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Original Research Communication

Thioredoxin Blood Level Increases After Severe Burn Injury

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ABSTRACT

We have investigated the thioredoxin (TRX) levels in severely burned patients and the possible origin of TRX, based on the recent understanding that TRX is a potent antioxidant with cytoprotective functions. Serum and plasma samples from burns patients and healthy blood donors were collected during the first 10 post-burn days and analyzed in a sandwich TRX enzyme-linked immunosorbent assay (ELISA). The TRX levels found were correlated to a panel of blood tests. The presence of TRX in platelets was investigated by immunoelectron microscopy and Western blotting. TRX serum levels of the severely burned patients showed a significant increase, with a mean serum TRX concentration on the day of injury of 76.5 ± 19.5 ng/ml (mean ± SD) and on post-burn day one 122.6 ± 66.9 ng/ml, compared to control blood donor levels of 22.7 ± 12.2 ng/ml ($p = 0.0041$ and 0.0117, respectively). A second peak of increase was found on post-burn days 7 to 9 with a four- to five-fold rise in concentration compared to controls. TRX elevation correlated well with increased platelet ($p = 0.007$) and leukocyte counts ($p = 0.002$). We also demonstrated by immunoelectron microscopy and Western blotting the presence of TRX in platelets. In conclusion, our demonstration of TRX release in burn injuries indicates that the TRX system is involved in a rapid antioxidant defense, coagulation processes, cell growth, and control of the extracellular peroxide tone intimately linked to cytoprotection and wound healing in burns. One of the cell types that delivers TRX promptly and efficiently into the blood may be the platelet. Antiox. Redox Signal. 2, 707–716.

INTRODUCTION

CELLS THROUGHOUT OUR BODY have multiple sophisticated mechanisms for maintaining an intracellular reduced state in an oxidizing environment (Halliwell, 1999). Thioredoxin (TRX) plays a key role in regulating the redox environment of cells (Holmgren and Björnstedt, 1995; Nakamura *et al.*, 1997) acting as potent reducing agents for disulfide bonds in many proteins (Holmgren, 1984). TRX was first recognized in *E. coli* as a hydrogen donor for ribonucleotide reductase (Laurent *et al.*, 1964), important for DNA synthesis and repair.

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There is a growing body of evidence that redox regulation by TRX is involved in various biological events, including cell growth (Deiss and Kimchi, 1991; Rosén *et al.*, 1995), and apoptosis (Nilsson *et al.*, 2000). TRX is also important in immunological responses (Rosén *et al.*, 1995), and in the control of the DNA binding properties of transcription factors including NF-κB (Matthews *et al.*, 1992). TRX expression in lymphocytes and other cells such as keratinocytes can be induced by oxidative stress (Sachi *et al.*, 1995), viral infection (Nakamura *et al.*, 1996), mitogen stimulation (Ericson *et al.*, 1992), hydrogen peroxide exposure (Nakamura *et al.*, 1994), UV light (Danno *et al.*, 1995; Sachi *et al.*, 1995), and malignant transformation (Nakamura *et al.*, 1992; Sahaf *et al.*, 1997). It is noteworthy that human immunodeficiency virus (HIV)-infected individuals have a decreased level of intracellular glutathione (GSH) in their peripheral blood mononuclear cells that is associated with elevated plasma TRX, suggesting TRX may be released in response to oxidative stress in HIV infection (Nakamura *et al.*, 1996). Oxidative stress generated during a normal physiological/inflammatory response will also induce cellular overexpression and release of the selenoprotein TRX-reductase (TRXR), which is required for reduction of oxidized TRX (Söderberg *et al.*, 2000).

Severe burns lead to a strong oxidative attack as well as a multitude of organ system complications (Kucan, 1994). The major hematological complications are anemia and coagulopathy due to initial hemolysis, blood losses, and disturbances in clotting mechanisms (Kucan, 1994). Patients with severe burns demonstrate increased levels of oxygen free radicals, together with a loss of certain plasma antioxidants (Nguyen *et al.*, 1993). When tissue damage occurs, platelets adhere to damaged vessel walls, aggregate, and release their contents of various proteins, including the vascular regulators tromboxane and serotinin; regulators of cell proliferation and healing processes such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). The purpose of this study was to determine the levels of TRX in plasma and serum of severely burned patients and possible association of platelets with plasma TRX.

