TrxR release *in vitro*.

These results add a quantitative aspect to previous findings that tumors of certain lineage express TrxR protein (35) or TrxR mRNA (36). Investigating TrxR in normal blood cells, we found the highest expression in monocytes, whereas platelets showed low or no expression (Table 2). Comparing our previous data on Trx expression and release (19, 20, 32), we conclude that Trx and TrxR expression levels are incongruent and do not correlate in several normal and tumor-transformed cell lines (Table 1; Fig. 1*E*).

During the course of this study, we obtained evidence for an adjustable and inducible secretion of TrxR in normal PBMCs, monocytes, and tumor-transformed U-937 monocytic leukemia and melanoma $FM55_{M2}$ cell lines. In monocytes, the secreted TrxR had an identical molecular weight with placenta TrxR (TrxR1; Fig. 3). $FM55_{M2}$ cell lines secreted TrxR at a slightly lower molecular weight of M_r 55,000 (Fig. 4). U-937 secreted TrxR of the same molecular weight as $FM55_{M2}$ (data not shown). The reason for the lower molecular weight of the secretory form is presently unknown, but may be explained by an induction of minor conformational changes during the secretory pathway, which results in slightly altered electrophoretic

Table 5 *TrxR level in plasma of healthy blood donors as determined by sandwich ELISA*

Donor no.	TrxR (ng/ml)[a]
1	18.0 ± 3.4
2	23.2 ± 2.3
3	29.0 ± 1.6
4	53.2 ± 1.5
5	20.8 ± 1.2
6	27.0 ± 2.9
7	18.3 ± 1.0
8	12.4 ± 0
9	5.7 ± 0.3
10	130 ± 0
11	26.5 ± 1.3
12	10.0 ± 0.6
13	12.3 ± 1.6
14	<2
15	<2
16	18.9 ± 1.6
17	11.8 ± 1.9
18	578.5 ± 17.5
19	11.7 ± 0
20	4.9 ± 0
21	9.5 ± 0.9

[a] The results represent mean values ± SE. Median = 18.0 ng/ml.

mobility. The immunological epitope structure is, however, unchanged because the mAb recognized only one protein band, regardless of whether the proteins were derived from cell extract or from supernatant (Fig. 4).

We studied the regulation of TrxR secretion in the PBMC and purified monocyte at the single cell level (Fig. 2, *A* and *B*). The number of cells secreting TrxR increased significantly after stimulation by a combination of IFN-γ, LPS, IL-1α, and PMA/ionomycin (Fig. 2*A*). In purified monocytes, stimulation by IFN-γ, LPS, and IL-1α resulted in a 4-fold increase in the number of TrxR-secreting cells (Fig. 2*B*). There was a direct correlation between the amount of reactive oxygen species released by the PBMCs after stimulation and the number of TrxR-releasing cells (Table 4). The presence of TrxR in healthy blood donor plasma strengthens our findings of extracellular TrxR. Because TrxR is light-sensitive, thermosensitive, and proteolytic-sensitive, methodological attention was paid to the careful blood sampling procedure (0°C, dark, proteolytic inhibitors) to avoid rapid degradation as well as hemolysis, which may cause TrxR and Trx release because it is known that erythrocytes contain TrxR as well as Trx (37, 38). These precautions in combination with our use of sensitive ELISA for TrxR may explain why plasma TrxR has not been reported until now. The secretory form of TrxR, which we found in human plasma, was biologically active as determined by the insulin reduction assay. The TrxR activity in the plasma of donor 18 corresponded to 18.8 nmol of oxidized NADPH/ml (ELISA value, 578.5 ng/ml). Due to the lower sensitivity of the insulin assay compared to the TrxR ELISA (0.95 pM *versus* 0.02 pM, *e.g.*, 110 ng/ml *versus* 2 ng/ml), most healthy blood donor plasma were below the detection limit. Median concentration by ELISA was determined as 18.0 ng/ml, *e.g.*, 0.16 pM (Table 5). These findings are in line with early observations by Apffel and Walker in 1973 (39), who reported that NADPH-dependent protein-disulfide reductase activity was found in ascites and sera from tumor-bearing mice, whereas normal mouse sera contained no activity. Holmgren, 1977 (40) pointed out that the protein-disulfide reductase activity found in those tumor-bearing animals was, by several criteria, identical to TrxR. Our findings in this study that TrxR can be detected in healthy blood donor plasma using sensitive sandwich ELISA highlights the importance of secreted TrxR during normal physiological responses in addition to its importance in tumor growth regulation.

