dozen different mRNAs are known to be regulated by this pathway, and sequences in the 5′-untranslated region of the respective mRNAs may determine the degree to which the translation of any particular mRNA can be modulated by mTOR signaling. HIF-1α protein expression is likely to be particularly sensitive to changes in the rate of synthesis because of its extremely short half-life under nonhypoxic conditions.

HIF-1 activity is regulated not only by HIF-1α protein expression but also by HIF-1α transcriptional activity. Our data analyzing transactivation mediated by Gal4-HIF-1α-TAD fusion proteins demonstrate that NOC18 treatment also induces HIF-1α TAD activity under nonhypoxic conditions. A regulatory switch controlling TAD activity involves $O_2$-dependent hydroxylation of Asn-803 by FIH-1. NOC18 treatment did not promote dissociation of FIH-1 and HIF-1α. TAD activity is also regulated by a MAPK-dependent mechanism (41). The MEK-1 inhibitor PD98059 blocked NOC18-induced HIF-1 activation and NOC18-induced MAPK activation, suggesting a link between NOC18, MEK/ERK, and HIF-1α. Published data suggest that the direct target of MEK/ERK may be the coactivators CREB-binding protein and p300, which interact with the TADs (42).

The action of NO in biological systems can be mediated directly by NO or by conversion of NO to $NO^-$ or $NO^+$ equivalents (43). Because two enzymes in the ubiquitin-proteasome pathway, E1 and E2, contain thiols in their active sites, these thiols were *a priori* candidates as targets of NO donors. However, our experimental results do not support this mechanism of action for NOC18. Another potential target is HIF-1α itself, since there is a report that GSNO induces nitrosylation of HIF-1α (44). However, our results indicate that if NOC18 induces nitrosylation of HIF-1α, this modification does not lead to accumulation of the protein. NOC18 treatment had no effect on the interaction of HIF-1α and $VHL_1$ whereas GSNO partially inhibited the interaction, and SNP dramatically augmented the interaction. SNP may stimulate the prolyl hydroxylation-ubiquitination system and promote increased HIF-1α degradation. Consistent with this hypothesis, SNP inhibited HIF-1α accumulation induced by DFX. Thus, different NO donors activate or inhibit HIF-1 through different molecular mechanisms..

Recent studies have demonstrated that NO donors stimulate cellular signaling cascades (45–47). Overexpression of a dominant negative form of Ras significantly inhibited NOC18-induced HRE-dependent gene expression, and the tyrosine kinase inhibitor genistein almost completely abolished NOC18-induced HIF-1α expression, suggesting that one or more protein-tyrosine kinases or phosphatases may be regulated by nitrosative modification. NOC18 treatment also induced phosphorylation of both AKT and ERK. Thus, NOC18 treatment modulates protein kinase signaling pathways similar to the effects of growth factor treatment. Determination of the extent to which NO signaling to HIF-1 participates in physiological and pathophysiological processes will require further investigation.

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# TMX, a human transmembrane oxidoreductase of the thioredoxin family: the possible role in disulfide-linked protein folding in the endoplasmic reticulum

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**Abstract**

Various proteins sharing thioredoxin (Trx)-like active site sequences (Cys–Xxx–Xxx–Cys) have been found and classified in the Trx superfamily. Among them, transmembrane Trx-related protein (TMX) was recently identified as a novel protein possessing an atypical active site sequence, Cys–Pro–Ala–Cys. In the present study, we describe the properties of this membranous Trx-related molecule. Endogenous TMX was detected as a protein of approximately 30 kDa with a cleavable signal peptide. TMX was enriched in membrane fractions and exhibited a similar subcellular distribution with calnexin localized in the endoplasmic reticulum (ER). The examination of membrane topology of TMX suggested that the N-terminal region containing the Trx-like domain was present in the ER lumen, where protein disulfide isomerase (PDI) was found to assist protein folding. Recombinant TMX showed PDI-like activity to refold scrambled RNase. These results indicate the possibility that TMX can modify certain molecules with its oxidoreductase activity and be involved in the redox regulation in the ER.


*Keywords:* Thioredoxin; Redox; Endoplasmic reticulum; Disulfide bond; Protein disulfide isomerase

Thioredoxin (Trx) is a small multifunctional protein, conserved from prokaryotes to higher eukaryotes [1–3]. Trx functions as one of the key molecules controlling cellular reduction/oxidation (redox) balance and is involved in various biological processes, including gene expression, cytoprotective action, signal transduction, and proliferation [4]. Trx is characterized by a conserved active site motif, Cys–Gly–Pro–Cys, and fulfills its function via reversible reduction and oxidation of thiols on these two cysteines (dithiol–disulfide interchange). Trx-like active site motif, Cys–Xxx–Xxx–Cys, has been found in several proteins classified in the Trx superfamily [5]. Various members of Trx superfamily can be found in each cellular compartment, such as mitochondria [6,7], the nucleus [8], and the endoplasmic reticulum (ER) [9]. They seem to participate in various redox regulations in these organelles. In the ER, newly synthesized secretory and membrane proteins are folded and undergo several post-translational modifications, including assembly, glycosylation, and disulfide bond formation to attain their proper structures and activities [10]. A variety of ER-resident proteins are involved in

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the protein folding process within the ER. Molecular chaperones that reside in the ER such as BiP/GRP78 and GRP94 facilitate the folding and prevent the aggregation of proteins. Formation of disulfide bonds is also essential for protein function and stability [11]. Until recently, the efficient disulfide bond formation in the ER has been considered to depend on the oxidizing environment in the ER lumen, where the concentration of oxidized glutathione (GSSG) is relatively high as compared with the cytosol, and GSSG was thought to provide oxidizing equivalents to allow disulfide bond formation. To support efficient oxidative protein folding, however, ER-resident oxidoreductases such as protein disulfide isomerase (PDI),[1] ERp57, and ERO1 are required. The activity of PDI depends on domains homologous to Trx with CGHC redox active site sequences [12]. When the cysteines in the active site exist in oxidized form (disulfide), PDI can catalyze the disulfide bond formation of substrate proteins through thiol–disulfide exchange. For efficient disulfide bond formation, reduced PDI is re-oxidized and regenerated continuously by ERO1, a recently identified protein localized in the ER [13–16]. ERp57, another ER-resident oxidoreductase, facilitates the formation of the correct disulfide bonds in glycoproteins and participates in the assembly of MHC class I molecules [17,18]. In addition to these enzymes, a number of proteins with CXXC motifs are found in the ER, but their precise functions are not well characterized. TMX (transmembrane Trx-related protein) was identified as a novel protein encoded by a gene isolated as a TGF-β-responsive gene in retrovirus-mediated gene trap screening [19,20]. TMX possesses a Trx-like domain with a CPAC active site sequence, which is unique to this protein. There are putative TMX orthologs with identical active site sequences in other species. Here, we report the molecular characterization of this novel Trx-related protein. Our results suggest that TMX is an integral membrane protein localized in the ER and its possible role as an oxidoreductase in the cellular redox regulation will be discussed.