MATERIALS AND METHODS

This study was carried out according to the principles of the Declaration of Helsinki, and approved by the local ethical committee.

Burns patients

Eight severely burned patients, aged 15 to 74 years (Table 1), were included in this study. Sera and plasma samples were collected at the time of admission to the Burns Unit at the Department of Biomedicine and Surgery, Division of Plastic Surgery, University Hospital, Linköping, Sweden. Then samples were collected every second day. The patients were admitted either on the day of injury or post-burn day 1 or 2. We selected only patients with thermal skin injuries caused by flame-burns or electricity. None of the patients with thermal injuries caused by electricity displayed gross muscular and/or periosteal injuries. The deep dermal and/or full-thickness burns was recorded as burned surface area.

Control blood donors

As controls, blood samples were collected from 20 healthy voluntary blood donors. The age and sex distribution of the donors were 13 males, 32–61 years of age, and 7 females, 27–46 years of age.

Blood sampling

Blood samples were drawn, using standard venipuncture technique. Plasma was prepared using cooled citrate-tubes, which were centrifuged immediately for 30 min at 2,000 × *g*. Serum was prepared after the blood had been coagulating at room temperature for 1 hr.

TABLE 1. DATA OF THE BURNS PATIENTS

Sex	*Age*	*Burned body surface area (%)*	*Cause of injury*
Male	29	26	Electricity
Male	74	17.5	Flame burn
Male	30	29	Flame burn
Male	53	12.5	Flame burn
Female	15	50	Electricity
Male	51	19	Electricity
Male	58	38	Flame burn
Male	39	75	Flame burn

Isolation of platelets

Peripheral blood was obtained by standard venipuncture technique from healthy volunteers, using tubes containing acid citric dextrose (ACD) (71 m*M* citric acid, 85 m*M* sodium citrate, 111 m*M* glucose, pH 4.4), giving a 6:1 vol/vol ratio of blood to ACD. After addition of apyrase (1 U/ml), the mixture was centrifuged at 220 × *g* for 20 min at room temperature. The uppermost layer was removed and mixed with acetylsalicylic acid (final concentration of 0.1 m*M*) and centrifuged at 480 × *g* for 20 min at room temperature. The pellet obtained was then resuspended and washed with an isotonic HEPES buffer, pH 7.4 (140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* HEPES, 10 m*M* glucose) before counting in a Bürker chamber.

Preparation of platelets for electron microscopy

Isolated platelets were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 m*M* phosphate-buffered saline (PBS), pH 7.4, for 3 hr at room temperature, and then washed three times with PBS. Subsequent dehydration was performed using a step-gradient of ethanol (50%; 70%; 95% 2 × 99.5%). The platelets were embedded in Unicryl (British Bio Cell Int., Cardiff, UK), and ultrathin sections (50 nm) were mounted on Formvar-coated copper grids (Analytical Standards AB, Kungsbacka, Sweden).

Immunogold electron microscopy

The sections of embedded platelets were incubated in 0.5% bovine serum albumin (BSA; Sigma) for 30 min to block nonspecific binding, followed by incubation for 1 hr with a mouse anti-human TRX monoclonal antibody (mAb) (produced at the Department of Biomedicine and Surgery, Linköping University, Sweden) (Sahaf *et al.*, 1997). After rinsing (3 × 10 min in PBS), sections were incubated with a gold-labeled goat anti-mouse immunoglobulin G (IgG) antibody (AuroProbe EM GAM IgG G10, gold particle size of 10 nm, RNP425, Amersham, Buckinghamshire, England) for 1 hr at room temperature. As a positive control, sections were incubated with rabbit anti-human PDGF B-chain antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by appropriate gold-labeled goat anti-rabbit antibody (Auroprobe EM GAR G15, Amersham). As a negative control, an irrelevant mouse IgG_1 was used (Dako, Copenhagen, Denmark). The sections were rinsed (3 × 10 min in distilled water), and counterstained with 2% uranyl acetate for 10 min and in lead-citrate for 3 min. Examination was performed with a JEOL 1200 electron microscope (JEOL Ltd., Tokyo, Japan).