First, it was previously shown that TrxR is an efficient electron donor to human plasma GSH peroxidases, which are important enzymes in the detoxification of hydroperoxides (41). GSH peroxidase is a selenoprotein considered to be very specific for GSH as an electron donor. However, plasma GSH is very low (<0.5 μM), suggesting that the function of extracellular GSH peroxidase is completely dependent on other electron donors, such as plasma TrxR. But the presence of plasma TrxR has not been shown until now.

Secondly, the release of TrxR from monocytes suggests that secretion of TrxR has a cytoprotective antioxidant role for monocytes, avoiding suicide by reactive oxygen species released during an inflammatory response, including oxidative burst. We tried to mimic physiological stimuli by exposing the cells to IFN-γ, LPS and IL-1α, FMLP, and CD23-ligation. Previously, it was shown that a cytotoxic peptide, NK-lysin, which is derived from NK cells and T cells, is effectively neutralized by TrxR through reduction of a disulfide that is necessary for its biological activity (11, 42). One may postulate that secreted Trx and TrxR membrane expression may dampen an immune attack delivered by NK or T cells via neutralization of NK-lysin, thereby avoiding self-destruction.

The reverse side of the coin is that this mechanism may be used by neoplastically transformed cells during tumor progression. It would favor immune escape variants of tumor cells that overexpress TrxR. Our results correlate with findings by Koishi *et al.* (16), who describe a novel reductase released from COS cells and human lung adenocarcinoma cell line A549. The reductase secretion was also inducible by PMA, LPS, and IL-1β. These authors did not report on the identity of KM-102-derived reductase-like factor with TrxR. Miranda-Vizuete and Spyrou (17) brought this identity to attention, however.

We included in this study a more detailed analysis of the intracellular distribution and localization of TrxR by three-dimensional laser scanning confocal microscopy because abundant plasma membrane TrxR was observed. Electron microscopic studies using these anti-TrxR mAbs also confirmed a plasma membrane enrichment of TrxR.[5] Optical sectioning of the melanoma cell line $FM55_{M2}$ showed enrichment of the TrxR at cell-matrix adhesion points when compared to the rest of the cell cytoplasm and membrane (Fig. 1*B*). TrxR may be important in keeping the Trx reduced at these sites. It has been reported that at these attachment sites, focal adhesion complexes are formed, which contain redox-regulated focal adhesion kinases important for the cytoskeleton formation (43). Whether Trx/TrxR are involved in their redox-regulation would be important to investigate. It was also recently shown that transfection of the breast cancer cell line MCF-7 with a dominant-negative redox-inactive Trx construct inhibited anchorage independence in these cells, but it did not affect monolayer growth *in vitro* and almost completely inhibited tumor growth *in vivo* (44).

The role of Trx/TrxR in promoting tumor progression is multiple. First, Trx is a proton donor for ribonucleotide reductase (45). Secondly, Trx was proposed to have an antiapoptotic function through reduction of intracellular hydrogen peroxide, which is a mediator of apoptosis. This reduction operates via a novel inhibitor of apoptosis, termed Trx-peroxidase (46), a pathway distinct from that of Bcl-2. In addition, a recent finding by Saitoh *et al.* (47) showed that mammalian Trx is a direct inhibitor of apoptosis signal-regulating kinase. Thirdly, Trx can induce cytokine release (48), which confers maintenance of Bcl-2 expression (49). Because Trx regeneration depends on proper reduction by TrxR and NADPH, the two proteins act in synergy. It is therefore reasonable and logical that there should be TrxR available in extracellular fluids for maintenance of reduced and active Trx ($Trx-SH_2$).

