

# Oxidation of a Specific Methionine in Thrombomodulin by Activated Neutrophil Products Blocks Cofactor Activity

## A Potential Rapid Mechanism for Modulation of Coagulation

Charles B. Glaser,\* John Morser,† Jeffrey H. Clarke,\* Eric Blasko,\* Kirk McLean,\* Irene Kuhn,§ Ray-Jen Chang,\* Jiing-Huey Lin,† Laura Vilander,§ William H. Andrews,§ and David R. Light\*

\*Department of Protein Chemistry, †Department of Cardiovascular Research, and §Department of Molecular Biology, Berlex Biosciences, South San Francisco, California 94080

### Abstract

Endothelial thrombomodulin (TM) plays a critical role in hemostasis as a cofactor for thrombin-dependent formation of activated protein C, a potent anticoagulant. Chloramine T, H<sub>2</sub>O<sub>2</sub>, or hypochlorous acid generated from H<sub>2</sub>O<sub>2</sub> by myeloperoxidase rapidly destroy 75–90% of TM cofactor activity. Activated PMN, the primary *in vivo* source of biological oxidants, also rapidly inactivate TM. Oxidation of TM by PMN is inhibited by diphenylene iodonium, an inhibitor of NADPH oxidase. Both Met291 and Met388 in the six epidermal growth factor-like repeat domain are oxidized; however, only substitutions of Met388 lead to TM analogues that resist oxidative inactivation. We suggest that in inflamed tissues activated PMN may inactivate TM and demonstrate further evidence of the interaction between the inflammatory process and induction of thrombotic potential. (*J. Clin. Invest.* 1992. 90:2565–2573.) Key words: diphenyleneiodonium • oxidants • leukocytes • thrombin • protein C

### Introduction

As has been known for some time, coagulation resulting in thrombosis can occur as a result of inflammatory disease (1). Mechanistic aspects of the link between inflammation and coagulation can be divided into two groups: inflammatory processes that activate the coagulation system and inflammatory processes that remove natural anticoagulants. Leukocytes play a procoagulant role in septicemic patients (2) and may act through the rapid, tissue factor-independent activation of factor X bound to the integrin, Mac-1 on activated monocytes (3–5). In addition, tissue factor itself is induced, albeit more slowly, on activated monocytes by endotoxin (4) or P-selectin (6), which is upregulated on thrombin-activated platelets (7). Tumor necrosis factor  $\alpha$ , produced by activated macrophages, plays a dual role in vascular endothelial cells (8) by inducing a tissue factor-like procoagulant activity (9) and downregulating

the anticoagulant, thrombomodulin (TM)<sup>1</sup> (10). Direct inactivation of natural anticoagulants can also occur during inflammation. Examples include the inactivation of protein S by activated PMN (11), the oxidative inactivation of antithrombin III (12), and the increase in the protein S inhibitor, C4b-binding protein, during inflammation (13).

TM is a glycoprotein present in the membrane of endothelial cells that regulates the coagulation pathway by modifying the action of thrombin. As a cofactor for thrombin-catalyzed activation of protein C, TM enhances the reaction rate by > 1,000-fold (14). The resultant serine protease, activated protein C (APC), then inactivates factors Va (15) and VIIIa (16), thus inhibiting further thrombin generation. TM also directly inhibits the procoagulant activities of thrombin, which include cleavage of fibrinogen to fibrin (17) and platelet activation (18).

Mouse TM (19), bovine TM (20, 21), and human TM (22, 23) genes have been cloned and the predicted sequence consists of five domains, an NH<sub>2</sub>-terminal domain homologous to some lectins, a domain with six tandem repeats of epidermal growth factor (EGF)-like structures, a serine- and threonine-rich domain modified by O-linked sugars, a transmembrane domain, and a cytoplasmic domain. A soluble human TM analogue consisting of only the six EGF-like domains, TM<sub>E</sub> (Sf9)<sup>2</sup> from Cys227 to Cys462 expressed in insect cells is active both *in vitro* and *in vivo* (24).

During attempts to label TM with radioactive iodine we found that chloramine T destroyed the cofactor activity of TM (25). In this paper we investigate the mechanism of the oxidative inactivation of TM and the potential for physiological oxidants released during inflammation to achieve this inactivation. We show that oxidation of a methionine at position 388 is solely responsible for the inactivation and suggest that this oxidation may occur during the inflammatory process.

### Methods

**Materials.** Rabbit TM and hirudin were from American Diagnostica Inc. (Greenwich, CT). Human recombinant protein C was from Gen-

Address correspondence and reprint requests to Dr. David R. Light, Berlex Biosciences, 213 East Grand Ave., South San Francisco, CA 94080.

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1. **Abbreviations used in this paper:** APC, activated protein C; CNBr, cyanogen bromide; DIP, diisopropylphosphoryl; EGF, epidermal growth factor; HOCl, hypochlorous acid; HUVEC, human umbilical vein endothelial cells; NEM-Ac, *N*-ethylmorpholine acetate; SLPI, secretory leukocyte protease inhibitor; TM, thrombomodulin.

2. In the nomenclature used here the subscript refers to the domains contained in the TM analogue: L, the lectin domain; E, the six EGF-like domains; O, the O-linked sugar domain; M, the transmembrane domain; and C, the cytosolic domain. Thus, TM<sub>LEO</sub> is equivalent to TMD123 (58) and TMD1 (34).

zyme Corp. (Boston, MA). Bovine thrombin was from Miles Laboratories Inc. (Dallas, TX).  $\alpha$ 1-Protease inhibitor was prepared as described (26). D-Val-L-Leu-L-Arg-p-nitroanilide (S-2266) was from Kabivitrum Inc. (Franklin, OH). Human  $\alpha$ -thrombin (4,000 NIH U/mg), bovine serum albumin (fraction V), citrated human plasma, chloramine T, 30% hydrogen peroxide, human leukocyte myeloperoxidase (100 U/mg), horse heart cytochrome *c* (type VI), bovine liver catalase (48,600 U/mg), and bovine erythrocyte superoxide dismutase (3,000 U/mg) were from Sigma Chemical Co. (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem Corp. (La Jolla, CA). Diphenylene iodonium was a gift from Dr. Dennis Stuehr (Cornell University Medical College, New York) (27, 28). Secretory leukocyte protease inhibitor (SLPI) was a gift from Dr. Robert Thompson (Synergen Inc., Boulder, CO) (29). Q-Sepharose fast flow, Mono Q, Sephadex G-25, Superose 6, and NAP-10 resins were from Pharmacia Fine Chemicals, Div. of Pharmacia Inc. (Piscataway, NJ). Excell 400 serum-free medium was from JR Scientific (Irvine, CA). An altered sites mutagenesis kit was from Promega Corp. (Madison WI). The mammalian expression vector pRc/CMV was from Invitrogen (San Diego, CA). All other reagents were of the highest grade available.