Sandwich ELISA for TRX

TRX levels were measured using a sandwich ELISA for human TRX and performed as previously described (Nakamura *et al.*, 1996). Briefly, 96-microwell plates were precoated with anti-TRX monoclonal antibody, ADF-21, blocked with 3% BSA and incubated for 2 hr at room temperature with samples diluted 1:10. A horseradish peroxidase-labeled anti-TRX monoclonal antibody, ADF-11, was used as conjugate with 2 hr of incubation. The substrate, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), was incubated for 1 hr. Absorption at 405 nm was measured by an ELISA reader (Molecular Devices, Menlo Park, CA). Data were analyzed by SOFTmax version 2.31 (Molecular Devices). Recombinant human TRX (rTRX) was used as a standard, using two-fold dilutions from 320 to 5 ng/ml.

Measurement of hemolysis

To measure the degree of red blood cell lysis, serum and plasma levels of hemoglobin were determined according to the procedure described by the supplier (Sigma, St. Louis, MO).

Panel of blood tests

At the time of blood sampling, a full panel of blood tests were performed, including measurement of platelets, leukocytes, red blood cells, hemoglobin, C-reactive protein, alkaline phosphatase, liver enzymes, blood coagulation parameters, creatinine, urea, fibrinogen, and anti-thrombin III.

Statistical analysis

Statistical differences between each group were evaluated by using Wilcoxon/Kruskal-Wallis non-parametric testing. Non-parametric Kendall-tau b analysis was used for assessment of correlations between TRX and clinical blood parameters. All statistical evaluations were performed with JMP Version 3 (SAS Institute Inc., Cary, NC) software and an Apple Macintosh microcomputer (Apple Computer, Cupertino, CA).

RESULTS

Burns patients were found to have significantly elevated levels of TRX both in serum (Fig. 1) and plasma. On the day of injury, the TRX serum concentration was 76.5 ± 19.5 ng/ml (mean ± SD), rising to 122.6 ± 66.9 ng/ml on post-burn day 1, as compared to control blood donor values of 22.7 ± 12.2 ng/ml (p values of 0.0041 and 0.0117, respectively). The following 4–5 days displayed normal TRX lev-

