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Monoclonal Antibodies to Human Thioredoxin Reductase

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The thioredoxin system consisting of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH is an electron donor for ribonucleotide reductase but has also been implicated in other cellular events, including secretion, growth promotion, regulation of transcription factors, protection against oxidative stress, and apoptosis. Mammalian TrxR is a dimeric flavoprotein with 58 kDa subunits each with a catalytically active selenocysteine residue. To study the function and expression of TrxR, we have produced and characterized, for the first time, monoclonal antibodies against human TrxR. Native placenta TrxR was used for immunization of BALB/c mice, followed by hybridization, cloning, and establishment of hybridomas producing specific antibodies against human TrxR. Three clones of IgG_1, κ subclass, termed anti-TrxR1, anti-TrxR2, and anti-TrxR3, were studied in detail. The isoelectric points (pIs) of the mAbs were 6.5, 6.0, and 6.5, respectively. The affinities (K_a) of the mAbs were 2×10^8 M^{-1}. Inhibition ELISA using biotin-labeled versus nonconjugated mAb IgG revealed that all three mAbs recognized one immunodominant epitope. Western blot analysis showed that the antibodies specifically bound to a 58 kDa protein, representing the subunit of TrxR. A Trx-dependent insulin reduction assay was used for analysis of enzymatic activity and the antibodies neutralized the reductase activity.

Mammalian TrxR catalyzes the reduction of the active site disulfide in Trx by NADPH and is a member of the pyridine-nucleotide disulfide oxidoreductase family (1, 2). Reduced Trx is a hydrogen donor for ribonucleotide reductase and a general disulfide reductase with numerous functions in redox-regulation by thiol redox control (3-5). TrxR from mammalian cells was first purified to homogeneity from rat liver (6) and showed subunits of 58 kDa in contrast to the well-characterized *E. coli* TrxR with 35 kDa subunits (1, 2). Recently the human placenta enzyme (hTrxR) was shown to be a selenoprotein with a selenocystein (SeC) residue in the C-terminal end (7). All mammalian TrxR are homologous to glutathione reductase but with a C-terminal elongation containing a catalytically active penultimate SeC residue in the conserved sequence: -Gly-Cys-SeC-Gly- (8). The content of SeC in TrxR explains the activity of the enzyme as a hydrogen peroxide and lipid hydroperoxide reductase (9). Since the SeC residue is required for catalytic activity this may also explain the wide substrate specificity of TrxR reducing e.g. selenite (SeO_3^{2-}), alloxan, vitamin K. S-nitrosoglutathione (GSNO) or lipoic acid (1).

The thioredoxin system plays a key role in maintenance of a reducing intracellular milieu and has an important role in protection of the cells against cytotoxic and oxidative stress inducing agents (3-5). DNA synthesis and repair, protein folding, DNA binding of transcription factors, and apoptosis control, all appear to involve TrxR-dependent redox-regulatory mechanisms (3-5). Some clinically used drugs such as 13-cis-retinoic acid inhibit TrxR and it is important to be able to measure the protein. We have therefore developed monoclonal antibodies.

MATERIALS AND METHODS

Reagents. Native human TrxR was prepared from human placenta according to previously described methods (10), or purchased from IMCO, Stockholm, Sweden. TrxR had a specific activity of 1200 A_{412} units/mg. Insulin was purchased from Novo Nordisk A/S, Bagsvaerd, Denmark. Human Trx was bought from IMCO Corporation LTD AB, Stockholm, Sweden or prepared from *E. coli* BL21 or BL21/pACATrx as described (11).

Cell lines and culture conditions. SP2/0 cells (12) were derived from a mouse myeloma and cultured in Iscove's modified Dulbecco's medium (Gibco BRL) and Opti-Mem (Gibco BRL) 50:50, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco BRL), and 10% fetal calf serum (FCS) (Gibco). The cells were grown in humidified air with 5% CO_2 at 37°C. U-937, a human histiocytic/monocytic leukemia cell line (13) was grown in

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RPMI 1640 with 10% FCS, as described before (11). HAT and HT (Gibco BRL) supplements were used for the selection procedure.