**Expression and mutagenesis of  $TM_E^2$  in Sf9 cells.** The DNA sequence encoding the six EGF-like repeats of TM (amino acids 227–462) was linked to the signal sequence of the insect protease, hypodermis A, and the hybrid gene placed under control of the polyhedron gene promoter (30) in the baculovirus shuttle vector pTMHY101. Recombinant virus was generated using standard techniques (24, 31). Mutant analogues described were prepared by use of a mutator site-specific mutagenesis kit (Stratagene, Inc., La Jolla, CA) and virus was prepared for expression in the baculovirus expression system by the same methods.

**Purification of  $TM_E$  (Sf9).** All procedures were performed at 4°C. Insect cell harvest was filtered (1.2  $\mu$ M Serum Capsule 12168, 0.2  $\mu$ M Culture Capsule 12140; Gelman Sciences Inc., (Ann Arbor, MI) and diluted 1:1 with water, titrated with acetic acid to pH 5.2, and loaded on a Q-Sepharose fast-flow resin (25 mM Na acetate, pH 5, 0.1 M NaCl, 0.02% NaN<sub>3</sub>). Active fractions were eluted with 0.3 M NaCl in the same buffer; pooled; diluted to 0.3 M NaCl, 20 mM Tris-HCl, 0.5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>; adjusted to pH 7.5 with NaOH; loaded on a 120-ml diisopropylfluorophosphate-inactivated thrombin. Affigel-10 resin (DIP-thrombin resin); and eluted with 2 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM Na<sub>2</sub>EDTA, 0.02% NaN<sub>3</sub>. Active fractions were pooled, desalted, and buffer exchanged on a Sephadex G-25 column into 0.2% NEM-Ac, pH 7, loaded on a Mono-Q HR10/10 column, and eluted with a gradient of 0–1 M NaCl in the same buffer. High specific activity fractions (by APC assay, see below) were pooled, desalted on Sephadex G-25 into PBS or 0.2% NEM-Ac, pH 7, and stored frozen or lyophilized.

**Expression of soluble  $TM_{LEO}$  and full-length TM in Cos 7 and CHL1 cells.** DNA coding for both the wild-type and M388L mutant forms of TM, amino acids 1–497, and full-length TM, amino acids 1–557 was expressed in Cos 7 and CHL1 cells using the mammalian expression vector pRc/CMV. For transient expression, Cos 7 cells expressing full-length TM were harvested 48–72 h posttransfection.

**Purification of  $TM_{LEO}$  (CHL1).** Media containing the  $TM_{LEO}$  (CHL1) analogue with chondroitin sulfate, expressed and secreted by CHL1 cells, was made up to 0.01% Tween 80; filtered as above; loaded on a 100-ml Q-Sepharose column; washed with 50 mM Tris-HCl, pH 7.8, 0.2 M NaCl, 0.1 mM EDTA, 0.01% Tween 80; eluted with a NaCl gradient (0.2–2 M) in the same buffer (elutes  $\sim$  1 M NaCl by APC assay); diluted threefold with H<sub>2</sub>O; loaded on a second 5-ml Q-Sepharose column; washed with the same buffer except with 0.001% Tween 80 and 0.7 M NaCl; and eluted with a second gradient (0.7–1.6 M NaCl).  $TM_{LEO}$  (CHL1) was further purified by molecular exclusion chromatography on Superose 6 in PBS.

**Measurement of TM cofactor activity (APC assay).** All samples and reagents were diluted in APC assay diluent (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 0.5% BSA). Samples and TM

standards (0–1 nM) were incubated for 60 min in 60  $\mu$ l total volume at 37°C in a 96-well plate with 0.5  $\mu$ M protein C and 1 nM thrombin to generate APC before being quenched with 20  $\mu$ l of hirudin (0.16 U/ $\mu$ l, 570 nM). The amount of APC formed was determined by monitoring the hydrolysis of S-2266 (100  $\mu$ l of 1 mM) at 1-min intervals at 405 nm using a plate reader (Molecular Devices Corp., Menlo Park, CA). 1 U of activity generates 1 pmol of activated protein C/min (37°C).

**Polyacrylamide gel electrophoresis.** We used precast 10–20% tricine gradient gels (NOVEX, Encinitas, CA). Nonreduced or reduced (2%  $\beta$ -mercaptoethanol [BME]) samples in 1% SDS were heated at 90°C for 15 min and run with molecular weight standards (Pharmacia).

**Amino acid analysis.** Samples hydrolyzed in a vapor phase (110°C, 22 h) with 6 N constant boiling HCl and 1% phenol were dried, derivatized with phenylisothiocyanate, and analyzed by the Pico-Tag method (Waters Associates, Milford, MA). Total cysteine plus cystine was determined after hydrolysis of a performic acid-treated sample. Tryptophan was analyzed by a separate acid hydrolysis using 4 M methanesulfonic acid.

**NH<sub>2</sub>-terminal sequence analysis.** We reduced TM samples with a fivefold molar excess of DTT in 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.5, 1 mM EDTA, under N<sub>2</sub> for 3 h at 37°C in the dark, then alkylated cysteines with iodoacetamide (2.2-fold molar excess over DTT) at room temperature for 15 min before termination of this reaction with  $\beta$ -mercaptoethanol. Protein was separated from excess reagents on a C-4 RP-HPLC column (DeltaPak; Waters Associates, linear gradient to 75% CH<sub>3</sub>CN in 0.1% trifluoroacetic acid), dried, and sequenced (model 477 with a 900 A data module or model 470A with chart recorder; Applied Biosystems, Foster City, CA). Phenylthiohydantoin amino acids were identified on a 120 A phenylthiohydantoin analyzer (Applied Biosystems) by RP-HPLC (Brownlee PTH-C18 cartridge, 2.1  $\times$  222 mm).

**Cyanogen bromide (CNBr) reaction.**  $TM_E$  (Sf9) (7.4 nmol) was treated with CNBr before or after oxidation with chloramine T or H<sub>2</sub>O<sub>2</sub>. Each sample was incubated at 37°C in the dark with 1 ml of 1 M CNBr in 70% (vol/vol) formic acid under N<sub>2</sub> for 45–55 h. The excess reagent was removed by repeated cycles of drying and resolubilization in H<sub>2</sub>O and analyzed by NH<sub>2</sub>-terminal sequencing.

**Oxidation of soluble TM analogues with chloramine T.** Purified  $TM_E$  (Sf9) at 1 mg/ml (38  $\mu$ M) in PBS was incubated with 1 mg/ml (4.4 mM) chloramine T (10 min, room temperature) and the reaction was terminated by desalting on a Sephadex G-25 column into 0.2% NEM-Ac, pH 7, or by the addition of *N*-acetylmethionine. To test whether TM was protected from oxidative inactivation by being bound to thrombin, the chloramine T reaction was performed on  $TM_E$  (Sf9) bound to a 0.5 ml DIP-thrombin affinity column. The bound TM was equilibrated either in equilibration buffer (0.3 M NaCl, 20 mM Tris-HCl, 0.5 mM CaCl<sub>2</sub>, pH 7.5) or in a modification of this buffer containing 0.1 M NaCl. After treatment for 10 min at room temperature with 1 mg/ml chloramine T in either the 0.1 M NaCl buffer or the 0.3 M buffer, the column was washed with the same buffer and eluted with 2 M NaCl, 20 mM Tris-HCl, 1 mM Na<sub>2</sub> EDTA, 0.02% NaN<sub>3</sub>, pH 7.5. The recovered protein was desalted into NEM-Ac, pH 7.0, and samples taken for APC assay and treatment with CNBr followed by sequence analysis.