## Materials and methods

### *Preparation of anti-TMX polyclonal antibody*

A truncated form of TMX (amino acid 27–180) was expressed as glutathione *S*-transferase (GST)-fusion protein in *Escherichia coli* and cleaved from GST portion [20]. The recombinant protein was extracted from SDS–polyacrylamide gel and used to immunize rabbits. IgG fractions were purified from the antiserum using protein A–Sepharose and used as the antibody against TMX.

### *Immunoblot analysis*

Cells were washed with PBS and lysed in PBS containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. Whole cell extracts (40 μg of protein) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane, followed by blocking in TBS containing 5% skim milk. For detection of TMX, the blotted membrane was incubated with anti-TMX at the concentration of 5 μg/ml at room temperature for 1 h. The membrane was washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit Ig. Immunodetection was carried out using ECL detection system (Amersham Biosciences). Anti-human calnexin antibody, AF8 [21], was a kind gift from Dr. Michael B. Brenner (Brigham and Women's Hospital, Harvard Medical School, Boston, MA). Anti-PDI antibody was obtained from BD Biosciences. Anti-c-Myc monoclonal antibody (9E10) was obtained from Santa Cruz Biotechnology.

### *Cell fractionation and membrane extraction*

Cells were disrupted by nitrogen cavitation using cell disruption bomb (Parr Instrument) in homogenization buffer consisting of 0.25 M sucrose, 10 mM Hepes–KOH, pH 7.5, 50 mM KCl, and 2 mM $MgCl_2$, and the homogenate was centrifuged at 1000*g* for 10 min. The post-nuclear supernatant was centrifuged at 100,000*g* for 1 h, and the resulting supernatant (cytosolic fraction) and the precipitate (membrane fraction) were collected. The membrane fractions were treated with 0.1 M $Na_2CO_3$, pH 11.7, for alkali extraction or 1% Triton X-100 plus 0.1% SDS. After incubation on ice for 15 min, samples were centrifuged again at 100,000*g* for 1 h to separate supernatant from pellet. These samples were subjected to SDS–PAGE and analyzed by immunoblotting.

### *In vitro transcription and translation*

TMX cDNA subcloned into pcDNA3.1 (–) (Invitrogen) was translated in the presence or absence of canine pancreatic microsomal membranes (Promega) using TNT T7 quick coupled transcription/translation system (Promega) and transcend non-radioactive translation detection system (Promega) with incorporation of biotinylated lysine residues. After SDS–PAGE and electroblotting, the translated products were visualized using streptavidin–HRP and ECL detection system.

[1] *Abbreviations used:* PDI, protein disulfide isomerase; PNS, post-nuclear supernatants; CMM, canine pancreatic microsome membrane; ERAD, ER-associated degradation.

*Immunofluorescence analysis*

A549 cells grown on multiwell chamber slides were transfected with pcDNA3.1-TMX-Myc using FuGENE 6 transfection reagents (Roche). The cells were fixed with 4% paraformaldehyde at room temperature for 15 min and then permeabilized with 0.2% Triton X-100 at room temperature for 4 min. After blocking in 5% bovine serum albumin at room temperature for 30 min, the cells were reacted with primary antibodies at room temperature for 1 h, followed by incubation with Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes). For detection of TMX-Myc, the cells were stained with FITC-conjugated anti-c-Myc (9E10) mouse monoclonal antibody (Santa Cruz Biotechnology).

*Topological studies of TMX by trypsin digestion*

Postnuclear supernatants or membrane fractions prepared as described above were treated with trypsin (Nacalai Tesque) at room temperature for 15 min. Digestion was terminated by addition of SDS sample buffer followed by boiling for 4 min. Samples were separated by SDS–PAGE and analyzed by immunoblotting.

*Reactivation of scrambled RNase*

Recombinant TMX [TMX(27–180)] and its mutant [TMX/CS(27–180)], in which two cysteines in the CPAC motif were substituted to serines, were prepared as described previously [20]. Assays for reactivation of scrambled RNase catalyzed by protein disulfide isomerase activity were performed as described elsewhere (assay protocol for PDI provided by TaKaRa) [22,23] with slight modifications. The assay was started by addition of scrambled RNase (Sigma) (final 0.2 mg/ml) to the reactivation mixture (50 mM sodium phosphate, pH 8.0, 1 mM EDTA, 2 mM GSH, and 0.2 mM GSSG) containing either 0.1 mg/ml (5.5 μM) TMX(27–180) or 0.1 mg/ml (5.5 μM) TMX/CS(27–180), and the mixture (final volume of 0.2 ml) was incubated at room temperature. A control sample without recombinant TMX was also examined. Aliquots of 50 μl were removed every 30 min from the reactivation mixture and assayed for RNase activity by mixing with 650 μl of 0.1 M Mops containing 0.3 mM cytidine 2′,3′-cyclic monophosphate (cCMP) as a substrate. RNase activity of each sample was monitored by the change in the absorbance at 284 nm ($A284$) that resulted from the hydrolysis of cCMP. The increase in $A284$ was recorded for 3 min in a spectrophotometer and the rate of increase in $A284$ ($\Delta A284$/min) was obtained. The rate of cCMP hydrolysis determined from $\Delta A284$/min was plotted against time of withdrawal of the aliquot from the reactivation mixture.

## Results

*TMX is expressed in various cell types*

To analyze the expression of endogenous TMX, we raised polyclonal antiserum against the N-terminal region of TMX (amino acids 27–180). After repeated immunization of rabbits with recombinant TMX expressed in *E. coli*, antiserum was collected and IgG fractions were purified. This antibody recognized a single band of approximately 30 kDa in immunoblot analysis. The intensity of this band was enhanced when using cells transfected with an expression vector encoding the full-length TMX, confirming the specificity of this antibody (Fig. 1, right panel). We examined TMX expression by immunoblotting using lysates prepared from several human cell lines, derived from different organs. TMX was expressed in all the cell lines tested (Fig. 1).

*TMX possesses a cleavable signal peptide*

Computational analysis predicted that the N-terminus of TMX contained a hydrophobic signal peptide for translocation into the ER, with a potential cleavage site between residues 26 and 27. To test whether the N-terminal hydrophobic region of TMX can function as a

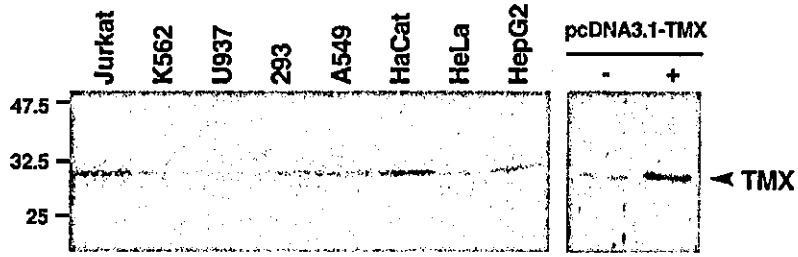


Fig. 1. Expression of endogenous TMX in human cell lines. Left panel: cell lysates (40 μg of protein) prepared from the following human cell lines were analyzed by immunoblotting with anti-TMX polyclonal antibody; Jurkat (T cell leukemia cell), K562 (proerythroblastic leukemia cell), U937 (promonocytic cell), 293 (embryonal kidney cell), A549 (lung adenocarcinoma cell), HaCat (keratinocyte cell), HeLa (cervix carcinoma cell), and HepG2 (hepatocarcinoma cell). right panel: The intensity of the immunoreactive band of TMX was enhanced in A549 cells transfected with pcDNA3.1-TMX (+) as compared with mock transfectants (−).