From a cancer therapeutic point of view, it is known that TrxR activity can be irreversibly inhibited by several agents, such as 13-*cis* retinoic acid (50), and these agents are presently being explored in cancer therapy. The blocking of TrxR activity in HTLV-1-transformed T-cell lines, which is known to express high Trx levels, inhibits cell cycle progression (51). In addition, Schallreuter and Wood (35) reported good prognosis after nitrosourea treatment of TrxR-producing melanomas. On the other hand, Ueda *et al.* (52) have shown that a cellular-reducing environment maintained by the Trx system, as well as GSH, is required for caspase-3 activity to induce apoptosis.

Selenium compounds like selenite have dual and opposing effects on cell growth. Strong inhibitory effects, particularly on mammalian tumor cell growth, are observed at doses >10 μM, in contrast to the stimulatory effects of both normal and tumor cells found at low (nM range) concentrations (53). The mechanisms are yet incompletely characterized, but the known inhibitory effects include NADPH depletion, competitive inhibition of TrxR, and oxidation of catalytic and structural cysteines in human Trx (54). The stimulatory effect of selenite at low doses may be explained by facilitated synthesis of selenoproteins like TrxR and GSH peroxidase (54). Dietary supplementation of selenium had preventive effects on certain cancers (55).

[5] M. Björnstedt, personal communications.

In this study, we found an overexpression followed by release of TrxR from monocytes exposed to selenite up to 10 μM concentration (Fig. 5).

In summary, we have obtained direct evidence that TrxR is secreted by normal blood monocytes, PBMCs, and by malignant leukemia and melanoma cell lines, and the most important message from this study is that we show for the first time that TrxR can be found in healthy blood donor plasma at the ng per ml level. These findings may cast new light both on the regulation of the antioxidant defense system during inflammation and on the complex issue of immune escape strategies observed in several types of cancer, in that TrxR release and overexpression may counteract apoptosis induced by oxidative stress (56). This information may also be important for designing TrxR inhibitors for cancer therapy.

ACKNOWLEDGMENTS

We thank Dr. Giannis Spyrou for helpful comments on the experiments and for testing our anti-TrxR monoclonal antibodies for reactivity against mitochondrial TrxR. We extend our thanks to Dr. Mikael Björnstedt for helpful comments on the manuscript; Anita Lönn and Inga-Lill Scherling for skillful technical assistance; Joacim Nilsson, B.Sc., for help with the fluorescence-activated cell-sorting analysis; Dr. Kajsa Peterson-Holmgren for help with the confocal microscopy; and Dr. Christina Ekerfeldt for help with ELISPOT.GSD.