**Oxidation of soluble TM analogues with H<sub>2</sub>O<sub>2</sub>.**  $TM_E$  (Sf9) was incubated with 0.21 M H<sub>2</sub>O<sub>2</sub> (93 mM Na phosphate, 0.28 M NaCl, 9.3% mannitol; pH 7.3; 23°C). Samples (65  $\mu$ l, 78  $\mu$ M) were withdrawn at various times and diluted into 325  $\mu$ l of buffer containing 333 U catalase (32). Samples (5  $\mu$ l) were assayed for activity in the APC assay and the remainder was reacted with [<sup>14</sup>C]iodoacetamide at low pH to selectively label the remaining nonoxidized methionines. Controls without peroxide were run simultaneously. To measure the oxidation rate, catalase-quenched samples were adjusted to pH 3 with 5.5  $\mu$ l of 6 N HCl. 1.2 ml of 6 N guanidine HCl, pH 3.8, containing 2 mM [<sup>14</sup>C]-iodoacetamide (444-fold molar excess over methionine; 1.13 Ci/mol) was added and the solutions were incubated (37°C; 48 h; in the dark),

dialyzed (4°C; 4.5 M guanidine·HCl, pH 3.5), and 0.5-ml samples were counted (Ecolume scintillation cocktail, ICN Biomedicals Inc., Costa Mesa, CA). Radioactivity in the catalase-quenched buffer blank was subtracted from radioactivity associated with the samples containing TM protein.

**Chloramine T oxidation of full-length TM on cells.** A549 cells (human lung carcinoma, ATCC CCL185), Cos7 cells (African green monkey kidney), or CHL1 cells (human melanoma) expressing full-length recombinant TM were grown to a confluent monolayer in 150-ml T-flasks (10% FBS/McCoys). Primary cultures of normal human umbilical vein endothelial cells (HUVEC) (Clonetics Corp., San Diego, CA) were incubated for 10 d, harvested, and cryopreserved in liquid nitrogen. Cells recovered from cryopreservation were established and grown in endothelial growth medium (Clonetics Corp.) supplemented with 8% FBS. Confluent cultures of all cell types were washed with PBS, harvested by scraping, washed in PBS, and resuspended in PBS at  $7 \times 10^6$  cells/ml. After incubation of  $3.5 \times 10^5$  A549 cells or HUVEC cells or  $10^5$  CHL1 or Cos7 cells with chloramine T (25°C,  $\leq 30$  min, 100  $\mu$ l), the excess reagent was removed from the cells by centrifugation, the cells were washed with APC assay diluent, and were assayed for cell-bound TM (33). These cell numbers were used because, on the basis of prior testing with the APC assay, they gave values in the appropriate range.

**Flow cytometry.** Chloramine T-treated or control cells were fixed with cold 70% ethanol, incubated with a mouse monoclonal antibody specific for human TM (mAb 531; Berlex Biosciences, South San Francisco, CA), washed with PBS supplemented with 1% FBS, and stained with FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG (CALTAG Laboratories, South San Francisco, CA). Stained cells were analyzed on the FACScan® system (Becton Dickinson Immunocytometry Systems, San Jose, CA) using an air-cooled argon ion laser (488-nm excitation) and data were analyzed with LYSYS II Acquisition and Analysis Software (Becton Dickinson Immunocytometry Systems).

**Solubilization of full-length TM.** CHL1 cells expressing full-length human TM with or without the M388L mutation were washed three times with ice-cold PBS, and then were resuspended in ice-cold extraction buffer (100 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1% Triton X-100; containing 65 kIU aprotinin and 1 mM *N*-tosyl-L-lysine chloromethylketone at a density of  $4 \times 10^7$  cell/ml; vortexed; incubated 15 min on ice; vortexed again; incubated an additional 15 min on ice; clarified by centrifugation for 15 min at 14,000 rpm; and desalted into PBS on NAP-10 columns (Pharmacia Inc.). Desalted supernatants contain  $\sim 1,000$  U/ml of TM, and control supernatants from untransfected cells contain no activity in the APC assay.

**Isolation and activation of human PMN.** Human PMN were isolated from 30–60 ml of blood from healthy male donors between 20 and 40 yr old. The granulocytes were isolated using Histopaque-1077 and Histopaque-1119 as described (procedure 1119; Sigma Diagnostics, St. Louis, MO) at room temperature and resuspended in 2 ml PBS. The cell suspension was diluted with 6 ml of H<sub>2</sub>O; inverted gently for 30 s to lyse the erythrocytes; 2 ml of 4 $\times$  PBS was added quickly; the PMN were centrifuged (200 g, 10 min); washed once with PBS; resuspended in 1 ml of PBS; and counted.

**Oxidation of TM by activated PMN.** TM<sub>E</sub> (Sf9) (50  $\mu$ l; 40–400 U/ml final) was added to isolated neutrophils (50  $\mu$ l;  $1-4 \times 10^5$  cells) in PBS previously treated with catalase (10 U/ml), superoxide dismutase (1,000 U/ml), or diphenylene iodonium (15  $\mu$ M), and the mixtures were activated with 1  $\mu$ l of PMA (20 ng/ml) as indicated. The reactions were incubated for 30 min at room temperature, separated by centrifugation (200 g, 4 min), and the supernatant was diluted  $\geq 10$ -fold in APC assay diluent before determination of TM cofactor activity in the APC assay. The ability of PMA to activate the oxidative burst was assessed qualitatively by nitro blue tetrazolium reduction (0.05% wt/vol) and inspection in a hemacytometer. For quantitative measurement of superoxide production, the same conditions were used except that the incubation included 0.1 mM cytochrome *c* (34). The reaction was terminated after 10 min by removing the PMN by centrifugation

and diluting the supernatant 20-fold into PBS. The spectrum of the cytochrome *c* in the supernatant was scanned and compared with a sodium hydrosulfite-reduced sample. The amount of reduced cytochrome *c* was calculated from the change in absorbance at 550 nm ( $E_{550} = 21,100$  M/cm). In experiments with whole cells,  $10^5$  CHL1 cells expressing either full-length TM<sub>LEOMC</sub> or TM<sub>LEOMC</sub> M388L were mixed with  $10^5$  PMN in 100  $\mu$ l PBS; activated with 1  $\mu$ l of PMA (20 ng/ml) in the presence of SLPI (10  $\mu$ M) and diphenyleneiodonium (15  $\mu$ M) as indicated. After 30 min at 37°C the cells were washed with 10 mM Hepes, pH 7.2, 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, and then assayed as described above except that thrombin and protein C were diluted in the same buffer. In experiments with attached cells, CHL1 cells were grown to confluence in 96-well tissue culture plates (Corning Glass Inc., Corning, NY), gently washed with PBS, PMN were added in 100  $\mu$ l PBS, activated with PMA for 30 min, washed with PBS and APC assay diluent, and assayed in the plate.