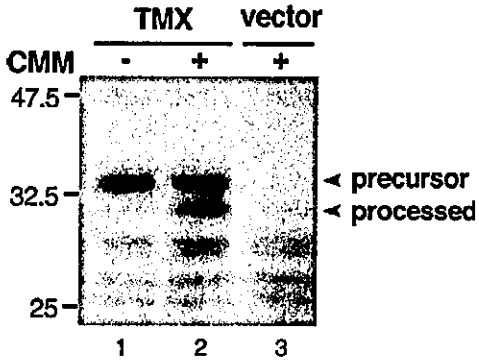


Fig. 2. In vitro translation of TMX cDNA. TMX cDNA was transcribed and translated in vitro using pcDNA3.1-TMX as a template and rabbit reticulocyte lysates with incorporation of biotinylated lysines in the presence or absence of canine pancreatic microsomal membranes (CMM). Control reaction was carried out with an empty vector. Samples were resolved by SDS–PAGE and transferred to a PVDF membrane. Translated products were detected using streptavidin–HRP.

cleavable signal peptide, TMX was in vitro translated in the presence of canine pancreatic microsome membrane (CMM). CMM has a signal peptidase activity and a protein with a cleavable signal peptide can be processed in the presence of CMM, resulting in the production of peptides with smaller molecular weights. In the absence of CMM, only a premature form of TMX was produced (Fig. 2, lane 1), and when CMM was added to the reaction, processed peptides were also detected (Fig. 2, lane 2), indicating the presence of a functional and a cleavable signal peptide which enabled the protein to enter into the membrane.

*TMX is an integral membrane protein*

Cell fractionation was carried out to monitor the subcellular localization of TMX. Cells were disrupted by nitrogen cavitation and resulting lysates were subjected to sequential centrifugation. As shown in Fig. 3A, TMX was recovered in the membrane fractions. Successful preparation of each fraction was confirmed by enrichment of marker proteins; calnexin (ER) and Trx (cytosol). When the membrane fractions were treated with 0.1 M sodium carbonate (pH 11.7), which should extract peripheral membrane proteins and luminal proteins such as PDI (Fig. 3B, lane 4), TMX was recovered in the insoluble pellet and not released from the membranes (Fig. 3B, lane 3). Only detergents could extract TMX and it was released into the supernatant (Fig. 3B, lane 6). These results indicate that TMX is an integral membrane protein.

*TMX-Myc colocalizes with calnexin in the ER*

In the immunofluorescence analysis using anti-TMX, we could not detect the signal showing the localization

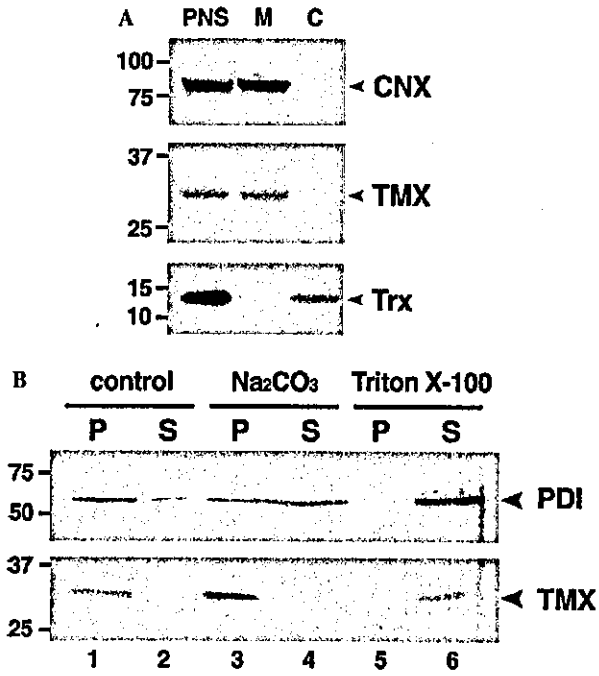


Fig. 3. Membrane association of TMX. (A) 293 cells were disrupted by nitrogen cavitation and fractionated into membrane (M) and cytosolic fractions (C). They were analyzed by immunoblotting using anti-calnexin (CNX: ER-resident membrane protein), anti-TMX, and anti-thioredoxin (Trx: mainly cytosolic protein) as indicated. Post-nuclear supernatants (PNS) were used as controls. (B) Membrane fractions prepared from 293 cells were suspended in homogenization buffer (control), 0.1 M sodium carbonate, pH 11.7, or 1% Triton X-100 plus 0.1% SDS. After incubation on ice for 15 min, samples were separated into pellet (P) and supernatant (S) by centrifugation and analyzed by immunoblotting with anti-TMX or anti-PDI.

of the endogenous protein. To visualize the intracellular location of TMX, A549 cells were transiently transfected with Myc-tagged TMX and analyzed by immunofluorescence microscopy. A reticular staining of the cytoplasm was observed in cells expressing TMX-Myc. A similar staining pattern was obtained when a C-terminus-tagged EYFP fusion TMX was examined (data not shown). These results implied that TMX was associated with subcellular membranous structure, such as the ER and the Golgi apparatus [24]. On this account, we compared the expression pattern of TMX-Myc with those of marker proteins localized in these organelles. When cells transiently transfected with TMX-Myc were doubly stained with anti-Myc antibody and anti-calnexin antibody, TMX-Myc was found to colocalize with calnexin (Fig. 4). Calnexin is a well-characterized transmembrane-type chaperone localized in the ER. In contrast, the staining pattern of TMX-Myc was entirely different from that of GM130 associated with the *cis*-compartment of the Golgi body (data not shown). From these results, it is suggested that TMX is localized in the ER membrane.