REFERENCES

1. Holmgren, A., and Björnstedt, M. Thioredoxin and thioredoxin reductase. Methods Enzymol., *52:* 199–208, 1995.
2. Nakamura, H., Nakamura, K., and Yodoi, J. Redox regulation of cellular activation. Annu. Rev. Immunol., *15:* 351–369, 1997.
3. Luthman, M., and Holmgren, A. Rat liver thioredoxin and thioredoxin reductase: purification and characterization. Biochemistry, *21:* 6628–6633, 1982.
4. Gasdaska, P. Y., Gasdaska, J. R., Cochran, S., and Powis, G. Cloning and sequencing of a human thioredoxin reductase. FEBS Lett., *373:* 5–9, 1995.
5. Gladyshev, V. N., Jeang, K. T., and Stadtman, T. C. Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene. Proc. Natl. Acad. Sci. USA, *93:* 6146–6151, 1996.
6. Zhong, L., Arnér, E. S. J., Ljung, J., Åslund, F., and Holmgren, A. Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. J. Biol. Chem., *270:* 11761–11764, 1998.
7. Sun, Q-A., Wu, Y., Zappacosta, F., Jeang, K-T., Lee, B. J., Hatfield, D. L., and Gladyshev, V. N. Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. J. Biol. Chem., *274:* 24522–24530, 1999.
8. Gasdaska, P. Y., Berggren, M. M., Berry, M. J., and Powis, G. Cloning, sequencing and functional expression of a novel human thioredoxin reductase. FEBS Lett., *442:* 105–111, 1999.
9. Miranda-Vizuete, A, Damdimopoulos, A. E., Pedrajas, J. R., Gustavsson, J-Å., and Spyrou, G. Human mitochondrial thioredoxin reductase: cDNA cloning, expression and genomic organization. Eur. J. Biochem., *261:* 405–412, 1999.
10. Lee, S-R., Kim, J-R., Kwon, K-S., Yoon, H. W., Levine, R. L., Ginsburg, A., and Ree, S. G. Molecular cloning and characterization of a mitochondrial selenocysteine-containing thioredoxin reductase from rat liver. J. Biol. Chem., *274:* 4722–4734, 1999.
11. Andersson, M., Holmgren, A., and Spyrou, G. NK-lysin, a disulfide containing effector peptide of T-lymphocytes is reduced and inactivated by human thioredoxin reductase. J. Biol. Chem., *271:* 10116–10120, 1996.
12. Rozell, B., Hansson, H. A., Luthman, M., and Holmgren, A. Immunohistochemical localization of thioredoxin and thioredoxin reductase in adult rats. Eur. J. Cell Biol., *38:* 79–86, 1985.
13. Schallreuter, K. U., Pittelkow, M. R., and Wood, J. M. Free radical reduction by thioredoxin reductase at the surface of normal and vitiliginous keratinocytes. J. Invest. Dermatol., *87:* 728–732, 1986.
14. Bertini, R., Howard, O. M. Z., Dong, H-F., Oppenheim, J. J., Bizzarri, C., Sergi, R., Caselli, G., Pagliei, S., Romines, B., Wilshire, J. A., Mengozzi, M., Nakamura, H., Yodoi, J., Pekkari, K., Guranath, R., Holmgren, A., Herzenberg, L. A., Herzenberg, L. A., and Ghezzi, P. Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells. J. Exp. Med., *189:* 1–8, 1999.
15. Powis, G., Briehl, M., and Oblong, J. Redox signaling and the control of cell growth and death. Pharmacol. Ther., *68:* 149–173, 1995.
16. Koishi, R., Kawashima, I., Yoshimura, C., Sugawara, M., and Serizawa, N. Cloning and characterization of a novel oxidoreductase KDRF from a human bone marrow-derived stromal cell line KM-102. J. Biol. Chem., *272:* 2570–2577, 1997.
17. Miranda-Vizuete, A., and Spyrou, G. The novel oxidoreductase KDRF is identical to human thioredoxin reductase. Biochem. J., *325:* 287–288, 1997.
18. Tamura, T., and Stadtman, T. C. A new selenoprotein from human lung adenocarcinoma cells purification, properties and thioredoxin activity. Proc. Natl. Acad. Sci. USA, *93:* 1006–1011, 1996.
19. Söderberg, A., Sahaf, B., Holmgren, A., and Rosén, A. Monoclonal antibodies to human thioredoxin reductase. Biochem. Biophys. Res. Commun., *249:* 86–89, 1998.
20. Sahaf, B., Söderberg, A., Spyrou, G., Barral, A. M., Pekkari, K., Holmgren, A., and Rosén, A. Thioredoxin expression and localization in human cell lines: Detection of full-length and truncated species. Exp. Cell Res., *236:* 181–192, 1997.
21. Kintner, C., and Sugden, B. Identification of antigenic determinants unique to the surfaces of cells transformed by Epstein-Barr virus. Nature (Lond.), *294:* 458–460, 1981.
22. Sundström, C., and Nilsson, K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer, *17:* 565–577, 1976.
23. Kirkin, A., Petersen, T., and Olsen, A. Generation of human-melanoma-specific T lymphocyte clones defining novel cytolytic targets with panels of newly established melanoma cell lines. Cancer Immunol. Immunother., *41:* 71–81, 1995.