## Results

**Purification and characterization of TM<sub>E</sub> (Sf9) and TM<sub>LEO</sub> (CHL1).** We used either a standard DIP-thrombin affinity column-based purification for TM<sub>E</sub> (Sf9) or a two-step ion-exchange purification for TM<sub>LEO</sub> (CHL1) to purify both proteins to > 95% on the basis of reduced SDS-PAGE gels and N-terminal analysis. The two-step ion-exchange protocol takes advantage of the highly acidic chondroitin sulfates on TM<sub>LEO</sub> (CHL1) (35). TM<sub>E</sub> (Sf9) analyzed by SDS-PAGE gave a single band at 32 kD under nonreducing conditions and at 38 kD under reducing conditions. TM<sub>LEO</sub> (CHL1) contains chondroitin sulfate and ran as a broad band with or without reduction above the 116-kD protein standard, *Escherichia coli*  $\beta$ -galactosidase. A close correlation with their expected amino acid compositions was found for both analogues. Labeling of nonreduced TM<sub>E</sub> (Sf9) with [<sup>14</sup>C]iodoacetamide in the presence or absence of 6 M guanidine-HCl resulted in the incorporation of < 0.1 mol of label/mol of protein, as anticipated for a protein devoid of free cysteine. Reduced-alkylated wild-type TM<sub>E</sub> (Sf9) has the NH<sub>2</sub>-terminal sequence AVVPRSCSVENGG(C/E)EHA(C/E)NAIPGAPR(C/E)QXPAGAXLQADG . . . verifying the first 39 amino acids predicted for mature TM<sub>E</sub> (Sf9) expressed in Sf9 cells. X indicates an unassigned cycle. At the indicated cycles the PTH derivative of *S*-carboxamidomethyl-L-cysteine was not distinguished from the PTH derivative of glutamic acid. The first six amino acids are derived from the hypodermis A signal sequence and the remainder from human TM starting at Cys227. When secreted by CHL1 cells, TM<sub>LEO</sub> (CHL1) showed NH<sub>2</sub>-terminal heterogeneity due to processing at two sites APAEPQPGXSQXVEXDXFALYPXPATFLX-AXQ . . . , beginning with Ala1, and FPAPAEPQ . . . , beginning with phenylalanine two amino acids upstream in the signal sequence, in a ratio of 56:44, similar to that seen in HEK293 cells (35).

**Inactivation of TM by methionine oxidation.** When TM<sub>E</sub> (Sf9) is treated with an oxidant it rapidly loses its cofactor activity. In a typical experiment, chloramine T-oxidized TM<sub>E</sub> (Sf9) retained only  $\sim 20\%$  cofactor activity, as measured in the APC assay. Fully oxidized TM<sub>E</sub> (Sf9) has residual cofactor activity because no further decrease occurred with repeated chloramine T oxidations. TM<sub>E</sub> (Sf9) contains no tryptophan nor free cysteine, therefore we suspected the oxidation occurred at one of the two methionines. We analyzed oxidized TM<sub>E</sub> (Sf9) by CNBr cleavage because this reagent cleaves spe-

cifically at methionine and neither methionine sulfoxide nor methionine sulfone will react. The CNBr-treated, nonoxidized control  $TM_E$  (Sf9) gave four  $NH_2$ -terminal sequences. Three sequences are expected after cleavage at methionine, the  $NH_2$ -terminal sequence of the intact protein, (S1) AVVPRS . . . , sequence after Met291, (S2) XETGY . . . , and Met388, (S3) FXNQTA . . . . The fourth sequence, found in  $\sim 50\%$  yield relative to the  $NH_2$  terminus whether or not  $TM_E$  (Sf9) is oxidized, is an Asp-Pro cleavage (36) after Asp349 (PXFRA-), which occurs during the prolonged formic acid incubation. The average molar ratio of S1/S2/S3 from separate lots of CNBr-cleaved  $TM_E$  (Sf9) was 1.0:0.87:1.1 ( $n = 3$ ), showing that the methionines are not oxidized during purification. When chloramine T-treated  $TM_E$  (Sf9) samples were cleaved with CNBr and sequenced, no cleavage at either methionine was detected. Both Met291 and Met388 were  $\geq 95\%$  oxidized because neither of the possible CNBr fragments were formed in  $> 5\%$  yield relative to the  $NH_2$  terminus after oxidation with either chloramine T or  $H_2O_2$ .

*Site-specific mutagenesis to locate critical methionine.* Site-specific mutations of Met291 and Met388 demonstrate that inactivation of  $TM_E$  (Sf9) is due to oxidation of a single methionine. Mutant and wild-type  $TM_E$  (Sf9) were expressed in insect cells, treated with 10 mM chloramine T, assayed for cofactor activity, and the results compared (Table I). Derivatives that retain Met388 were inactivated by chloramine T to a similar extent ( $> 80\%$ ) whereas mutants at Met388 were resistant. Mutants in which Met291 is replaced were active but were not resistant to oxidative inactivation.

*Kinetics of oxidation of  $TM_E$  (Sf9) by  $H_2O_2$ .* We measured the rate of oxidation of  $TM_E$  (Sf9) by 210 mM  $H_2O_2$ . After quenching the reaction with catalase we monitored the cofactor activity in the APC assay and followed methionine loss by derivatizing nonoxidized methionines with [ $^{14}C$ ]iodoacetamide at low pH in denaturant (32). The extent of total methionine oxidation of  $TM_E$  (Sf9) reaches 87% of the theoretical possible within 30 min and 97% within 60 min. No label incorporation from [ $^{14}C$ ]iodoacetamide is observed at longer time points and the protein can no longer be cleaved by CNBr. In

this experiment cofactor activity decreased to a constant level of 16% of control, again confirming that fully oxidized TM retains activity.

Methionine oxidation exhibited pseudo first-order kinetics as expected for the ratio of  $H_2O_2$  to methionine used in this study. We determined a half-life of 10 min for methionine oxidation based on the kinetics of label incorporation from [ $^{14}C$ ]iodoacetamide. This translates to an apparent second-order rate constant of 0.35 M/min for both methionines in 210 mM  $H_2O_2$ . The log plot of label incorporation with time was monophasic. Thus, both methionine residues are equally reactive and accessible to  $H_2O_2$  within the limits of the assay. Loss of cofactor activity in the APC assay was also first order, 17 min, which translates to an apparent second-order rate constant of 0.20 M/min at 210 mM  $H_2O_2$ .