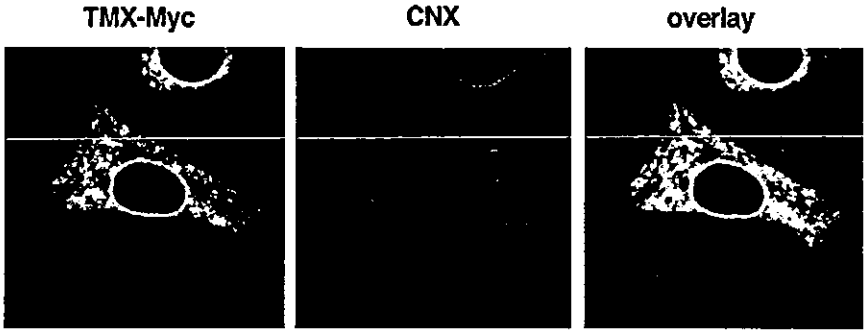


Fig. 4. Confocal immunofluorescence analysis of cells expressing TMX-Myc. A549 cells transiently transfected with pcDNA3.1-TMX-Myc were co-stained with anti-Myc (green) and anti-calnexin (red). A merged image is shown in the right panel with yellow color indicating colocalization of TMX-Myc and calnexin.

*Membrane topology of TMX*

Hydropathy analysis and motif search revealed a hydrophobic stretch in the C-terminal half of TMX, which was likely to form a transmembrane domain. To confirm the orientation of TMX in the membrane, the 1000*g* supernatant fraction prepared from 293 cells was treated with trypsin, followed by immunoblotting. After digestion with trypsin, the amount of intact TMX protein was decreased and fragments of 20 kDa were newly detected by anti-TMX (Fig. 5A). The size of these fragments corresponded to that of the N-terminal region which could be recognized by anti-TMX. These results indicated that the N-terminal region of TMX was localized on the luminal side of the ER and protected from trypsin digestion. We further confirmed the membrane topology of TMX using epitope-tagged TMX. Membrane fractions prepared from NIH3T3 cells transfected with TMX-Myc were subjected to trypsin treatment and digested TMX-Myc was monitored using two antibodies, anti-TMX specific to the N-terminal region or anti-Myc recognizing the C-terminus. After trypsin treatment, anti-Myc antibody failed to detect the digested protein (Fig. 5B, lane 4), indicating that the C-terminal tail was accessible to the protease, whereas anti-TMX antibody recognized a band of 20 kDa in the same way as the above experiment using endogenous protein (Fig. 5B, lane 2). Together these results suggest that TMX is a type I integral membrane protein [25,26]; N-terminal region containing the Trx-like domain is present in the ER lumen and the C-terminus of TMX is oriented facing the cytoplasm.

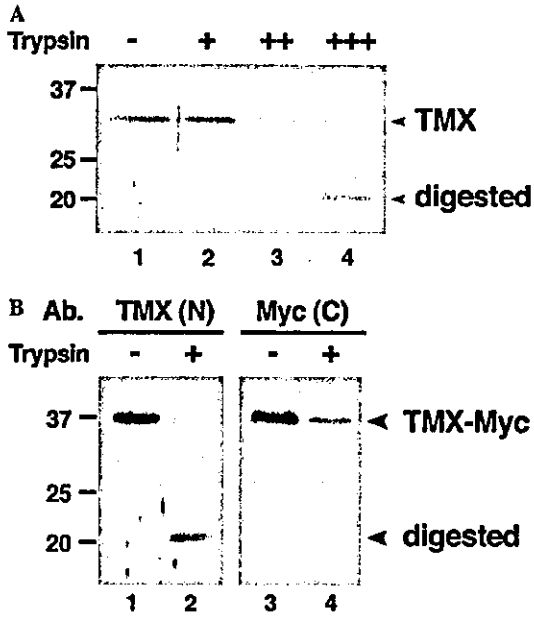


Fig. 5. Membrane topology of TMX. (A) Post-nuclear supernatants prepared from 293 cells were treated with increasing amounts of trypsin (lane 1, 0 μg/ml; lane 2, 5 μg/ml; lane 3, 15 μg/ml; and lane 4, 50 μg/ml) at room temperature for 15 min. Trypsin digestion was terminated by adding SDS sample buffer and samples were analyzed by immunoblotting with anti-TMX. (B) NIH3T3 cells were transiently transfected with pcDNA3.1-TMX-Myc. Membrane fractions were prepared and subjected to trypsin digestion (lanes 1 and 3, 0 μg/ml; lanes 2 and 4, 150 μg/ml), followed by immunoblotting with anti-TMX (lanes 1 and 2) recognizing the N-terminal region of the protein or anti-Myc (lanes 3 and 4) specific to the tag added at the C-terminus.

*Trx-like domain of TMX catalyzes refolding of scrambled RNase*

In the ER, newly synthesized proteins undergo several post-translational modifications. Among them, the formation of disulfide bonds is critical for the folding and stabilization of proteins on the secretory pathway. PDI is a physiological catalyst for native disulfide bond formation in the ER lumen. PDI possesses the sequences of CGHC within two domains homologous to Trx, which are required for its activity. This redox-active CXXC motif, characterized by two cysteines separated by two amino acids, is also found in TMX (CPAC). To test whether TMX has PDI-like activity in vitro, a Trx-like domain of TMX was expressed as recombinant protein in

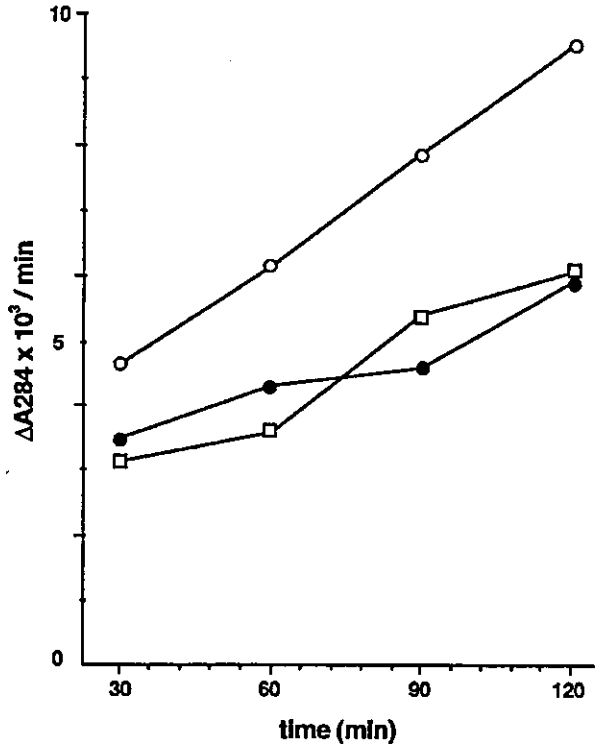


Fig. 6. Reactivation of scrambled RNase catalyzed by TMX. Enzymatically inactive scrambled RNase was incubated with TMX or its mutant (TMX/CS) expressed in *E. coli*. Aliquots were removed every 30 min and added to the reaction mixture containing cCMP as a substrate for RNase. Reactivation of RNase was monitored by the rate of increase in the absorbance at 284 nm (Δ*A*284/min) resulting from cCMP hydrolysis. Open circles, wild type TMX; closed circles, TMX/CS; and open squares, negative control without recombinant protein. The experiments were repeated three times with essentially the same results.