24. Tsuchida, S., Yambe, M., Yamaguchi, Y., Kobayashi, Y., Kanno, T., and Tada, K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer, *26:* 171–176, 1980.
25. Katamine, S., Otsu, M., Tsuchiya, S., Sato, T., Ishida, N., Honjo, T., and Ono, Y. Epstein-Barr virus transforms precursor B cells even before immunoglobulin gene rearrangements. Nature (Lond.), *309:* 369–371, 1984.
26. Lirvall, M., Ljungqvist-Höddelius, P., Wasteson, Å., and Magnusson, K-E. UVB radiation affects the mobility of epidermal growth factor receptors in human keratinocytes and fibroblasts. Biosci. Rep., *16:* 227–238, 1996.
27. Rheinwald, J. C., and Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell, *6:* 331–344, 1975.
28. Freundlich, B., and Avdalovic, N. Use of gelatin/plasma coated flasks for isolating human peripheral blood monocytes. J. Immunol. Methods, *62:* 31–37, 1983.
29. Czerkinsky, C. C., Tarkowski, A., Nilsson, L. A., Ouchterlony, O., Nygren, H., and Gretzer, C. Reverse enzyme-linked immunospot assay (RELISPOT) for the detection of cells secreting immunoreactive substances. J. Immunol. Methods, *72:* 489–496, 1984.
30. Johansson, A., and Dahlgren, C. Characterization of the luminol-amplified light-generating reaction induced in human monocytes. J. Leukoc. Biol., *45:* 444–451, 1989.
31. Sander, B., Hoiden, B., Andersson, U., Möller, E., and Abrams, J. S. Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen. Cytokine detection by immunoassay and intracellular immunostaining. J. Immunol. Methods, *166:* 201–214, 1993.
32. Ericson, M., Hörling, J., Wendel-Hansen, V., Holmgren, A., and Rosén, A. Secretion of thioredoxin after in vitro activation of human B cells. Lymphokine Cytokine Res., *11:* 201–207, 1992.
33. Rosén, A., Lundman, P., Carlsson, M., Bhavani, K., Srinivasa, B. R., Kjellström, G., Nilsson, K., and Holmgren, A. A CD4+ T-cell line-secreted factor, growth promoting for normal and leukemic B cells, identified as thioredoxin. Int. Immunol., *7:* 625–633, 1995.
34. Rubartelli, A., Bajetto, A., Allavena, G., and Sitia, R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. J. Biol. Chem., *267:* 24161–24164, 1992.
35. Schallreuter, K. U., and Wood, J. M. New aspects in the pathophysiology of cutaneous melanoma: a review of the role of thioproteins and the effect of nitrosourea. Melanoma Res., *1:* 159–167, 1991.
36. Berggren, M., Gallegos, A., Gasdaska, J. R., Gasdaska, P. Y., Warneke, J., and Powis, G. Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. Anticancer Res., *16:* 3459–3466, 1996.
37. Nakamura, H., De Rosa, S., Roederer, M., Anderson, M. T., Gregson Dubs, J., Yodoi, J., Holmgren, A., Herzenberg, L., and Herzenberg, L. Elevation of plasma thioredoxin in HIV-infected individuals. Int. Immunol., *8:* 603–611, 1996.
38. Cha, M. K., and Kim, I. H. Thioredoxin-linked peroxidase from human red blood cell: evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. Biochem. Biophys. Res. Commun., *217:* 900–907, 1995.
39. Apffel, C. A., and Walker, J. E. Tumor growth and disulfide reduction: Possible dependence on protein-disulfide reductase. J. Natl. Cancer Inst., *51:* 575–583, 1973.
40. Holmgren, A. Bovine thioredoxin system. Purification of thioredoxin reductase from calf liver and thymus and studies of its function in disulfide reduction. J. Biol. Chem., *252:* 4600–4606, 1977.
41. Björnstedt, M., Xue, J., Huang, W., Åkesson, B., and Holmgren, A. The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. J. Biol. Chem., *269:* 29382–29384, 1994.
42. Andersson, M., Gunne, H., Agerberth, B., Boman, A., Bergman, T., Sillard, R., Jörnvall, H., Mutt, V., Olsson, B., Wigzell, H., Dagerlind, Å., Boman, G. H., and Gudmundsson, H. NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. EMBO J., *14:* 1615–1625, 1995.
43. Gozin, A., Franzini, E., Andrieu, V., Da Costa, L., Rollet-Labelle, E., and Pasquier, C. Reactive oxygen species activate focal adhesion kinase, paxillin and p130cas tyrosine phosphorylation in endothelial cells. Free Radic. Biol. Med., *25:* 1021–1032, 1998.
44. Gallegos, A., Gasdaska, J. R., Taylor, C. W., Paine-Murietta, G. D., Goodman, D., Gasdaska, P. Y., Berggren, M., Briehl, M. M., and Powis, G. Transfection with human thioredoxin increased cell proliferation and dominant negative mutant thiore-

