Peroxidasin Is Secreted and Incorporated into the Extracellular Matrix of Myofibroblasts and Fibrotic Kidney

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Mammalian peroxidases are heme-containing enzymes that serve diverse biological roles, such as host defense and hormone biosynthesis. A mammalian homolog of *Drosophila* peroxidasin belongs to the peroxidase family; however, its function is currently unknown. In this study, we show that peroxidasin is present in the endoplasmic reticulum of human primary pulmonary and dermal fibroblasts, and the expression of this protein is increased during transforming growth factor- β 1-induced myofibroblast differentiation. Myofibroblasts secrete peroxidasin into the extracellular space where it becomes organized into a fibril-like network and colocalizes with fibronectin, thus helping to form the extracellular matrix. We also demonstrate that peroxidasin expression is increased in a murine model of kidney fibrosis and that peroxidasin localizes to the peritubular space in fibrotic kidneys. In addition, we show that this novel pathway of extracellular matrix formation is unlikely mediated by the peroxidase activity of the protein. Our data indicate that peroxidasin secretion represents a previously unknown pathway in extracellular matrix formation with a potentially important role in the physiological and pathological fibrogenic response. (*Am J Pathol* 2009, 175:725–735; DOI: 10.2353/ajpath.2009.080693)

Peroxidases are heme-containing enzymes with highly conserved structure, serving diverse functions in the plant and animal kingdom.¹ Peroxidases catalyze the oxidation of various substrates in the presence of H₂O₂. Mammalian peroxidases have an important role in several physiological processes including host defense and hormone biosynthesis. The family of mammalian peroxidases consists of myeloperoxidase, eosinophil peroxidase, lactoperoxidase, thyroid peroxidase, and the mammalian peroxidasin. Myeloperoxidase, eosinophil peroxidase, and lactoperoxidase have antimicrobial activity and serve in the first line of host defense, while thyroid peroxidase has an essential role in the biosynthesis of thyroid hormones.^{2–4} The function of the mammalian peroxidasin is currently unknown. Peroxidases in plants and in lower animal species frequently participate in extracellular matrix (ECM) formation. In the presence of H₂O₂, peroxidases enzymatically cross-link extracellular proteins through tyrosine residues.⁵ ECM stabilization by dityrosine bridges is well-documented during sea urchin fertilization, where secreted ovoperoxidase is responsible for the formation of cross-links.⁶ Dityrosine formation is also involved in the stabilization of *C. elegans* cuticle, where dual oxidases, carrying both NADPH oxidase and peroxidase-like domains, provide hydrogen peroxide for the crosslinking reaction.⁷

Peroxidasin (PXDN), a unique form of peroxidase was first identified in *Drosophila melanogaster*.⁸ Beside containing a peroxidase domain, which is highly homologous to other animal peroxidases, peroxidasin also contains protein domains characteristic for proteins of the ECM. *Drosophila* PXDN was found to be expressed in several stages of development, but the exact function remained

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Table 1. siRNA Sequences

Name	Sequence
PXDN-1	5'-CCUCCAUCUAGAUCUUCGCUUUA-3'
PXDN-1control	5'-CCUCCCUCAUAGAUGUCCCUUUA-3'
PXDN-2	5'-GCAUAACAACCGGAUUAACAUUUA-3'
PXDN2control	5'-GCAUAAAAACCGGAUUAACAUUUA-3'