*E. coli* and its ability to catalyze disulfide interchange was assessed using scrambled RNase containing randomly formed disulfide bonds. Scrambled RNase is inactive and requires interchange of incorrectly paired disulfide bonds to regain its native conformation and activity toward its substrate. The recombinant TMX could refold scrambled RNase resulting in the recovery of its activity observed as an increase in the rate of cCMP hydrolysis (Fig. 6). When the two cysteines in the active site were substituted to serines, this mutant TMX showed no effect on the recovery of RNase activity, as compared with a control, revealing that two cysteine residues in the CPAC motif were essential for the catalytic activity.

## Discussion

In this study, we describe the properties of TMX, a novel member of the Trx superfamily. Various members of the Trx superfamily have been found in each cellular compartment and seem to act as redox regulators with specialized functions depending on their localization. Subcellular localization of TMX was practically identical to that of calnexin, a transmembrane protein localized in the ER. Calnexin is a membrane-bound chaperone that interacts with many glycoproteins on the secretory pathway during their maturation within the ER [21,27]. The C-terminal charged residues of calnexin are required for its retention in the ER, while no consensus motif for localization is found in TMX. Due to the weak reactivity of the antibody to the protein with native conformation, we could not obtain the fluorescence image showing the localization pattern of endogenous TMX. When several TMX constructs containing tag sequence (c-Myc or EYFP) were expressed in cells, they showed typical ER staining pattern. These observations strongly suggest that TMX is localized primarily in the ER, but we could not exclude the possibility that overexpression of the tagged protein might lead to the mislocalization. Further studies are needed to elucidate the precise mechanism for the retention in the ER.

In the ER, various types of Trx-like molecules can be found [28], but many of these ER-resident Trx-like molecules are less well characterized. As reported previously, Trx-like domain of TMX showed reducing activity toward interchain disulfides of insulin [20]. Here we show that TMX can also catalyze the renaturation of inactive RNase containing incorrect disulfide bonds. The topological studies suggested that the Trx-like domain of TMX was present in the ER lumen, where TMX might modify a certain target molecule and participate in the disulfide-linked protein folding. The active site sequence of TMX (CPAC) is unique to this protein and TMX orthologs with identical active site sequence have been found in other species. They are similar in overall organization: a signal peptide; a luminal Trx-like domain; a transmembrane domain; and a C-terminal tail. Among them, *Caenorhabditis elegans* DPY-11 has been reported to be expressed exclusively in the hypodermal cells [29]. *Dpy-11* mutants and RNAi-mediated knockdown worms show dumpy body phenotype and the sensory ray abnormalities, suggesting that DPY-11 is required for body and sensory organ morphogenesis. These studies raise the possibility that, unlike classical PDI as a general foldase, TMX may be required for the modification of specific substrates involved in the developmental process.

In our previous study, overexpression of TMX in 293 cells significantly delayed onset of ER stress-induced cell death [20]. The perturbations of ER function lead to the accumulation of misfolded or abnormal proteins in the ER, which causes ER stress [30]. Cells can adapt to ER stress by activating the stress response pathway [31,32], but excessive and prolonged ER stress causes apoptotic cell death [33]. ER-resident molecular chaperones and folding enzymes including PDI are induced by ER stress to increase folding capacity in the ER. Involvement of luminal oxidoreductases is also suggested in the

degradation of misfolded proteins [34]. Terminally misfolded proteins in the ER are translocated back to the cytosol through the translocon complex and degraded by the ubiquitin–proteasome system defined as ER-associated degradation (ERAD) [35]. During this process, reduction of inter- and intramolecular disulfide bonds of misfolded proteins should occur to unfold the polypeptides and make them transported through the translocon [36,37]. Such enzymatic reduction of disulfide bonds is observed in the endocytic pathway. Many proteins internalized by endocytosis are degraded in lysosomes into small peptides. Lysosomal thiol reductase named GILT catalyzes the reduction of disulfide bonds of these proteins to promote efficient proteolysis, and facilitates processing and presentation of antigenic proteins [38,39]. If the disulfide reduction of proteins is a general strategy equipped with cells to achieve efficient protein degradation, ER-resident oxidoreductases can be implicated in unfolding of proteins destined to be degraded by ERAD, although none has been found to catalyze disulfide reduction in the oxidizing environment of the ER. Recent studies have suggested that ER stress-induced cell death is associated with neuronal degenerative disorders such as Alzheimer's disease [40], Parkinson's disease [41], and polyglutamine disease [42]. These diseases are associated with accumulation of abnormal protein aggregates. Thus, the defects of the protein-folding system could lead to the pathogenesis of various diseases [43]. As compared with molecular chaperones, which promote protein folding and prevent aggregation, less is known about the manipulation of disulfide bonds catalyzed by oxidoreductases in the ER. It will be of great interest to determine the specific activities and their preferred substrates in disulfide bond modification, and further studies to clarify their physiological functions will provide us a biochemical basis for understanding of the importance of redox regulation in protein folding.

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# 9 革新的診断・治療へのアプローチ

## ―膜透過性・標的特異性を有する融合タンパク質を用いたイメージング・ターゲティング―

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1989年大阪大学で医学博士修得。90年学振特別研究員（がん）、93年ERATO国由細胞変換プロジェクト研究員。99年京都大学医学研究科助手。2004年京都大学医学研究科COEプロジェクト"病態解明を目指す基礎医学研究拠点"特任助教授。
研究テーマは、低酸素がん細胞・アポトーシスの解析と病態イメージング。趣味は旅行。

Key words：低酸素がん細胞、膜透過ドメイン（PTD；Protein transduction domain），HIF-1，酸素濃度依存的分解（ODD; Oxygen-dependent degradation)

Abstract

低酸素が問題になる疾患には、脳・心筋梗塞や閉塞性末梢動脈硬化症といった虚血性疾患のみならず、難治性固形腫瘍が含まれる。固形腫瘍には、細胞増殖と血管新生の不均衡に起因する『低酸素領域』が存在する。低酸素領域にあるがん細胞は、放射線や抗がん剤に感受性が低く、治療効果不良の主因であるばかりでなく、浸潤・転移・再発の温床となっている。しかし、低酸素環境は正常組織には存在しないため治療標的となりうる。我々は、低酸素環境下にある細胞内で特異的に安定化する融合タンパク質を開発し、それを用いることにより、低酸素細胞のイメージング・ターゲティング研究を行っている。本稿では、固形腫瘍を対象にした研究について紹介する。

## はじめに

医学の進歩に伴い、がんの診断・治療技術も年々進歩してきている一方で、我が国では、世界のどの国も経験したことのない速度で人口の高齢化が進行している。これが我が国でがんが増える第一の要因になっている。血液検査、内視鏡、画像診断等の技術の向上により、多くのがんが早期に発見できるようになってきたが、「手軽に、どのがんでも」というところまでは至っていない。我々の研究は、早期診断・早期治療に革新的イメージング・ターゲティング技術を提供することにより、がん撲滅に寄与することを目的としている。