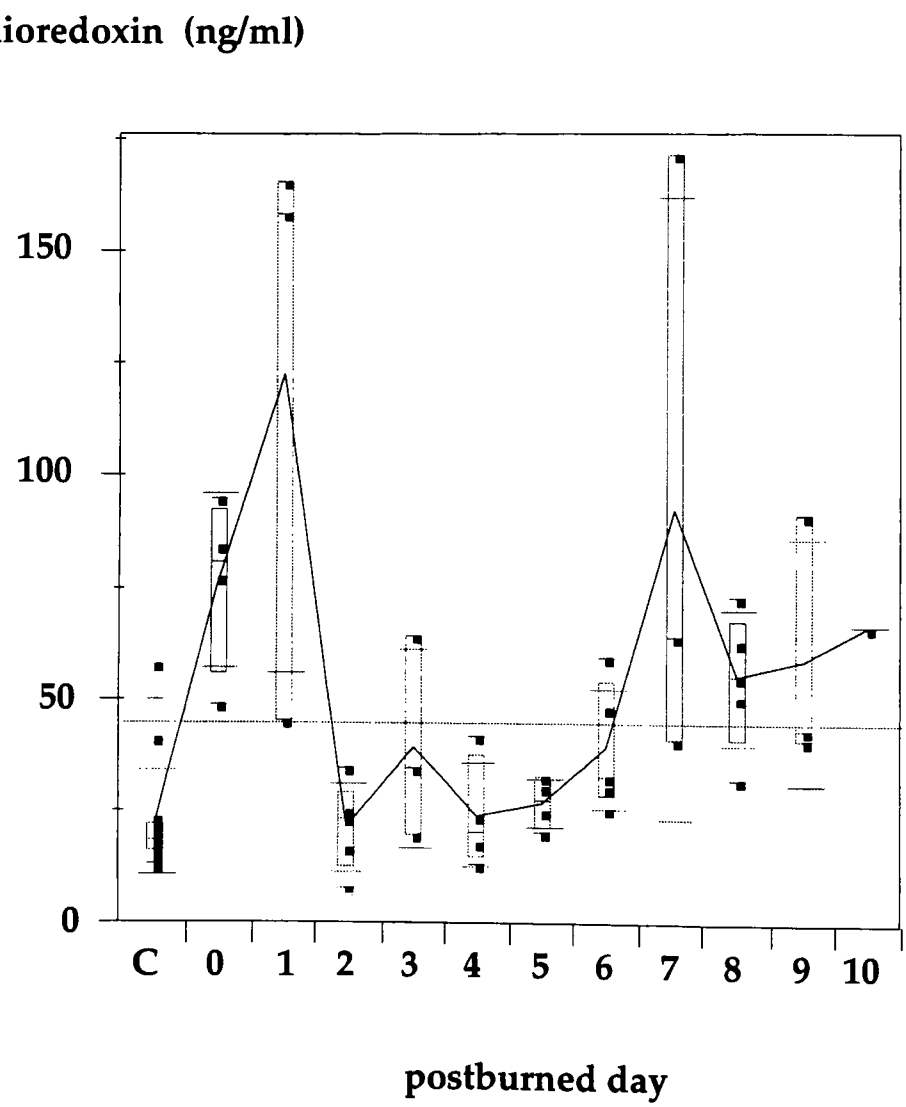

FIG. 1. Serum TRX levels in severely burned patients. Levels are shown from day of entry to the Burns Unit (day 0), then on post-burn days 1–10. C, Control blood donors (n = 20); (■) value of individual serum samples; (●) mean value; (filled vertical bar) SEM; (dotted horizontal bar) SD. Because blood samples were collected every other day, and admission of patients to the Burn Care Unit varied (4 patients were admitted on the day of burn injury, 3 on post-burn day 1, and 1 on post-burn day 2), four, three, or five samples are plotted each day in the diagram.

els, prior to a second peak (days 7–9) of four to five times higher concentration compared to control level (p values ranging from 0.0253 to 0.009). The plasma TRX concentrations were slightly (~30%) lower than the serum levels but followed the same pattern of significant increase (data not shown). The observed difference between plasma and serum levels of TRX was not found in the controls. The TRX levels in burns patients never fell below the normal range.

There was a significant correlation between the extent of burn (burn surface area) and TRX levels, as shown by linear regression analysis. Serum TRX versus burn surface area, $p = 0.009$; plasma TRX versus burn surface area, $p = 0.0027$.

Serum and plasma TRX values were compared with clinical parameters and positive correlations were summarized in Table 2; significant correlation was found to platelet and leukocyte counts, prothrombin (PT), and a liver enzyme, lactate dehydrogenase (LD). The elevated LD originated from liver, not from red blood cells (RBC), as indicated by LD correlation to aspartate-aminotransferase (ASAT) ($p = 0.0002$) and LD correlation to alanine-aminotransferase (ALAT) ($p = 0.0035$) LD correlation to hemoglobin (Hb) was not significant ($p = 0.1073$). Clinical laboratory data showed no unexpected values; all patients developed anemia and a transient thrombocytopenia. The TRX level did not correlate to the Hb level, indicating that elevated TRX was not a result of hemolysis (RBC-derived TRX). The transient thrombocytopenia was accompanied by a transient dip in TRX level (Fig. 1). No septic complications were recorded.

Table 2. Correlation of Serum and Plasma TRX with Clinical Parameters

	Parameters	*Significance (p value)*
Serum TRX vs.	Thrombocytes	0.007
	Leukocytes	0.002
	PT	0.005
	Plasma Trx	<0.000
	β-TG	0.691
	Hb	0.550
Plasma TRX vs.	Thrombocytes	0.007
	Leukocytes	0.027
	LD	0.017
	PT	0.029
	β-TG	0.337
	Hb	0.846
LD vs.	ASAT	0.0002
	ALAT	0.0035
	Hb	0.1073

p values are based on Kendall's non-parametric correlation analysis between plasma or serum TRX and the indicated parameter. PT, prothrombin; LD, lactate dehydrogenase; β-TG, β-thromboglobulin; ASAT, aspartate-aminotransferase; ALAT, alanine-amino-transferase; Hb, hemoglobin.

Isolated platelets were analyzed by immunochemical methods based on specific mAbs against TRX (Sahaf *et al.*, 1997). ELISA and dot-blot analyses detected 20–100 ng of TRX in 1.0×10^9 platelets. Immunogold electron microscopy directly detected TRX in human platelets (Fig. 2). PDGF labeling served as a positive control and an isotype mouse IgG_1 mAb as negative control (Fig. 3). PDGF labeling compared to TRX labeling appears stronger due to different Abs, polyclonal anti-PDGF and monoclonal anti-TRX, respectively. The size of TRX found in platelets was 12 kDa, as shown by Western blot (Fig. 4).