Oligonucleotides Used in Quantitative PCR Experiments		
Name	Species	Sequence
gabdh F	<i>H. Sapiens</i>	5'-AAGGTGAAGGTCGGAGTCAACGG-3'
gabdh R	<i>H. Sapiens</i>	5'-CCAAAGTTGTCATGGATGACCTTGG-3'
pxdn F	<i>H. Sapiens</i>	5'-CTCAGCTTCAGCACACGCTC-3'
pxdn R	<i>H. Sapiens</i>	5'-GAGTTCTGGGTGTTTCTCGGT-3'
gabdh F	<i>M. musculus</i>	5'-CTGAGTATGTCGTGGAGTCTACTG-3'
gabdh R	<i>M. musculus</i>	5'-AAGGCCATGCCAGTGAGCTTC-3'
pxdn F	<i>M. musculus</i>	5'-CGAGGCCGGGACCATGGCATC-3'
pxdn R	<i>M. musculus</i>	5'-CTGCAGGCTGGCAAGCTTCCAC-3'

unknown.⁸ Little is still known about the mammalian PXDN protein. A human homolog of *Drosophila* PXDN was originally identified as a p53-responsive gene product from a colon cancer cell line, but it was not characterized in detail.⁹ An independent cloning effort, using subtractive hybridization also led to the identification of the mammalian PXDN gene, which was originally named melanoma gene 50, based on the expression in melanoma samples.¹⁰ This latter study has characterized PXDN as a possible potent melanoma-associated antigen, but it did not examine the possible physiological role of the protein.

Here we demonstrate that peroxidase is expressed by human primary cells, including fibroblasts of different origin, where the protein is localized to the endoplasmic reticulum. On stimulation by transforming growth factor (TGF)- β 1, differentiating myofibroblasts show increased expression of peroxidase. The protein becomes secreted to the extracellular space where it is organized into a fibril-like network. We also show that this pathway of ECM formation is probably not mediated by the peroxidase activity of the protein. Our results suggest that beside the secretion of well-known constituents of the ECM, PXDN secretion by myofibroblasts is a novel way of ECM modification in wound repair and tissue fibrosis.

Materials and Methods

Materials

We used the following antibodies in our studies: Alexa488- and Alexa568-labeled anti-rabbit and anti-mouse Fab (Molecular Probes, Eugene, OR), protein disulfide isomerase (PDI) antibody (RL90), and fibronectin antibody (IST-9) (Abcam, Cambridge, UK), lamin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), β -actin antibody, and smooth muscle actin (SMA) antibody (Sigma Chemical Co., St. Louis, MO).

Anti-PXDN Antibody

PXDN polyclonal antibody was purified from rabbit serum following intracutaneous injections of glutathione

S-transferase-PXDN (amino acids 1329 to 1479). The antibody was affinity purified using Affigel 10 beads (BioRad Laboratories, Richmond, CA) loaded with the antigen.

Cell Culture and Treatments

COS-7 cells were grown in Dulbecco's Modified Eagles Medium with Glutamax I (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum, 50 U/ml penicillin (Sigma), and 50 μ g/ml streptomycin (Sigma). Human pulmonary and dermal fibroblasts (PromoCell, Heidelberg, Germany) were grown in fibroblast basal medium supplemented with 2% fetal calf serum, 5 μ g/ml insulin, and 1 ng/ml basic fibroblast growth factor. Cells were grown in a humidified atmosphere of 5% CO₂ in air at 37°C. Before TGF- β 1 treatment, primary fibroblasts were serum-deprived in the presence of 0.05% serum. Cells were treated with TGF- β 1 (R&D Systems, Minneapolis, MN) for 24 to 72 hours in the absence of serum. In some experiments the medium was supplemented with 500 μ mol/L δ -aminolevulinic acid (Sigma).

Transient Transfections

PXDN encoding pcDNA 3.1 plasmid was transfected by using Eugene6 (Roche Diagnostics GmbH, Mannheim, Germany) or Lipofectamine2000 (Invitrogen). Small interfering (si)RNA was transfected in 100 nmol/L concentration using the Interferin siRNA transfection reagent (Polyplus Transfection, Illkirch, France) or RNAiMAX (Invitrogen).

siRNA Sequences

Sequence-specific and control Stealth siRNAs were obtained from Invitrogen. The sequences of PXDN-specific and control siRNAs are provided in Table 1.