Hybridoma establishment and characterization. Two to three weeks old female BALB/c mice were immunized by subcutaneous injection of 30 μg hTrxR in complete Freund's adjuvant (Sigma Chem. Co., St. Louis, MO). The animals were boosted three times at 2-week intervals with 15 μg hTrxR in incomplete Freund's adjuvant (Sigma Chem. Co.). Three days before the mice were sacrificed, they were injected intravenously with 5 μg hTrxR. The splenocytes were fused with SP2/0 myeloma cells according to Köhler and Milstein (14) and were cultured in Iscove's Modified Dulbecco's medium (Gibco BRL) and Opti-Mem (Gibco BRL) 50:50, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco BRL), 10% fetal calf serum (FCS) (Gibco), and 1% HAT grown in humidified air with 5% CO_2 at 37°C. Screening was performed by enzyme-linked immunosorbent assay (ELISA) for anti-TrxR. ELISA plates (Costar, Cambridge, MA) were coated for 16 h at 4°C with 50 μl of hTrxR (0.6 μg) per well. The plates were rinsed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) and blocked with 100 μl PBS containing 3% BSA (Sigma Chem., Co.) for 1h. The wells were then incubated with 50 μl of hybridoma supernatant, 1:4 diluted, in PBS containing 0.1% BSA and 0.05% Tween 20, of for 16 h at 4°C, washed three times and incubated with 50 μl alkaline phosphatase-conjugated goat-anti-mouse IgG (Mabtech, Stockholm, Sweden) diluted 1:1000 in PBS containing 0.1% BSA and 0.05% Tween 20. The plates were washed three times in PBS-Tween and incubated with 50 μl p-nitrophenyl phosphate (Sigma Chemical Co.) in dietanolamine, pH 9.0, containing 0.5 mM $MgCl_2$, 0.02% NaN_3 for 30 min. The absorbance was measured at 405 nm in a Multiskan RC photometer (Labsystems, Stockholm, Sweden). Cells derived from positive wells were cloned by limiting dilution. Three anti-TrxR mAbs were investigated in detail as described below. Purification and characterization of anti-TrxR mAbs was performed in the following way. Supernatants containing mAbs were adjusted to pH 8.6 and 3 M NaCl. The supernatants were then filtered through 0.22 μm Millipore filters. Protein-A Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) was suspended in distilled water. The Protein-A column was equilibrated with 0.05 M Tris-HCl containing 3 M NaCl, pH 8.6. IgG from the culture supernatants were bound to Protein-A and the column was washed with three column volumes with 0.05 M Tris-HCl containing 3 M NaCl, pH 8.6. The mAbs were eluted with 0.5 ml batches of 0.1 M glycine-HCl, pH 3.0 and immediately neutralized with 50 to 100 μl of 1 M Tris-HCl, pH 9.0. Protein-A purified monoclonal antibodies were biotinylated with NHS-Biotin (Pierce, Rockford, IL) according to the manufacturer's recommendation. The isoelectric point of the antibodies was determined by isoelectric focusing. 25 ng of protein-A purified IgG from the different clones were run on an isoelectric focusing gel, pH range 3-10 (Phastgel, Pharmacia, Uppsala, Sweden). Proteins were detected by silver staining.

Mouse monoclonal antibody isotyping kit for class and subclass determination (Dipstick format) were used according to the method recommended by the manufacturer (Life Technologies, Gaithersburg, MD). Criss-cross tests for epitope determinations were performed. Unlabeled mAb IgG were mixed with biotin-labeled mAb. Unlabeled IgG was tested in 1-, 10-, or 100-fold molar excess compared to labeled IgG. The assay was performed according to the description for quantitative competitive inhibition ELISA except that the visualization was carried out by the avidine-biotin detection system with alkaline phosphatase-conjugated avidine (Dako, Glostrup, Denmark) in dilution 1:1000.

The neutralization capacity of the mAbs was tested in the insulin reduction assay (2). 60 μl (0.15 μM) of pure placenta hTrxR was preincubated with equimolar and a 10-fold molar excess of IgG from protein-A purified anti-TrxR mAbs for 45 min at 0°C. We used an irrelevant mouse IgG_1 mAb (M 705, Dakopatts) same concentration as a negative control. Five microliters of the Ab solution was then mixed after the pre-incubation with 20 μl of a 0.26 M HEPES buffer, pH 7.6, containing, 10 mM EDTA, 2.0 mM NADPH (Sigma Chemicals Co.), 0.75 μg of hTrx and 1.03 mM insulin (Novo Nordisk A/S, Bagsvaerd, Denmark) with H_2O adjusted to 60 μl, and incubated at 37°C for 20 min. The reaction was stopped by a solution of 5,5′-dithiobis-nitrobenzoic acid (DTNB) (Sigma Chemicals Co.), 0.4 mg/ml, and 6 M guanidine hydrochloride (GuHCl) (Sigma Chem. Co). OD was measured at 412 nm in a Multiskan RC photometer. Protein concentration was determined by the BCA method (Pierce) according to Smith *et al.* (15).