## 1. 腫瘍内低酸素領域

固形がんには、非常に小さながん（数ミリ以下）でも通常ではありえないような低酸素状態のがん細胞が存在する。それは、がん細胞の増殖に血管新生が追いつかないために、血流からの酸素や栄養が十分行き渡らない領域が生じるためで、酸素も栄養も枯渇して死んでしまったがん細胞と増殖しているがん細胞の境界の極めて限られた領域に存在する（図1）。この低酸素がん細胞は、過酷な環境に順応するために、増殖は停止し、代謝も解糖系を用いて省エネルギー化し、いわば冬眠状態にある。それだけであれば、これら低酸素がん細胞は注目に値しないのであるが、これらの細胞はがん治療の抵抗性の指標となるほど、抗がん剤や放射線治療に抵抗性を示す。従って、癌治療が終わった後も生き残り、再発の温床となる可能性が指摘されている。更

*Imaging and targeting with a novel fusion protein possessing membrane permeability and target-specificity:* Shinae Kondo, Hiroshi Harada, Masahiro Hiraoka, Department of Therapeutic Radiology and Oncology, Kyoto University Graduate School of Medicine

に、生き延びるために様々な因子（例えばＶＥＧＦ，ＴＧＦ－β，　ＦＧＦ，　ＩＧＦ）を分泌して、血管新生を促したり、転移や浸潤に関わったりして、腫瘍全体の悪性度を高める働きをする。故に、低酸素がん細胞は癌治療を行う上で、見逃すことができない標的であると同時に、低酸素がん細胞が存在する微小環境は「正常組織ではありえない」という点で絶好の標的（環境標的）となりうる。

## 2. 酸素濃度依存的制御機構

この低酸素がん細胞には、極めて興味深いタンパク質が存在する。そのタンパク質はＨＩＦ－１αと呼ばれ、転写因子ＨＩＦ－１を構成する２つのサブユニットのひとつで、低酸素環境で安定化し、通常の酸素環境下（有酸素環境）で速やかに分解される。そのためＨＩＦ－１は低酸素環境下で機能し、低酸素特異的に応答する一連の遺伝子の発現を誘導する。それらの遺伝子は、低酸素がん細胞が過酷な微小環境に順応するために必要な因子や上記のがん悪性化に関与する因子をコードしている。