Western Blot Experiments

Cells lysed in Laemmli sample buffer were boiled and run on 7.5% or 10% polyacrylamide gels. After blotting onto nitrocellulose membranes blocking was performed in PBS 5% milk and 0.1% Tween 20 for 1 hour at room temperature. We incubated the membranes with the first antibody for 1 hour at room temperature. Membranes were washed five times in PBS 0.1% Tween 20 and horseradish peroxidase-labeled anti-rabbit secondary antibody (Amersham Pharmaceuticals, Amersham, UK) was used in 1:5000 dilution and signals were detected on FUJI Super RX films using the enhanced chemiluminescence method. To precipitate PXDN from the cell culture medium, the medium was removed, and 1 volume of 100% (w/v) trichloroacetic acid was added to four volumes of medium. The samples were kept on ice for 10 minutes then the precipitate was separated by centrifugation (14,000 rpm, 5 minutes). The pellets were washed three times with 2 ml of cold acetone and dried by placing the tube in 95°C heat block for 5 minutes. The pellets were resuspended in 4× sample buffer then boiled for 10 minutes before they were loaded onto polyacrylamide gels.

Measurement of Peroxidase Activity

COS-7 cells expressing PXDN or primary fibroblasts were lysed in PBS containing 1% hexadecyltrimethylammonium bromide. Peroxidase activity of the lysates was immediately determined by the Amplex Red peroxidase assay (Molecular Probes). After 30 minutes incubation time with the Amplex Red reagent, resorufin fluorescence was measured at 590 nm.

Measurement of H_2O_2 Production

TGF- β 1-induced H_2O_2 production of pulmonary fibroblasts was measured with the Amplex Red assay (Molecular Probes). Attached cells were incubated in the presence of 50 μ Mol/L Amplex Red and 0.1 U/ml horseradish peroxidase in an extracellular medium containing 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.8 mmol/L $CaCl_2$, 5 mmol/L glucose, and 10 mmol/L HEPES. After 1 hour incubation at 37°C, resorufin fluorescence was measured at 590 nm.

Immunofluorescent Labeling and Confocal Laser Microscopy

Cells grown on coverslips were fixed in 4% paraformaldehyde in PBS then rinsed 5 times in PBS and incubated for 10 minutes in PBS containing 100 mmol/L glycine. Coverslips were washed twice in PBS and permeabilized in PBS containing 1% bovine serum albumin and 0.1% Triton X-100 for 20 minutes at room temperature. After 1 hour blocking in PBS containing 3% bovine serum albumin cells were incubated with the primary antibody in PBS plus 3% bovine serum albumin, washed thoroughly six times in PBS, and incubated with the secondary an-

tibody for 1 hour and finally washed six times in PBS again. Coverslips were mounted using Mowiol 4-88 anti-fade reagent (prepared from polyvinyl alcohol 4-88, glycerol, H_2O , and Tris pH 8.5).

Confocal images were collected on an LSM510 laser scanning confocal unit (Carl Zeiss) with a 63×1.4 numerical aperture plan Apochromat and a 40×1.3 numerical aperture plan Neofluar objective (Carl Zeiss). Excitation was with 25-mW argon laser emitting 488 nm, and a 1.0-mW helium/neon laser emitting at 543 nm. Emissions were collected using a 500- to 530-nm band pass filter to collect A488 and a 560-nm long pass filter to collect A568 emission. Usually images from optical slices of 1- to 2- μ m thickness were acquired. Cross talk of the fluorophores was negligible.