doxin reversed the transformed phenotype of breast cancer cells. Cancer Res., *56:* 5765–5770, 1996.
45. Thelander, L. Thioredoxin reductase. Characterization of a homogenous preparation from *Escherichia coli B.* J. Biol. Chem., *242:* 852–859, 1967.
46. Zhang, P., Liu, P., Kang, S. W., Seo, M. S., Rhee, S. G., and Obeid, L. M. Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of bcl-2. J. Biol. Chem., *272:* 30615–30618, 1997.
47. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazano, K., and Ichijo, H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J., *17:* 2596–2606, 1998.
48. Schenk, H., Vogt, M., Dröge, W., and Schulze-Osthoff, K. Thioredoxin as a potent costimulus of cytokine expression. J. Immunol., *156:* 765–771, 1996.
49. Nilsson, J., Söderberg, O., Nilsson, K., and Rosén, A. Thioredoxin prolongs survival of B-type chronic lymphocytic leukemia cells. Blood, *95:* 1420–1426, 2000.
50. Furuke, K., Sasada, T., Ueda-Taniguhi, Y., Yamauchi, A., Inamoto, T., Yamaoka, Y., Masutani, Y., and Yodoi, J. Role of intracellular redox status in apoptosis induction of human T-cell leukemia virus type I-infected lymphocytes by 13-cis-retinoic acid. Cancer Res., *57:* 4916–4923, 1997.
51. Taniguchi, Y. U., Furuke, K., Masutani, H., Nakamura, H., and Yodoi, J. Cell cycle inhibition of HTLV-I transformed T cell lines by retinoic acid: The possible therapeutic use of thioredoxin reductase inhibitors. Oncology Res., *7:* 183–189, 1995.
52. Ueda, S., Nakamura, H., Masutani, H., Sasada, T., Yonehara, S., Takabayashi, A., Yamaoka, Y., and Yodoi, J. Redox regulation of caspase-3 (-like) protease activity: regulatory roles of thioredoxin and cytochrome c. J. Immunol., *161:* 6689–6695, 1998.
53. Spyrou, G., Björnstedt, M., Skog, S., and Holmgren, A. Selenite and selenate inhibit human lymphocyte growth via different mechanisms. Cancer Res., *19:* 4407–4412, 1996.
54. Gladyshev, V. N., Stadtman, T. C., Hatfield, D. L., and Jeang, K. T. Levels of major selenoproteins in T cells decrease during HIV infection and low molecular mass selenium compounds increase. Proc. Natl. Acad. Sci. USA, *3:* 835–839, 1999.
55. Clark, L. C., Jr., Combs, J. J., Turnball, B. W., Slate, E., Chalker, D. K., Chow, J., Davis, L., Glover, R. A., Graham, G. F., and Gross, E. G. Effects of supplementation for cancer prevention in patients with carcinoma of the skin: a randomized clinical trial. J. Am. Med. Assoc., *276:* 1957–1963, 1996.
56. Matsuda, M., Masutani, H., Nakamura, H., Miyajima, S., Yamauchi, A., Yonehara, S., Uchida, A., Irimajiri, K., Horiuchi, A., and Yodoi, J. Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependent cytotoxicity on U937 cells. J. Immunol., *11:* 3837–3841, 1991.