*Oxidation of  $TM_E$  (Sf9) bound to thrombin.*  $TM_E$  (Sf9) bound to thrombin on the DIP-thrombin affinity resin is not protected from oxidation by chloramine T. After elution the recovered  $TM_E$  (Sf9) had lost 86–90% of its cofactor activity in the APC assay. SDS-PAGE analysis shows that chloramine T treatment does not desorb TM from the column, since the TM only appears in the eluant and not in the chloramine T-containing buffer. Eluted material treated with CNBr and analyzed by  $NH_2$ -terminal sequence analysis showed no significant cleavage at Met291 nor Met388 compared with sequence obtained from the  $NH_2$  terminus. Thus, neither methionine is protected from oxidation by chloramine T when bound to thrombin. If  $TM_E$  (Sf9) is first oxidized in solution it still binds the DIP-thrombin affinity resin under the usual conditions in 0.3 M NaCl equilibration buffer.

*Chloramine T sensitivity compared with  $\alpha 1$ -protease inhibitor.* We compared the relative sensitivity of soluble TM analogues with  $\alpha 1$ -protease inhibitor as a model compound (37). When treated with chloramine T at various concentrations, the wild-type forms of  $TM_E$  and  $TM_{LEO}$  were inactivated at the same chloramine T concentrations at which  $\alpha 1$ -protease inhibitor lost activity (Fig. 1). Both soluble TM analogues in which the Met388 is mutated to leucine remained fully active in 10 mM chloramine T for 30 min.  $TM_{LEO}$  (CHL1), the complete exodomain analogue, has two additional methionines in the lectin domain, however, as is the case for  $TM_E$  (Sf9), a single substitution of Met388 for leucine confers full resistance to oxidative inactivation. Subsequently, we expressed full-length wild-type TM ( $TM_{LEOMC}$ ) and its M388L analogue in CHL1 cells. When the detergent-solubilized full-length molecules  $TM_{LEOMC}$  and  $TM_{LEOMC}$  M388L are oxidized with chloramine T under the conditions used in Fig. 1, the same result is obtained (Table II). Thus,  $TM_E$ ,  $TM_{LEO}$ , and  $TM_{LEOMC}$  all are inactivated by chloramine T oxidation whereas the corresponding M388L analogues, including full-length  $TM_{LEOMC}$  M388L, are all completely resistant.

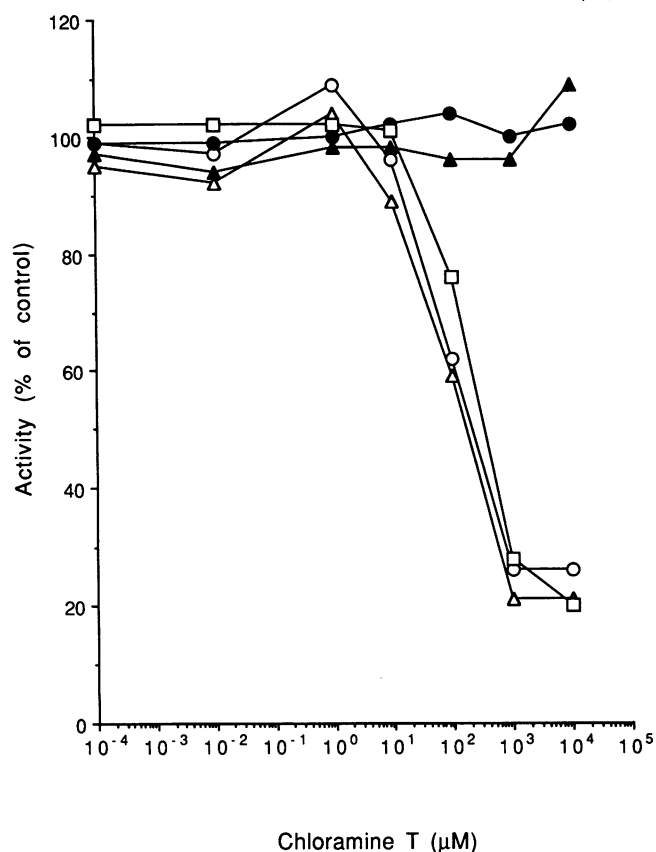
*Oxidation of TM by neutrophils.* Activated PMN effectively destroy the cofactor activity of wild-type  $TM_E$  (Sf9) but not the M388L analogue (Table III). Identical results were obtained with blood from six separate donors. This inactivation is completely prevented by prior treatment of the PMN with diphenylene iodonium (DPI), an inhibitor of the NADPH oxidase (28). Although SOD effectively intercepts all of the superoxide produced by the activated PMN as evidenced by the lack of cytochrome *c* reduction, neither SOD alone nor in combination with catalase protects  $TM_E$  (Sf9) from oxidation inactivation.

Table I. Chloramine T Oxidation of Site-Specific Mutant Analogues of  $TM_E$  (Sf9)

Thrombomodulin analogue	Percent activity remaining after oxidation*
Met291, Met388 (wild-type)	15 $\pm$ 6
Met291, Leu388	106 $\pm$ 13
Leu291, Met388	19 $\pm$ 0.3
Met291, Gln388	87 $\pm$ 14
Leu291, Leu388	111 $\pm$ 1

Growth media containing secreted mutants of  $TM_E$  (Sf9) was clarified by centrifugation, lyophilized, and redissolved in 1:10 volume of 0.2% NEM-Ac, pH 7, and 0.008% Tween 80. Aliquots (50  $\mu$ l) were treated with either 5  $\mu$ l of  $H_2O$  or 5  $\mu$ l of 100 mM chloramine T; incubated for 20 min at room temperature; oxidant removed by dilution; desalted on NAP-5 columns (20 mM Tris-HCl, 0.1 M NaCl, 2.5 mM  $CaCl_2$ , 5 mg/ml BSA, pH 7.4; Pharmacia Inc.); and assayed in the standard APC assay. The results after chloramine T treatment were expressed as a percentage of the activity after control treatment.

\* Average of duplicate determinations and deviation from the mean.



**Figure 1.** Relative susceptibility to oxidation of different TM analogues. Wild-type (Met388)  $TM_E$  (Sf9) and  $TM_{LEO}$  (CHL1) as well as their Leu388 site-specific analogues were treated with range of concentrations of chloramine T and compared with  $\alpha 1$ -protease inhibitor (37). Each of the soluble proteins were at  $9 \mu\text{g/ml}$  in  $0.2 \text{ M}$  Tris-HCl, pH 8.0, and  $0.01\%$  BSA. Samples were oxidized for 30 min at room temperature, and after removing excess reagent, analyzed by the standard APC assay.  $\alpha 1$ -Protease inhibitor activity was measured by inhibition of porcine pancreatic elastase (57). Values are plotted as a percent of untreated controls:  $\Delta$ ,  $TM_E$  (Sf9);  $\blacktriangle$ ,  $TM_E$  M388L (Sf9);  $\circ$ ,  $TM_{LEO}$  (CHL1);  $\bullet$ ,  $TM_{LEO}$  M388L (CHL1);  $\square$ ,  $\alpha 1$ -protease inhibitor.