Figure 1. Characterization of PXDN expression and activity. Detection of PXDN (A) mRNA expression by Northern blot analysis. Multiple-tissue (2 μ g of poly[A]⁺ RNA) Northern blot membranes were probed at 65°C with a randomly radiolabeled cDNA fragments corresponding to the 3'-untranslated regions of PXDN. B: Detection of PXDN by Western blot analysis. A polyclonal antibody raised against PXDN recognizes the protein in PXDN-expressing COS-7 cells (+, second lane), whereas it does not produce immunoreactive band in mock-transfected COS-7 cells (-, first lane). Loading controls developed for β -actin indicate that all lanes contained similar amounts of total protein. C: Detection of peroxidase activity in the lysates of PXDN-expressing COS-7 cells. COS-7 cells were transfected with PXDN cDNA; control cells were mock-transfected. After 48 hours cells were lysed in 1% hexadecyltrimethylammonium bromide and were assayed for peroxidase activity by the Amplex Red peroxidase assay. The fluorescent product, resorufin fluorescence was measured at 590 nm. D-F: Intracellular localization of PXDN in transfected COS-7 cells. PXDN-transfected, paraformaldehyde-fixed, permeabilized COS-7 cells were stained for PXDN (D) and the endoplasmic reticulum marker PDI (E). Merge of the fluorescent signals is shown in (F). Scale bar = 20 μ m.

Gene Expression Studies

For the human PXDN mRNA detection, human multiple-tissue (2 μ g of poly[A]⁺ RNA) Northern blot membranes (Clontech) were probed at 65°C with a randomly radio-labeled cDNA fragments corresponding the 3'-untranslated regions of PXDN mRNA following standard hybridization methods. For the quantitative PCR experiments RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 μ g total RNA using oligo(dt)₁₈ primers and RevertAid M-MuLV Reverse Transcriptase in 20 μ l reaction mix according to the manufacturer's (Fermentas) recommendations. One μ l of the first strand was amplified in 10 μ l total volume in a LightCycler 1.5 instrument (Roche) using LightCycler FastStart DNA Master SYBR Green I mix (Roche) with a final Mg²⁺ concentration of 2.25 mmol/L, and final primer concentration of 0.5 μ mol/L. To avoid amplification of the genomic region, primers were designed in separate exons neighboring long introns; the sequences of the primers are provided in Table 1. The following PCR protocol was used: 95°C for 10 minutes, then 40 cycles of 95°C 10 seconds, 60°C 5 seconds, 72°C 15 to 25 seconds (amplicon size [bp]/25), the last step was a melting curve analysis (from 65 to 95°C with a slope of 0.1°C/sec). The quantification was performed by the LightCycler Software 4.05 as follows. The crossing point was determined by the second derivative method. PCR efficiency and standard curve was calculated for each gene by performing amplification on serial dilutions of a mixture of the samples. In each sample the expression of the target gene was divided with the expression of the endogenous control, which was the housekeeping gene *GAPDH*. The relative expression lev-

els were finally normalized to the average expression level of the control samples (set to 1).

Animal Model

Animal experiments were authorized by the Institutional Animal Experiment Committee under permission No. 86/2006 SE TUKEB. Eight-week-old male BALB/c mice were obtained from the National Institute of Oncology. Animals were maintained on standard diet and given water *ad libitum*. Unilateral ureteral obstruction was performed using a standard procedure¹¹ Briefly, under ketamine (50 mg/kg) and xylazine (10 mg/kg) induced general anesthesia complete ureteral obstruction was performed by ligating the left ureter with 8-0 silk after a midline abdominal incision. Mice were sacrificed after 7 days of the procedure and the kidneys (both obstructed and control) were removed. One part of the kidneys was fixed in 4% phosphate-buffered paraformaldehyde followed by paraffin embedding for histological analysis. Goldner's trichome staining was used for the detection of fibrosis. Histological analysis was performed by Histopathology Ltd, Pécs, Hungary. Samples were coded and examined in a blinded fashion. The remaining kidneys were processed for RNA isolation and immunohistochemistry. We used acetone fixed, 8- μ m thick frozen sections for the immunolocalization of PXDN in control and fibrotic kidneys. After blocking in 1% albumin and 2% normal goat serum we incubated the sections overnight with anti-PXDN and anti-fibronectin antibodies. Sections were then stained with fluorophore-labeled secondary antibodies for 1 hour at room temperature.