Competitive inhibition ELISA. ELISA plates (Costar) were coated with 50 μl of hTrxR (0.5 μg/ml) for 16 h at 4°C. The plates were rinsed with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked with PBS containing 3% BSA for 1 h. The wells were then incubated with 25 μl of sample diluted in PBS containing 0.1% BSA and 0.05% Tween 20 and Protein A- purified anti-TrxR mAb (25 ng/ml) for 16 h at 4°C. The plates were then washed three times and incubated with 50 μl of alkaline phosphatase-conjugated goat anti-mouse IgG (Mabtech, Stockholm), diluted 1:1000. The plates were washed three times in PBS-Tween and incubated with p-nitrophenyl phosphate (Sigma Chem., Co.) in dietanolamine, pH 9.0, containing 0.5 mM $MgCl_2$, 0.02% NaN_3 for 30 min. The absorbance was measured at 405 nm in a Multiskan RC photometer.

Western blot. A minor part of a human placenta was collected and immediately frozen and stored at −70°C. Placenta extract was prepared on ice by homogenization in 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.2 mM AEBSF protease inhibitor, 2 ml/g fresh weight. Homogenization was achieved using a Polytron tissue disrupter. The homogenates were centrifuged for 1 h at 150,000*g*. The supernatant were kept frozen at −70°C in small aliquots. Cell extracts of U-937 was prepared from 20×10^6 cells. Cells were washed twice in PBS and lysed in 100 μl RIPA-buffer containing AEBSF, aprotinin and sodium orthovanadate for 30 min on ice, then disrupted by repeated aspiration through an 18 gauge needle and then further incubated on ice for 30 min. SDS-PAGE was carried out in 12% polyacrylamide gels. All samples were run on SDS-PAGE under reducing conditions. After electrophoresis, transfer of proteins to nitrocellulose membranes (0.2 μm, Pharmacia). The membranes were blocked with 5% milk powder and incubated over night at +4°C with 10 ml of anti-TrxR mAbs (10 μg/ml). The bound antibodies were visualized with horseradish peroxidase labeled anti-mouse IgG and ECL detection kit (Amersham Life Science) according to the manufacturer's recommendations.

RESULTS

For the monoclonal antibody production and characterization, two female BALB/c mice were immunized with human placenta TrxR and fused with SP2/0 myeloma cells. Cells from three positive wells, which were derived during the initial screening, were further cloned by limiting dilution. The limiting dilution was repeated three times. Then we selected three clones, anti-TrxR1, anti-TrxR2, and anti-TrxR3, which were subjected to further investigations. Isotype determinations showed IgG_1 subclass with kappa light chains. The isoelectric points were 6.5, 6.0, and 6.5, respectively. The apparent affinities were measured by ELISA inhibition technique and were recorded to 2×10^8 M^{-1}. Epitope determinations were performed in criss-cross tests, using unlabelled mAb in competition with biotin-labeled mAb for binding to TrxR-coated plates. All clones were competitively inhibited by each other, proving that the three mAbs bound to an identi-

TABLE 1

Specificities of Anti-human TrxR mAb Paratope: Comparisons in Competitive Inhibition ELISA

Biotin-labeled mAb	Competing mAb		
	Anti-Trx1	Anti-TrxR2	Anti-TrxR3
Anti-TrxR1	0.06	0.17	0.21
Anti-TrxR2	0.09	0.11	0.12
Anti-TrxR3	0.06	0.07	0.08

Note. Inhibition of binding of biotin-labeled mAb by 10-fold molar excess of unlabeled mAb. Results are expressed as absorbances relative to that without competition (diluent only).

cal epitope (Table 1), alternatively, the mAb-binding caused steric hindrance in such a way that the active site function failed. Since the three mAbs bound to an identical epitope, we used the clone anti-TrxR3 in the following experiments. In the Trx-dependent insulin reduction assay, the anti-TrxR mAb neutralized and inhibited the enzyme activity of TrxR (Fig. 1), whereas an irrelevant control mouse IgG_1 mAb did not. TrxR and the Ab were tested at equimolar concentration (0.15 μM) and a 25% inhibition was found. At 10-fold molar Ab-excess 60% inhibition of enzyme activity was found. The specificity of the anti-TrxR mAb was shown by Western blot analysis, in which the mAb exclusively bound to a 58 kDa protein of placenta tissue extracts