DISCUSSION

Recent studies have shown that patients with infections, including HIV (Nakamura *et al.*, 1996), tumors such as hepatocellular carcinoma and malignant melanoma (Nakamura *et al.*, 1992; Sahaf *et al.*, 1997; Barral *et al.*, 2000), and inflammatory diseases such as rheumatoid arthritis (Maurice *et al.*, 1997, 1999) have displayed elevated TRX levels in serum, presumably as a response to cellular activation and increased oxidative stress. Studies by us and others have revealed that TRX is secreted by a number of cells, including lymphocytes (Ericson *et al.*, 1992; Rubartelli *et al.*, 1992; Rosén *et al.*, 1995), epidermal cells (Schallreuter and Wood, 1998), monocyte/macrophages (Martin and Dean, 1991; Sahaf *et al.*, 1997; Söderberg *et al.*, 1998), hepatocytes (Holmgren and Luthman, 1978; Nakamura *et al.*, 1992; Rubartelli *et al.*, 1995), human cytotrophoblast cell lines (Di Trapani *et al.*, 1998), and is present in erythrocytes (Holmgren and Luthman, 1978; Cha and Kim, 1995). Early reports also demonstrated TRX-like activity in extracts of human platelets

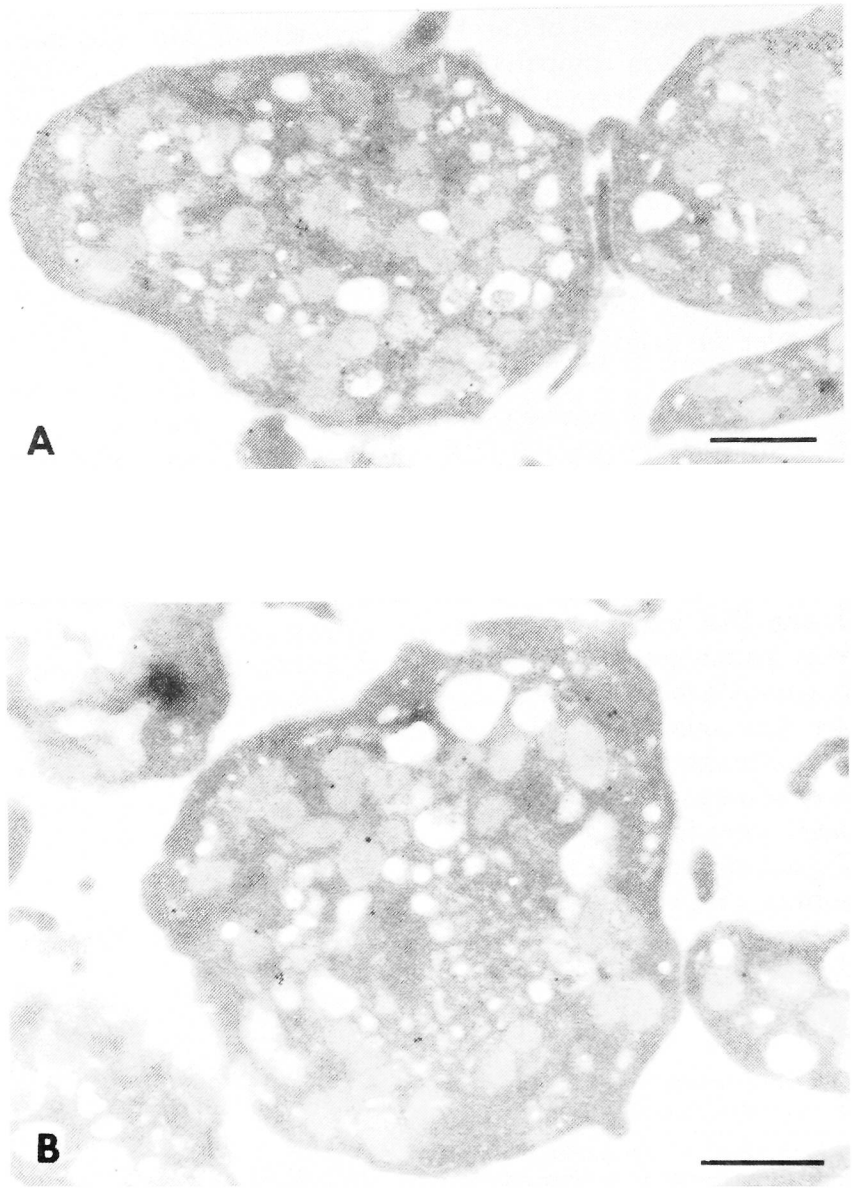

FIG. 2. Immunogold electron micrograph of human platelets stained for TRX. (**A** and **B**) Two different fields of vision of the same slide. TRX labeling was performed by using monoclonal mouse anti-human TRX (αTRX1/2G11) followed by 10-nm gold conjugate goat anti-mouse IgG. Scale bar = 500 nm.