Figure 2. Detection of PXDN protein expression in human pulmonary fibroblasts (HPF) and human dermal fibroblasts (HDF). **A:** Detection of PXDN by Western blot analysis in HPFs (first lane) and HDFs (second lane). A polyclonal, PXDN-specific antibody detects PXDN in both cell types. **B–G:** Intracellular localization of PXDN in human primary fibroblasts. Paraformaldehyde-fixed, permeabilized HPFs (**B–D**) or HDFs (**E–G**) were stained for PXDN (**B, E**) or for the endoplasmic reticulum marker PDI (**C, F**). Merge of red and green fluorescences is shown in (**D**) and (**G**). Note the significant overlap of the two fluorescent signals. Scale bar = 20 μ m.

Results

Characterization of the Expression Pattern of PXDN

Conflicting data have been published regarding the tissue expression pattern of PXDN. While Horikoshi et al suggested that PXDN is ubiquitously expressed in a variety of tissues,⁹ Mitchell et al proposed that it is primarily

a melanoma-specific protein.¹⁰ To clarify these conflicting data we have used the Northern blot technique to study the expression of PXDN mRNA. To exclude the potential cross-hybridization to mRNAs encoding other peroxidases, we have used the 3'-untranslated region of the PXDN cDNA as a probe. As it is shown in Figure 1A, PXDN mRNA was expressed in several human tissues including heart, skeletal muscle, colon, spleen, kidney, liver, small intestine, and placenta, while it was absent in brain, thymus, and leukocytes. A similar picture of expression was also suggested by the analysis of PXDN EST sequences deposited in GenBank.

Intracellular Localization and Enzymatic Activity of PXDN

To study the peroxidase activity of PXDN we have expressed the protein in COS-7 cells. Using polyclonal antibodies raised against the protein we could detect PXDN in transfected cells, while mock-transfected cells showed no detectable expression (Figure 1B). We used the Amplex Red assay to study the peroxidase activity of transfected cell lysates. Figure 1C shows that we could detect peroxidase activity only in PXDN-expressing cells, while lysates of mock-transfected cells showed no detectable enzymatic activity. Next we studied the intracellular localization of PXDN in transfected COS-7 cells. Figure 1, D–F show that PXDN colocalized with PDI, a well characterized marker protein of the endoplasmic reticulum.¹²

Next we sought to examine if we can detect endogenously expressed PXDN in human primary cells. Among the cells examined we could detect PXDN protein expression in human pulmonary fibroblasts (HPFs) and human dermal fibroblasts (HDFs) (Figure 2A) and vascular endothelial and smooth muscle cells (data not shown). We used confocal microscopy to study the intracellular distribution of endogenously expressed PXDN protein. In

Figure 3. TGF- β 1 increases PXDN expression in human pulmonary fibroblasts (HPF). **A:** Induction of PXDN mRNA expression by TGF- β 1 treatment. HPFs were serum-depleted in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for 24 hours. RNA was isolated from the cells and cDNA was synthesized (see *Materials and Methods*). Quantitative PCR experiments were performed with the SybrGreen method. Relative expression levels of PXDN are shown using GAPDH as internal control. The PXDN expression level in uninduced cells is defined as 1. Values are the mean $\pm$ SEM. **B:** Induction of PXDN protein expression by TGF- β 1 in HPFs. HPFs were serum-depleted in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 (+) for 24, 48, and 72 hours. In the medium of the control cells TGF- β 1 was omitted (-). Western blot analysis was used for PXDN detection (upper panel). Detection of Lamin A and C in loading controls indicate that each lane contained similar amount of protein. **C:** Detection of TGF- β 1-induced PXDN expression by immunofluorescence. HPFs were serum-depleted in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for 24 hours (**D**) or left untreated (**C**). Paraformaldehyde-fixed, permeabilized cells were stained for PXDN (red color in **C** and **D**) and for the myofibroblast marker SMA (insets in **C** and **D**). The appearance of SMA expression indicates myofibroblastic differentiation. Scale bars = 20 μ m. **E:** Detection of PXDN in cell culture medium. After 72 hours incubation time TCA was used to precipitate proteins from the medium of control (untreated) and TGF- β 1-treated HPFs. PXDN expression of the cells was analyzed in parallel experiments. Loading controls developed for β -actin indicate that cell lysates contained similar amounts of total protein, while the absence of β -actin in the precipitated samples suggests that PXDN in the medium does not originate from unattached cells.