**Oxidation of full-length TM on cells.** CHL1 and Cos7 cells expressing recombinant full-length wild-type  $TM_{LEO}$  and its oxidant-resistant M388L analogue, as well as A549 cells that express human TM constitutively, were used to model the oxidative inactivation of TM on vascular endothelial cells. In addition, human TM was tested on freshly cultured HUVEC cells in the third passage. In Table IV we compare the sensitivity of full-length TM in these cells to oxidation by chloramine T. Cell-bound cofactor activity of the M388L TM mutant is much less sensitive to chloramine T than is the activity of wild-type TM. Both Cos7 and CHL1 cells expressing  $TM_{LEO}$  M388L lost activity at longer times of exposure shown for Cos7 cells in Fig. 2 A and higher chloramine T concentrations as shown for CHL1 cells in Fig. 2 B. However, in  $10 \text{ mM}$  chloramine T, the oxidative inactivation of wild-type TM was 86% complete in 5 min in contrast to cells expressing the M388L mutant, which lost only 25% of their starting cofactor activity (Fig. 2 A). In addition, 100–1,000-fold higher concentrations of chloramine T are required to achieve the same level of inactivation (Fig. 2 B).

**Table II.** Oxidation of Solubilized Full-Length Thrombomodulin

Solubilized full-length analogue	Treatment	
	Control*	Chloramine T
	% activity <sup>‡</sup>	
Human $TM_{LEO}$ <sup>§</sup>	100±3	24±9
Human $TM_{LEO}$ M388L	100±4	97±15 <sup>  </sup>
Rabbit $TM_{LEO}$ <sup>§</sup>	100±5	14±2

CHL1 cells were grown and the full-length human TM was solubilized and assayed as described in Methods. Full-length rabbit TM (American Diagnostica Inc.) was used without further purification. Desalted supernatants were treated with  $1 \text{ mM}$  chloramine T for 30 min in PBS at room temperature, *N*-acetylmethionine was added to  $1.5 \text{ mM}$ , and samples were incubated an additional 10 min, followed by a 50-fold dilution into ice-cold assay diluent. \* Received all additions except chloramine T. <sup>‡</sup> Average±SD of data from two experiments assayed in triplicate. <sup>§</sup> Wild-type Met388. <sup>||</sup> Differs significantly from chloramine T-treated human TM M388 ( $P < 0.01$ ).

The cell number was not affected by the chloramine T treatment shown in Table IV and Fig. 2 B. In addition, CHL1 cells and HUVEC cells treated with  $1 \text{ mM}$  chloramine T as shown in Table IV were fixed and stained with a mouse monoclonal antibody to human TM and analyzed by flow cytometry. A population of cells that stained with the antibody was found in all three cell populations,  $33\pm 1\%$  in CHL1 cells expressing wild-type  $TM_{LEO}$ ,  $44\pm 1\%$  in CHL1 cells expressing the M388L mutant of  $TM_{LEO}$ , and  $15\pm 1\%$  in HUVEC cells compared with  $0.9\pm 0.04\%$  for vector-transfected control CHL1 cells. After chloramine T treatment, the population of cells expressing TM was lower than the pretreatment controls. When expressed as a percentage of these pretreatment controls, the amount of antigen that remains on the cell surface is not significantly different among the three cell types,  $68\pm 4\%$  in

**Table III.** Oxidation of  $TM_E$  (Sf9) by Activated PMN and Protection by DPI

PMN treatment (donors)	$TM_E$ (Sf9)	$TM_E$ M388L (Sf9)	Cytochrome c reduction
			% of unactivated control* nmol/min
Unactivated ( $n = 6$ )	100±5	100±13	0.019±0.004 (6)
+PMA ( $n = 6$ )	21±5	95±9 <sup>§</sup>	0.32±0.03 (100)
+PMA + catalase ( $n = 2$ )	27±6	99±3	ND
+PMA + dismutase ( $n = 2$ )	23±2	104±11	0.010±0.011 (3)
+PMA + dismutase + catalase ( $n = 2$ )	29±2	119±4	ND
+PMA + DPI ( $n = 2$ )	111±7	103±20	0.030±0.002 (9)

$TM_E$  (Sf9) samples were treated with activated PMN (30 min, room temperature) in PBS and assayed as described in Methods. \*  $TM_E$  (Sf9) samples reacted with PMN from each donor in duplicate or triplicate. Average of all samples±SD. <sup>‡</sup> Average of duplicate determinations±deviation from the mean. Values in parentheses are percents. <sup>§</sup> Differs significantly from  $TM_E$  (Sf9) ( $P < 0.01$ ).

Table IV. Oxidation of Cell-associated Full-Length Thrombomodulin Expressed in Mammalian Cells

Cell line	Full-length TM analogue	
	Met388*	Leu388
	% activity relative to untreated cells <sup>§</sup>	
A549 cells <sup>§</sup>	22±3	NA <sup>  </sup>
HUVEC cells	29±3	NA
Cos 7 cells (transient) <sup>¶</sup>	35±2	96±10
CHL1 cells (stable) <sup>¶</sup>	27±11	83±7

Cells were grown, treated with 1 mM chloramine T for 30 min in PBS, washed, and assayed as described in Methods. \* Wild-type.

† Average of three determinations±SD using the APC assay. § Human lung carcinoma cell line that constitutively expresses TM.

|| Not available. ¶ Using the APC assay for cell surface TM (33) both Cos7 and CHL1 cells expressing recombinant human TM have higher activity than A549 cells (8±3-fold) and the activity of untransfected control cells is low (2–4%).

CHL1 cells expressing wild-type TM<sub>LEOMC</sub>, 70±4% in CHL1 cells expressing the M388L mutant of TM<sub>LEOMC</sub>, and 80±9% in HUVEC cells. The percentage of retained cell surface antigen is similar to the percentage of APC activity retained in treated cells expressing the oxidation-resistant M388L mutant TM.

Neutrophil myeloperoxidase catalyzes the formation of hypochlorous acid, a powerful physiological oxidant, from Cl<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>, during the neutrophil oxidative burst. The fact that high concentrations of H<sub>2</sub>O<sub>2</sub> (200 mM) are required to rapidly oxidize Met388 allowed us to generate hypochlorous acid at low H<sub>2</sub>O<sub>2</sub> concentrations that are optimal for myeloperoxidase activity but do not directly inactivate TM. Peroxide in the presence of myeloperoxidase effectively inactivates the wild-type full-length TM on Cos7 and CHL1 cells and the M388L analogue is more resistant (Fig. 3). Hypochlorous acid levels were not quantitated in this experiment and therefore a quantitative estimate of the difference in sensitivity cannot be made.

Finally, we activated PMN in the presence of CHL1 cells expressing full-length TM at a cell/cell ratio of 1:1. The cell mixture was treated with PMA for 30 min before the cells were washed and assayed. As shown in Table V, TM<sub>LEOMC</sub> M388 retains significantly greater activity than does TM<sub>LEOMC</sub> in agreement with chloramine T (Fig. 2) and myeloperoxidase (Fig. 3) treatments. Similar results were obtained if attached CHL1 cells were treated with activated PMN. Inspection of the cells after treatment did not reveal selective cell loss compared with control wells. DPI fully protected against this inactivation and identical results were obtained whether or not the protease inhibitor SLPI (29) was included in the incubations. PMN have recently been reported to synthesize an inactive form of TM (38) and in control experiments we did not detect protein C activation by PMN, whether or not they have been activated with PMA.