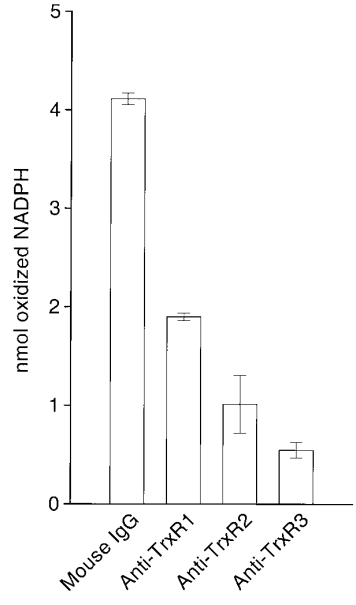

FIG. 1. Neutralization of TrxR by the three monoclonal antibodies against TrxR in the Trx-dependent insulin disulfide reduction assay. 0.15 μM of hTrxR was mixed with 1.5 μM of mAb IgG and tested. Negative control mAb was an anti-CD5 mouse IgG_1. Error bars represent standard error of the means, SEM. The A_{412} values representing SH-groups were recalculated to amount of NADPH oxidized by using a 27.2 mM extinction coefficient for DTNB (6).

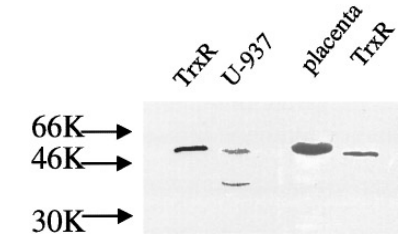

FIG. 2. Western blot analysis after a 12% SDS-PAGE gel separation. Lane 1, TrxR from human placenta; lane 2, U-937 cell lysate; lane 3, crude extract from human placenta; lane 4, TrxR from human placenta. The primary antibody was anti-TrxR3 and the conjugate antibody was an HRP-conjugated rabbit anti-mouse IgG. Development was performed by ECL.

(Fig. 2). Pure TrxR was used as a control. This protein band represents the monomer subunit of TrxR. In the U-937 cell extracts, we found, besides the 58 kDa band, a 35 kDa band (Fig. 2) representing a breakdown product of monomeric TrxR. In separate ELISA and Western blot experiments (data not shown), we found that the mAbs did not cross-react with rat and bovine TrxR.

DISCUSSION

In this study we describe the development and characterization of mAb developed for the first time against purified human placental TrxR for the purpose of detection and localization in human tissue, purified blood cells and cell lines. The mAb was able to neutralize the Trx-dependent protein disulfide reductase effect of TrxR in an insulin assay, which indicates that the mAbs binding interferes with the function of TrxR. It was recently shown that the C-terminal of TrxR contains a penultimate SeCys residue, the presence of which is necessary and may explain its potent antioxidant function (7). Trx/TrxR are redox proteins that have been implicated in the control of cell proliferation and transformation. Besides its function as the hydrogen donor for ribonucleotide reductase (1), Trx/TrxR system exert redox control over transcription proteins in mammalian cells by thiol/redox control via reversible disulfide formation modulating their DNA binding and, in this way, regulate gene expression. The transcription proteins directly modulated by the thioredoxin system include NF(B (16) TFIIIC (17), BZLF1 (18), and glucocorticoid receptor (19).

In summary, using our novel monoclonal antibody against TrxR, we found high level of TrxR expression in human placenta and in monocytic leukemia cell line U-937. These findings illustrate two important mechanistic aspects. First, in normal human placenta, the presence of TrxR as an antioxidant may have an important role in protection of the developing fetus. Second, in malignant tumor growth such as in the leukemia U-937, the overexpression of TrxR may favor out-

growth and progression of aberrant clones. Recently Powis *et al.* (20) reported that also solid tumors may overexpress TrxR. We believe that immune escape variants of leukemia cells at a progressive stage may resist cytotoxic attacks delivered by triggered macrophages and T cells. T cells are known to release cytotoxic peptides such as NK-lysin (21), which can be neutralized by TrxR (22). Our novel monoclonal antibodies against human TrxR may be helpful for studies on TrxR distribution pattern, tumor localization, including clinical situations in which TrxR inhibitory drugs are used for therapy.

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