Figure 4. Formation of PXDN-containing fibrils by TGF- β 1-treated HPFs and HDFs. **A–D:** HPFs (**A,B**) and HDFs (**C,D**) were serum-deprived in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for 72 hours in the presence of 500 μ mol/L ALA. TGF- β 1 was omitted from the medium of control cells (**A** and **C**). Paraformaldehyde-fixed, permeabilized cells were stained for PXDN. Note the intense staining of fibril-like structures (indicated by **arrows**) around the cells (**B**, **D**). **E:** Effect of siRNA treatment on PXDN expression detected by Western blot. HPFs were transfected with PXDN sequence-specific siRNAs or “minimally” changed control siRNAs or left untransfected. Myofibroblast differentiation was induced by 5 ng/ml TGF- β 1 for 72 hours in the presence of 500 μ mol/L ALA. Detection β -actin in loading controls indicate that each lane contained similar amount of protein. **F–I:** Effect of siRNA treatment on the development of PXDN-containing fibrils. HPFs (**F,G**) and HDFs (**H,I**) were transfected with PXDN sequence-specific siRNAs (**F,H**) or “minimally” changed control siRNA (**G,I**). siRNAs were transfected to the cells during serum deprivation for 24 hours and myofibroblastic differentiation was subsequently induced by 5 ng/ml TGF- β 1 in the presence of 500 μ mol/L ALA. Paraformaldehyde-fixed, permeabilized cells were stained for PXDN and for the myofibroblast marker SMA. **Insets (F–I)** show SMA staining from the same microscopic field. Scale bars = 20 μ m.

HPFs and HDFs we observed a reticular staining pattern (Figure 2, B and E). The specificity of staining was confirmed by two different PXDN-specific siRNAs (data not shown). The observed intracellular localization suggested that PXDN localized to the endoplasmic reticulum therefore we investigated the localization of PXDN in relation to this organelle. We examined the localizations of PXDN and PDI. When the cells were stained for PDI and PXDN, we observed significant overlap of the two signals suggesting that PXDN indeed localized to the endoplasmic reticulum (Figure 2, C–G). Besides its localization to the endoplasmic reticulum we have also observed perinuclear localization of PXDN in both HPF and HDF cells (Figure 2, D and G).

Induction of PXDN Expression by TGF- β 1 in Pulmonary and Dermal Fibroblasts

PXDN contains several domains, including leucine-rich repeats, immunoglobulin C2-type domains, which are usually found in proteins of the ECM.^{13,14} In accordance with its structural features the *Drosophila* homolog was described to be secreted by Kc cells.⁸ The protein localization software TargetP 1.1 predicts that human PXDN, which contains a signal peptide, goes through the secretory pathway as well. We therefore sought to find conditions when increased protein secretion occurs. When primary fibroblasts are stimulated by TGF- β 1 they go through a drastic phenotypic change and differentiate into myofibroblasts. Myofibroblasts possess contractile features and show intense synthesis of ECM proteins including fibronectin and different types of collagen.^{15,16} We studied the effect of TGF- β 1 on PXDN expression in HPFs. As shown in Figure 3A, we have observed a two-fold increase in PXDN mRNA expression after 10 hours

treatment with TGF- β 1. Figure 3B shows that after 24 hours of TGF treatment we detected an increased level of PXDN protein by Western blot and elevated PXDN level was still observed after 72 hours. We also confirmed this result by immunostaining experiments (Figure 3, C and D) where an increase in PXDN protein level was also evident after 24 hours and remained elevated for 72 hours (data not shown). Induction of the myofibroblast phenotype was confirmed by immunostaining of SMA, which was absent in fibroblasts (see inset in Figure 3C), but appeared during the course of TGF- β 1 treatment (inset in Figure 3D). We were interested if PXDN was secreted to the extracellular space, therefore we analyzed the cell culture medium for its PXDN content. Figure 3E shows that TGF- β 1 stimulated the secretion of PXDN to the cell culture medium. PXDN in the medium did not originate from unattached cells since we could not detect β -actin in the protein precipitate with a highly sensitive antibody.