(Blombäck *et al.*, 1974) and cultured human foreskin fibroblasts (Larsson *et al.*, 1978), using insulin disulfide reduction with TRX reductase and NADPH.

Our present study demonstrates that TRX is present in purified human platelets (20–100 ng of TRX/10^9 platelets), thus indicating that platelets are one of the main sources of TRX in burns patients. TRX seems to be one of the platelet constituents released in the platelet release reaction, which takes place during the first day after severe burn injuries (Abdiu and Sjöberg, manuscript in preparation). The mechanism of TRX liberation from platelets is not known, but parallel studies have indicated that TRX was not released after activation with well-known agents such as thrombin. Thiol-oxidants, however, will induce TRX release from platelets (Sahaf and Rosén, submitted for publication). The constantly high levels of TRX in burns patients indicate a continuous release, higher than the TRX

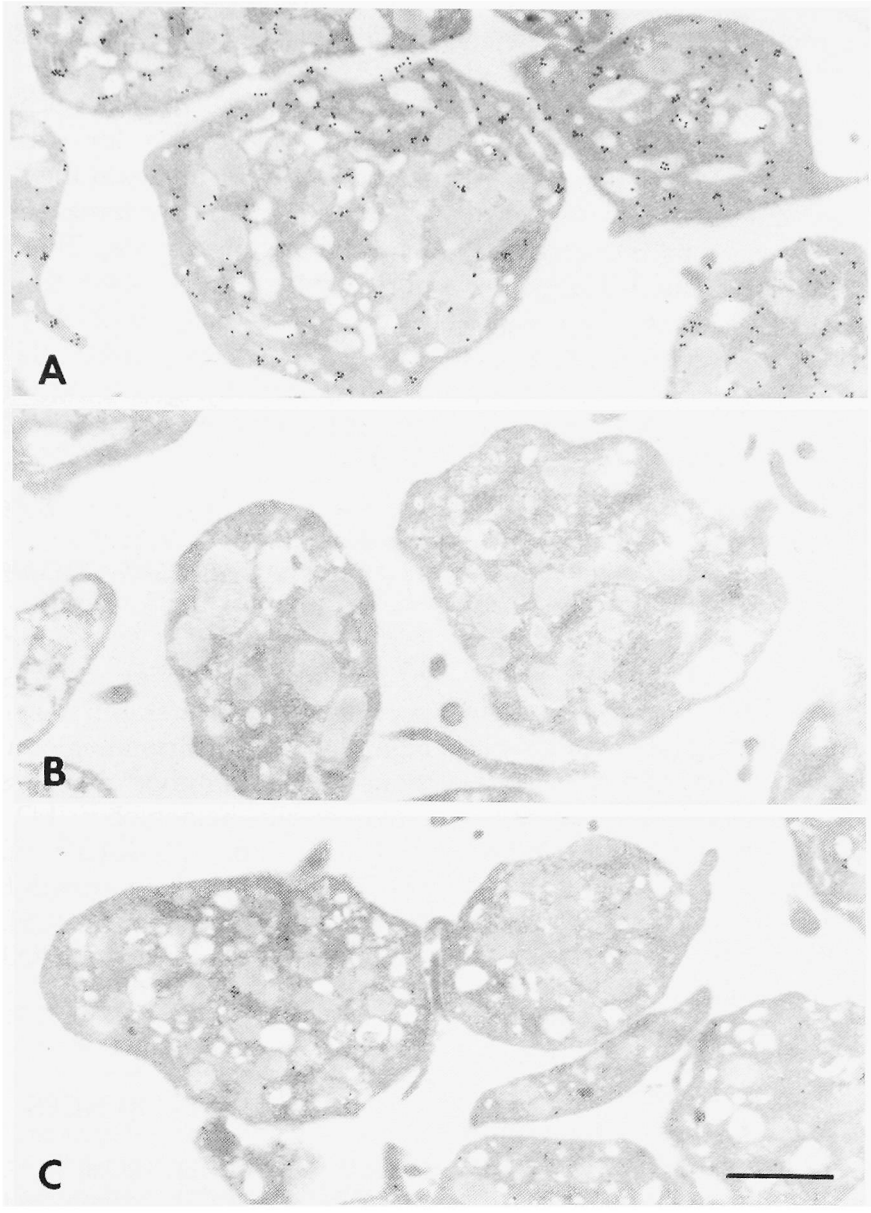

FIG. 3. Controls for immunogold electron micrographs of human platelets. (**A**) Positive control is PDGF labeled with rabbit anti-PDGF, B-chain followed by 15-nm gold conjugate goat anti-rabbit Ig. (**B**) Negative control is Mouse IgG_1 isotype control, followed by 10-nm gold conjugate rabbit anti-mouse IgG. (**C**) Same as Fig. 2A but at the same magnification as the positive and negative controls (**A** and **B**). Scale bar = 500 nm.

elimination by cellular uptake, degradation, or clearance to urine. There is, however, a transient dip in the TRX level, between the first and second peak (Fig. 1), in parallel with the transient thrombocytopenia, which indicates consumption of TRX. Erythrocytes are another possible source of TRX, albeit not likely in our patients, since very little hemolysis was detected in the samples investigated.

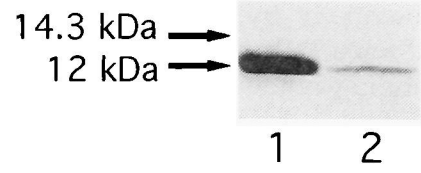

FIG. 4. Demonstration of TRX in human thrombocytes, using anti-human TRX mAb for development in ECR Western blot. Lane 1, Recombinant human TRX; lane 2, thrombocyte extract.

However, the correlation of TRX levels to leukocyte counts and liver proteins (PT and LD) in serum and plasma (Table 2) indicates that additional contributions of TRX are made by other organs and tissues, including leukocytes and liver parenchyma.