我々は、ＨＩＦ－１αタンパク質の酸素濃度依存的分解（ＯＤＤ）制御機構に着目した。2001年に新たなプロリン水酸化酵素が発見さ

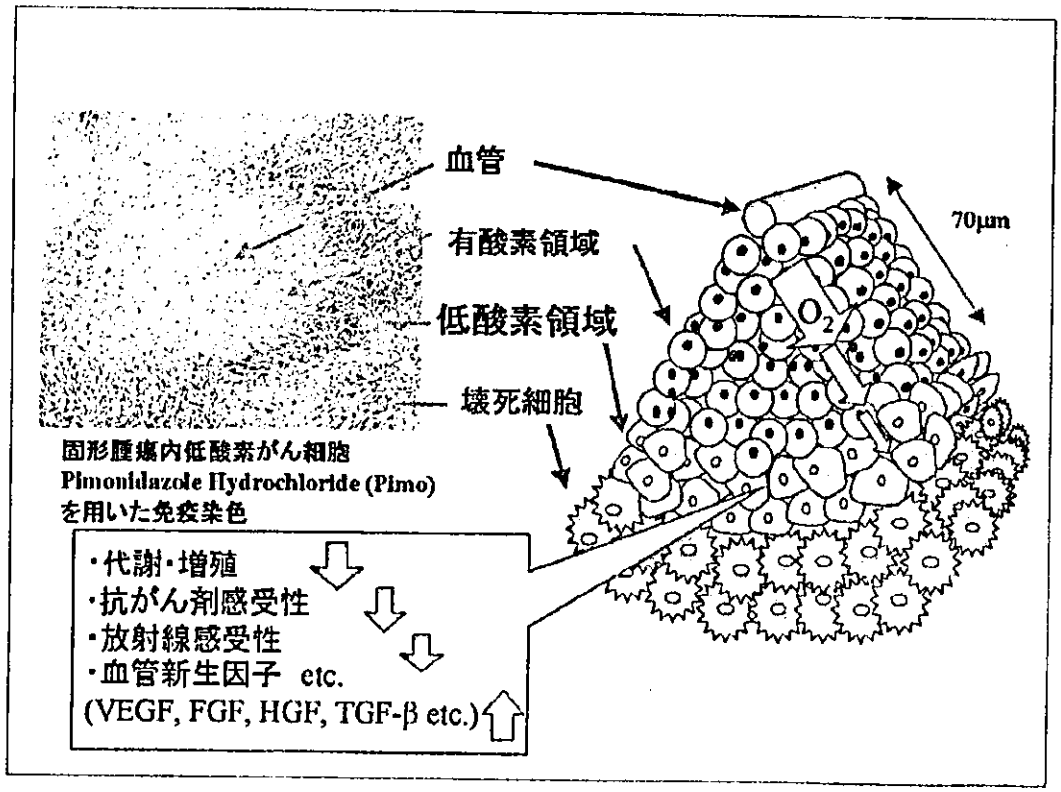


図1　固形腫瘍内低酸素領域

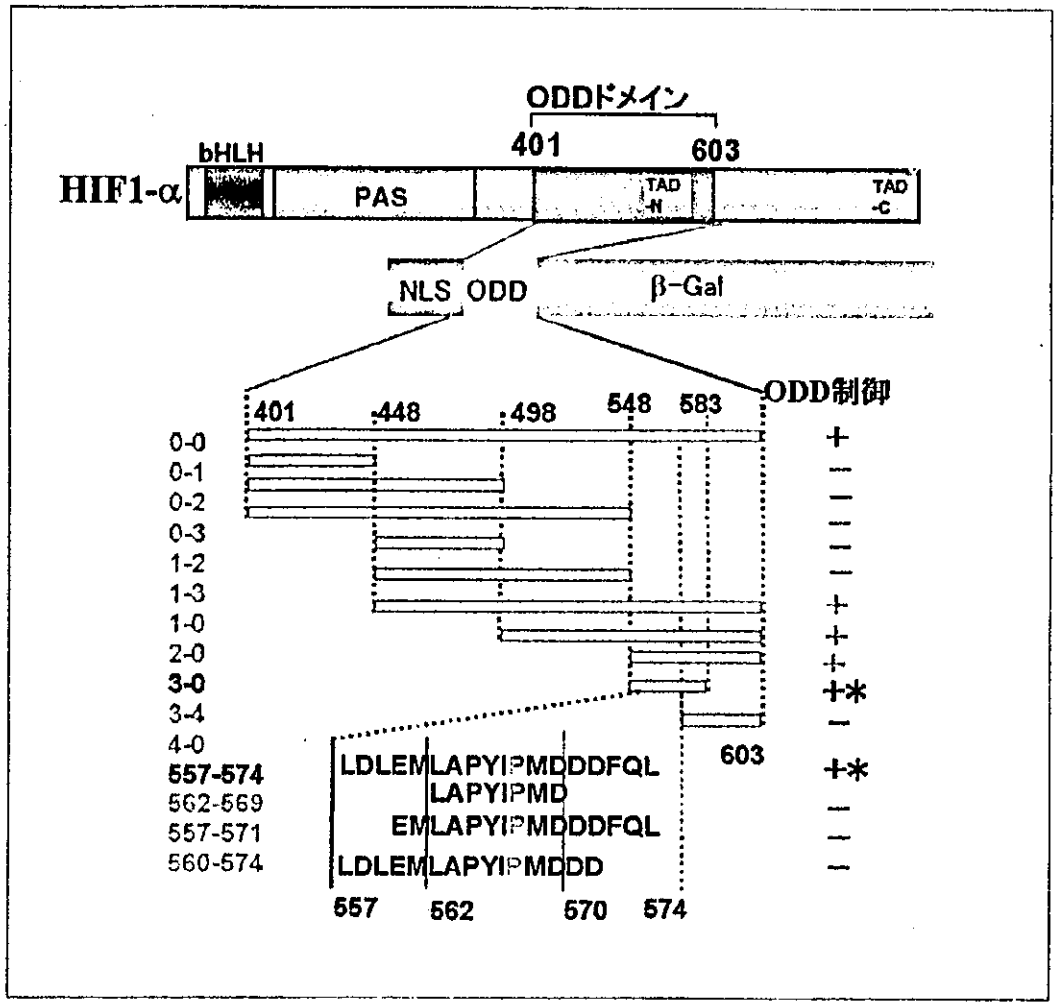


図2　ODD融合タンパク質のODD制御

れ、この制御機構は分子レベルで詳細が明らかにされている。即ち、プロリン水酸化酵素が、ＨＩＦ－１αタンパク質の中央付近にあるＯＤＤドメインのプロリン残基を水酸化し、これを目印にして結合するユビキチン連結酵素複合体Ｅ３により、ＨＩＦ－１αはユビキチン化されプロテオゾームに運ばれて分解される。このプロリン水酸化酵素が機能する際に、酸素を含んだ鉄分子を必要とすることが酸素依存性の中枢機構であった。このＯＤＤ制御は極めて厳密で、低酸素下で安定化したＨＩＦ－１αは、有酸素にすると数分以内に分解される。この極めて厳密なＯＤＤ制御機構を応用して、低酸素がん細胞特異的イメージング・ターゲティング材料の構築が始まった。

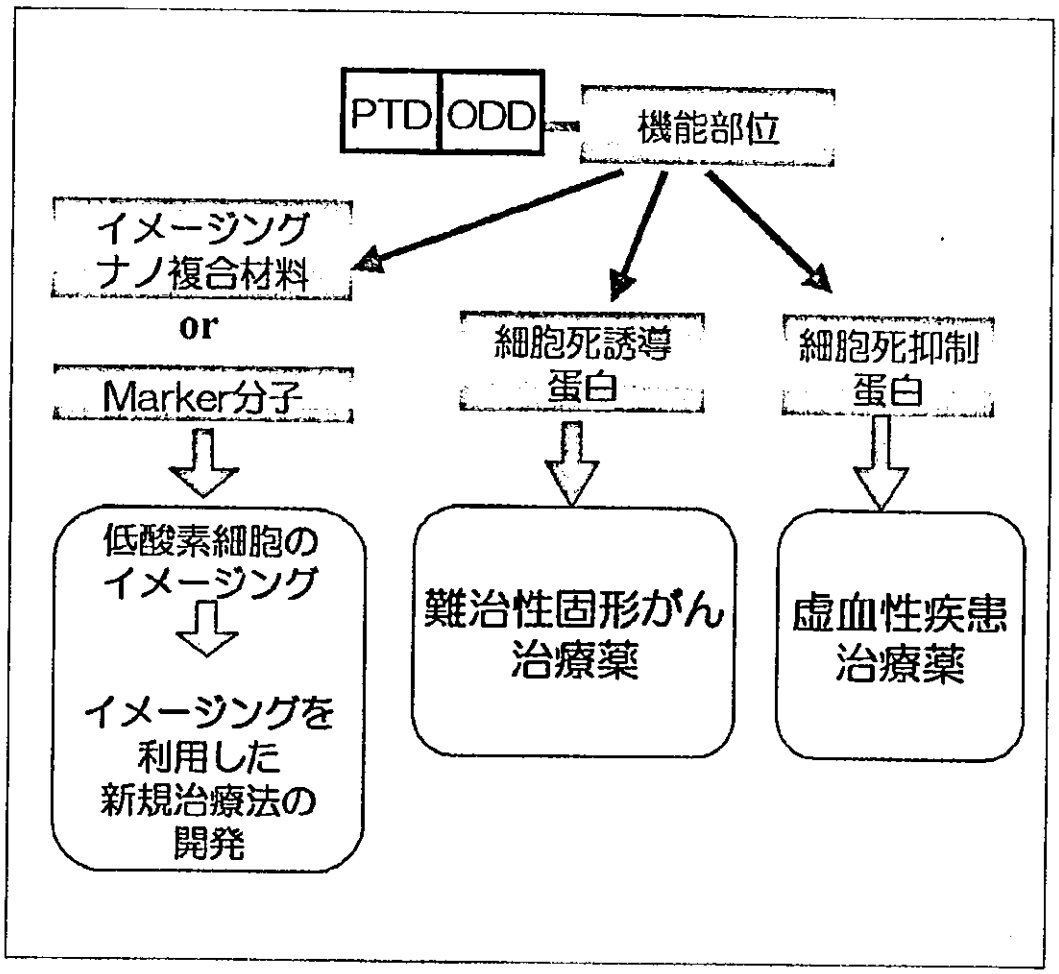


図3　PTD-ODD融合タンパク質の利用例

## 3. ＰＴＤ－ＯＤＤ融合タンパク質の構築

我々がまず行ったことは、上記のＨＩＦ－１αにあるＯＤＤドメインを任意のタンパク質に融合させることで、任意のタンパク質が持つ機能を酸素濃度依存的に制御することができるか否かの検証である。ＯＤＤドメイン全部（約２００アミノ酸）を付加すると、全体の分子量がかなり大きくなるので、最小のアミノ酸配列を決定するためにＯＤＤドメインを部分的にβ－ガラクトシダーゼに融合させ、β－ガラクトシダーゼ活性の酸素濃度依存性を調べた（図２）。その結果、少なくとも18個のアミノ酸があれば、任意のタンパク質の活性をＯＤＤ制御できること、最適なＯＤＤ制御のためには、約50個のアミノ酸配列からなるＯＤＤドメインが必要であることがわかった。