## Discussion

We investigated the mechanism of oxidative inactivation of human TM and the potential for activated neutrophils to affect this inactivation, after our observation that soluble TM<sub>E</sub> (Sf9)

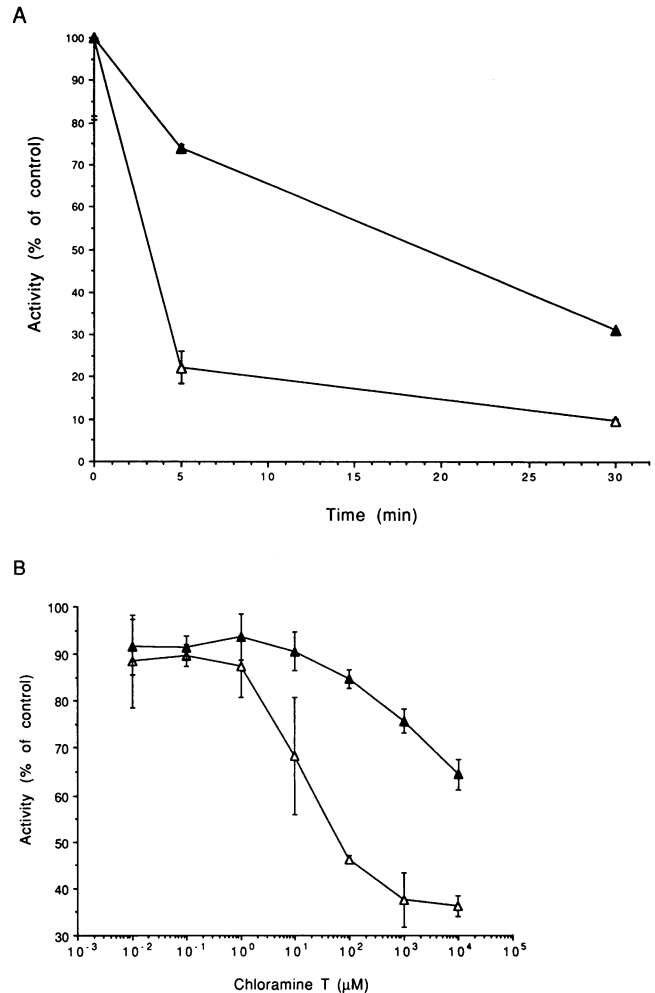


Figure 2. Chloramine T oxidation of TM on transformed Cos7 and CHL1 cells. (A) Time-dependent inactivation of TM on Cos7 cells by 10 mM chloramine T. (B) Chloramine T-dependent oxidation of TM on CHL1 cells after 30-min exposure. Cos7 cells or CHL1 cells transfected with a plasmid for the expression of either full-length human TM<sub>LEOMC</sub> (Δ) or TM<sub>LEOMC</sub> M388L (▲) were incubated for the indicated times and with the indicated concentrations of chloramine T (10<sup>5</sup> cells in 100 μl PBS, 25°C); excess reagent was removed by centrifugation; cells were washed with APC assay diluent and assayed for cell-bound TM (33). Untransfected Cos7 and CHL1 cells have < 4% of the activity of transfected cells (TM<sub>LEOMC</sub>).

lost > 80% of its cofactor activity under the oxidizing conditions used for radioactive iodination with chloramine-T (25). Inactivation is due to the oxidation of a single methionine at position 388. Met388 is in the short interdomain loop between the fourth and fifth EGF domains and is conserved in mouse, bovine, and human TM. Since, a TM fragment containing only the fourth through the sixth EGF-like domains is the smallest structure with cofactor activity (39), this loop is in a region of TM that is critical for cofactor activity.

Although any methionine in a protein will be oxidized under denaturing conditions, some proteins contain oxidant-sensitive methionines in their native state. Oxidative inactivation of proteins can have physiological importance (40). The biological properties of a number of proteins and peptides, including α1-protease inhibitor (41, 42), complement factor 5a

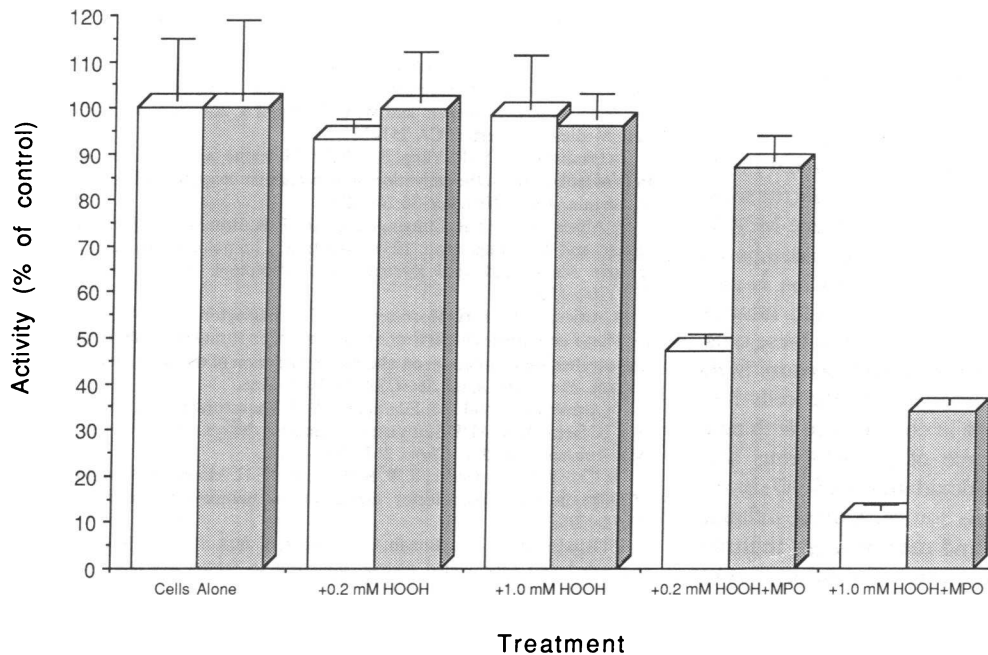


Figure 3. Treatment of TM on whole cells with myeloperoxidase and hydrogen peroxide. Cos7 cells transiently expressing either full-length human  $TM_{LEOMC}$  (open bars) or  $TM_{LEOMC}$  M388L (stippled bars) were incubated with the indicated concentrations of hydrogen peroxide (HOOH)  $\pm 1 \mu M$  myeloperoxidase (MPO) ( $8 \times 10^4$  cells in  $100 \mu l$  PBS,  $25^\circ C$ , 30 min); excess reagent was removed by centrifugation; cells were washed with APC assay diluent and assayed for cell-bound thrombomodulin (33). Parental Cos7 cells have  $< 2\%$  of the activity of transfected cells ( $TM_{LEOMC}$ ).