Extracellular TRX levels may reflect several functions of the protein in the inflammatory reactions and the oxidative stress accompanying severe burn injury. As a cytokine, TRX participates in the growth of lymphocytes (Rosén *et al.*, 1995). Together with TRXR, TRX may help to control the peroxide tone of the extracellular space via plasma glutathione peroxidase (Björnstedt *et al.*, 1994; Söderberg *et al.*, 2000). Because the level of GSH in plasma are below 5 μM, the system operates as a source of electrons for the selenium-containing plasma peroxidase (Björnstedt *et al.*, 1994). Additionally, reduced TRX is a major protein disulfide reductase operating intracellularly as well as extracellularly (Holmgren, 1985; Ericson *et al.*, 1992). Previous work has studied the role of TRX as a reductant of disulfides in proteins of the coagulation system (Blombäck *et al.*, 1974, 1986). Reduction of disulfide bonds in fibrinogen results in loss of clotting ability, but the reaction is reversible (Blombäck *et al.*, 1974). With Factor VIII (von Willebrand factor), reduction by TRX brings about a reversible loss of activity and a decrease in multimer size (Hessel *et al.*, 1984). Potentially, such as redox process at or in the vessel wall controls a reversible multimerization of Factor VIII, which in turn controls platelet adhesion to the endothelium or to subendothelial structures (Hessel *et al.*, 1984; Blombäck *et al.*, 1986). Factor XIIIa catalyzes the formation of fibrinogen oligomers by cross-linking of the γ-chains and the Aα-chain of fibrinogen promoters. This reaction is enhanced by thiols including TRX, potentially by keeping the active site thiol in Factor XIIIa active.

The finding of elevated plasma and serum levels of TRX in burns patients highlights its role in platelets and the coagulation process. In addition, antioxidative roles of TRX in oxidative stress accompanying burn injury is in agreement with recent results regarding the role of extracellular TRX and TRXR (Söderberg *et al.*, 2000).

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ABBREVIATIONS

Ab, Antibody; ACD, acid citric dextrose; ALAT, alanine-aminotransferase; ASAT, aspartate-aminotransferase; BSA, bovine serum albumin; β-TG, β-thromboglobulin; ELISA, enzyme-linked immunosorbent assay; GSH, glutathione; Hb, hemoglobin; LD, lactate dehydrogenase; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PT, prothrombin; RBC, red blood cell; SD, standard deviation; TRX, thioredoxin; TRXR, thioredoxin reductase.

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