ＯＤＤ制御できるタンパク質が作れても、細胞内に導入できなければ、細胞内で行われ

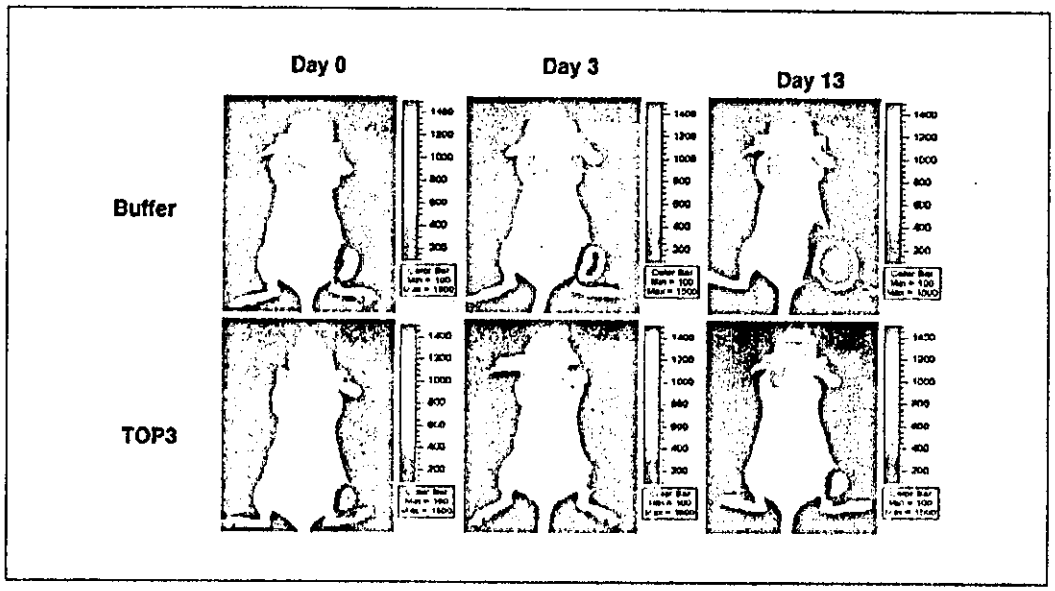


図4　TOP3による抗がん効果
光イメージングにより低酸素領域を可視化する系で同一マウス大腿部腫瘍内の低酸素がん細胞の量を経時的にモニターした。TOP3の投与は20mg/Kg，day0，5，10の3回。

るＯＤＤ制御を受けることができない。そこで我々は、タンパク質に膜透過性を付加する膜透過ドメイン（ＰＴＤ）をＯＤＤ融合タンパク質に付加することにより、培養細胞を用いた実験でほぼ100％の細胞にタンパク質を導入し、酸素依存的に機能させることに成功した。しかもこのＰＴＤ融合タンパク質は、ネズミの腹腔内に投与すると、脳を含む全身の組織細胞にデリバリーされることが示されている。そこで我々は、ＰＴＤ－ＯＤＤ－β－ガラクトシダーゼを腹腔内に投与し、この融合タンパク質の体内分布とβ－ガラクトシダーゼ活性を調べた。その結果、ＯＤＤを付加していないタンパク質を投与した場合、正常肝組織と腫瘍組織全体で、タンパク質と活性が確認できたのに対し、ＯＤＤを付加したタンパク質を投与した場合は、正常組織ではタンパク質も活性も確認できず、腫瘍組織でも一部でのみタンパク質と活性が確認できた。その部分が低酸素であるか否かを確認するために、低酸素マーカーとして知られている化合物（pimonidazole）を用いて腫瘍切片を染色したところ、低酸素マーカーと同一のところに、β－ガラクトシダーゼタンパク質が存在していることがわかった。これらの結果は、我々の設計通り、ＰＴＤ－ＯＤＤ融合タンパク質はＯＤＤ制御を受け、低酸素がん細胞特異的に分布・機能することを示しており、低酸素がん細胞特異的イメージング・ターゲティングが可能であることを示していた。

## 4．ターゲティングへの応用

ＰＴＤ－ＯＤＤ融合タンパク質は、機能ドメインに付加するものを変えることで、様々な機能を持たせることができる（図３）。即

ち、低酸素がん細胞に細胞死を誘導する機能を付加することで、低酸素がん細胞のターゲティングを行うことができる。モデルタンパク質として構築したＴＯＰ３は、低酸素細胞内で安定化し、活性化され細胞死を誘導するように設計されている。ＴＯＰ３を投与することにより、腫瘍サイズは小さくなった。ヒト膵臓がん細胞をネズミの皮下に移植した実験で、無処理群で腫瘍体積が２倍になる日数が4.8 ± 2.3日であったのに対し、ＴＯＰ３投与群では、12.6 ± 5.4日（＊ｐ＜０．０３）と約３倍にのびている。この明らかな腫瘍増殖抑制効果の原因が低酸素がん細胞を効率よく腫瘍から除いているためであることが、腫瘍内低酸素がん細胞を光イメージングで可視化し、経時的に観察することにより明らかになった（図４）。今後臨床応用にむけて検討を行っていく予定である。

## 5. イメージングへの応用

タンパク質を用いる場合は光イメージングが最も容易で種類も多いが、光の透過度が小さいことから光イメージングの臨床応用は現状では極めて難しい。しかしながら、基礎的データが容易に集められることから、現在機能ドメインに光イメージングに対応する物を融合させて動物実験を行っている。将来的には、磁性体ナノ粒子の利用を検討しており、ＭＲＩによる低酸素がん細胞の画像化をめざして研究を進めている。

## おわりに

「西暦2015年には、1年間に89万人ががんにかかる（がんセンターＩＩＰ）」と予測されている。1990年代で既に、男性では55%、女性では65％が5年以上生存している。つまり、毎年がん治療を受けて治った約50万人が、その後再発と転移の不安をかかえながら、長い人生を歩む時代が来たといえる。定期的なモニターによるがんの早期発見は、このようなハイリスク群のみならず、通常の検診でがんの早期発見を望んでいる予備群に対しても重要であることは言うまでもない。

現在の画像診断では、基本的に正常組織との違い（異常）を探し出すことで、がんを検出する手法が取られるため、小さな癌を見つけるためには、かなりの知識と経験が求められる。それでは、とても急増する需要に追いつくことができない。そのためにも誰が見ても明らかにがんの存在を示唆できるような鮮明な画像を提供できるプローブの開発が必須である。つまり、「がんが無ければ画面には何も写らず、イメージとして画面に写し出されたら、そこにがんがある」という極めて簡単な画像診断を提供でき、しかもがんの組織特異性に左右されず、どのようながんでも早期に検出が可能であるプローブ、そんな夢のようなプローブを我々は、これまでに医学の領域では使われることのなかった工学系の材料；ナノ複合材料を応用することで実現しようとしている。革新的診断・治療への挑戦である。

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