(43), plasminogen activator inhibitor I (37),  $\alpha 2$ -antiplasmin, antithrombin III (12), and *N*-formylmethionyl-leucyl-phenylalanine (45, 46), are altered as a consequence of methionine oxidation. The inactivation of  $\alpha 1$ -protease inhibitor by oxidation of a methionine to methionine sulfoxide is well characterized and occurs in patients with acute respiratory distress syndrome (46) and in smokers' lungs (43). We show that TM is as sensitive to oxidants as is  $\alpha 1$ -protease inhibitor (Fig. 1) and suggest that inactivation of TM occurs during inflammation.

A single methionine causes sensitivity to oxidants. In solution all wild-type forms of TM containing Met388 are readily inactivated by oxidation, including detergent-solubilized full-length human and rabbit TM. Two soluble analogues tested,  $TM_E$ , comprised of the six EGF-like domains, and  $TM_{LEO}$ , comprised of all of the exodomains, react similarly to detergent-solubilized full-length  $TM_{LEOMC}$ . The single mutation of Met388 to leucine renders all TM analogues resistant to inactivation. Compared with  $TM_E$ ,  $TM_{LEO}$  has two additional methionines and six potentially oxidant-sensitive tryptophans in the lectin domain and  $TM_{LEOMC}$  has one other methionine in the cytosolic domain. However, a single substitution of Met388 for

leucine confers full resistance to oxidative inactivation. Both  $TM_{LEO}$  (CHL1) and  $TM_{LEOMC}$  (CHL1) contain chondroitin sulfate, which enhances thrombin cofactor activity (35); however this highly charged polysaccharide does not affect the sensitivity of the wild-type forms to oxidative inactivation. Full-length TM is inactivated at similar concentrations of chloramine T whether or not it is on the cell surface or detergent solubilized, showing that insertion in the cell membrane does not protect Met388 from oxidation.

The detergent-solubilized M388L mutant of full-length TM is completely resistant to oxidative inactivation (Table II), however, cells expressing this mutant TM lose some cell-surface activity after exposure to oxidants. Cells expressing the full-length M388L mutant TM retain significantly more activity than cells expressing wild-type  $TM_{LEOMC}$  after all treatments (Figs. 2 and 3; Table IV). Loss of cofactor activity by cells containing  $TM_{LEOMC}$  M388L cannot be due to oxidation of the sensitive methionine in TM, but must be due to a different effect of exposure of the cells to oxidants. We cannot account for the loss of activity of cells expressing oxidant-resistant TM by lower cell counts nor do treated cells have a different morphology from control cells after treatment. Analysis of chloramine T-treated cells by flow cytometry shows that the percentage of retained cell surface TM antigen is high in HUVEC cells and CHL1 cells transfected with either wild-type or M388L mutant full-length TM (70–80%). This high percentage of antigen retention is similar to the percentage of APC activity seen after chloramine T treatment of the cells expressing the oxidation resistant M388L mutant TM (75–95%; Table IV and Fig. 2 B) and is significantly higher than the 20–35% APC activity that is retained by cells expressing full-length wild-type TM.

CHL1 and Cos7 cells were used to study full-length TM mutants because the parent cells have no TM cofactor activity. Vascular endothelial cells may not respond to oxidants in the same way as these cultured cells, however, HUVEC cells expressing wild-type human TM show no ability to resist oxidative inactivation of cell surface TM (Table IV). We are continu-

Table V. Oxidation of Cell-associated Full-Length Thrombomodulin Expressed in CHL1 Cells by Activated PMN

PMN treatment (donors)	$TM_{LEOMC}$ (CHL1)	$TM_{LEOMC}$ M388L (CHL1)
	% of unactivated control*	
Unactivated ( $n = 2$ )	100 $\pm$ 9	100 $\pm$ 9
+ PMA ( $n = 2$ )	25 $\pm$ 5	43 $\pm$ 7 <sup>†</sup>

CHL1 cells were grown and mixed with freshly isolated PMN in PBS in the presence of absence of PMA (20 ng/ml), incubated for 30 min ( $37^\circ C$ ), washed, and assayed as described in Methods. \* CHL1 cells treated with PMN from each donor in quadruplicate. Average of all samples  $\pm$  SD. <sup>†</sup> Differs significantly from treated cells expressing wild-type  $TM_{LEOMC}$  ( $P < 0.01$ ).



ing to test in vitro models for the PMN-dependent inactivation of endothelial cell surface TM. However, with respect to the oxidation of Met388, TM will be equally susceptible on any cell surface and exposure to PMN-derived oxidants will inactivate TM on vascular endothelium and other tissues.

**Inflammation and oxidative inactivation.** Activated PMN generate oxidants that inactivate wild-type TM but not the soluble M388L analogue (Table III) and the cell surface M388L mutant of full-length TM is resistant to inactivation compared with the wild-type TM (Table V). This inactivation is prevented by treatment of the PMN with DPI, which inhibits NADPH oxidase, the primary source of oxidant in these cells. Unlike DPI, catalase and SOD do not protect because SOD converts half of the superoxide produced by the cells into H<sub>2</sub>O<sub>2</sub>, which catalase must scavenge in competition with neutrophil myeloperoxidase, the source of hypochlorous acid (HOCl). DPI is a more effective antioxidant than SOD since it inhibits superoxide formation at its source. During inflammation, phagocytes, including PMN and macrophages, infiltrate the affected tissue. If activated, these cells enter the respiratory burst, producing superoxide and hydroxyl radicals, hydrogen peroxide, HOCl, and long-lasting N-chloramines, which are spontaneous reaction products of HOCl and amines (47). Other oxidants include superoxide produced by xanthine oxidase, which is released from tissues by Ca<sup>2+</sup>-triggered proteolysis of xanthine dehydrogenase (48), and oxidants produced by monocytes (49). Thus, in vivo oxidants with the potential to inactivate TM in tissues exceed the list of oxidants we tested in vitro.

Oxidant damage to tissue is important in the pathophysiology of human disease. Examples include acute respiratory distress syndrome, reperfusion injury, immune injury to the lung and kidney, cerebral trauma or ischemia, atherosclerosis, and rheumatoid arthritis (50–52).

Although TM is downregulated over a period of hours by cytokines (33, 53–55), we suggest that oxidative inactivation of TM is a more rapid mechanism for decreasing the anticoagulant potential of endothelial cells and will have a profound effect on the regulation of coagulation when endothelium is damaged. At the site of a cut, damaged endothelial cells result in the attraction and activation of PMN. In this context, rapid inactivation of TM could facilitate healing by allowing the formation of a localized clot. However, localized TM oxidation in reperfused ischemic tissue could allow thrombosis and blood vessel blockage; or a more generalized oxidation of the endothelium of the microvasculature during sepsis could result in a thrombogenic surface conducive to disseminated intravascular coagulation. TM has been detected on human blood monocytes and synovial tissue lining macrophages and is suggested to play a role in the control of increased coagulation associated with inflammatory synovitis (56).

In summary, TM is readily inactivated by oxidants and the oxidized molecule has ~ 10–25% of its original activity. Oxidation may play a crucial role in controlling the thrombogenic potential of the endothelium. In an inflamed area where PMN are activated, TM may be inactivated.

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