Localization of PXDN to Fibril-Like Structures in the Extracellular Space

During myofibroblast differentiation cells undergo a drastic phenotypic change that is accompanied by the increased expression of heme-containing proteins such as the NADPH oxidase Nox4¹⁷ and PXDN. Since we applied TGF- β 1 to serum-deprived HPFs we have hypothesized that the cells may lack enough heme to complete the synthesis of PXDN. We therefore supplemented the medium with δ -aminolevulinic acid (ALA), a precursor of heme biosynthesis.¹⁸ Supplementation of the cell culture medium with ALA during the differentiation process did not increase the PXDN content of the medium (data not shown), however we observed a drastic change in PXDN

localization (Figure 4). After 72 hours treatment, PXDN appeared in the form of a dense network extracellular deposition of PXDN (Figure 4, A and B). The appearance of these structures was also observed in myofibroblasts developed from HDFs, suggesting that the characteristic localization of PXDN was not specific for pulmonary cells (Figure 4, C and D). PXDN-specific siRNAs effectively inhibited PXDN expression (Figure 4E) and the development of PXDN-containing fibrils (Figure 4, F–I). Importantly, siRNA treatment did not affect the myofibroblastic differentiation, as judged by the expression SMA in the siRNA-treated cells (insets, Figure 4, F–I). In our next experiments we have performed a detailed characterization of the PXDN-containing structures. Figure 5, A and B shows that PXDN-containing fibrils are frequently formed between neighboring cells and anchor to the proximity of nucleus, however fibrils bridging larger distances were also observed. To confirm the extracellular localization of PXDN we compared the staining pattern of PXDN between nonpermeabilized and permeabilized cells. As shown in Figure 5C PXDN-containing fibrils were detected when cells were not permeabilized. Importantly

the intracellular localization of PXDN was undetectable and the cells did not stain for SMA, confirming the integrity of the plasma membrane (Figure 5D). Parallel experiment on permeabilized cells proved that SMA indeed appeared in the cells during differentiation (Figure 5, E and F). Next we sought to determine the relation of PXDN-fibrils to fibronectin. Figure 6 shows that PXDN fibrils partially colocalize with fibronectin, in cultures of TGF- β 1-treated HPFs (Figure 6, A–C) and HDFs (Figure 6, D–F). Colocalization was frequently observed in thick cable-like structures, while the two proteins did not colocalize in thinner fibrils.

Role of H₂O₂ and Peroxidase Activity in the Secretion and Localization of PXDN

We analyzed the peroxidase activity of fibroblast and differentiated myofibroblast cultures but despite the obvious difference in PXDN expression we could not detect the peroxidase activity of PXDN (data not shown). Since PXDN acts as a functional peroxidase when expressed in COS7 cells (Figure 1C) and TGF- β 1-stimulated fibroblasts were described to produce reactive oxygen species (ROS)¹⁹ we wanted to investigate if we can detect the H₂O₂-consuming activity of PXDN. Confirming the results of earlier reports, we measured increased H₂O₂ production by TGF- β 1-stimulated fibroblasts (Figure 7A). We have hypothesized that if PXDN used the produced H₂O₂, then decreased PXDN expression should result increased release of H₂O₂. As shown in Figure 7A, inhibition of PXDN expression by PXDN-specific siRNA had no effect on the TGF- β 1-induced H₂O₂ production. Next we measured stimulated H₂O₂ production at different time points after the TGF- β 1 stimulus (Figure 7B). H₂O₂ production has peaked at 24 hours and no stimulated ROS-release was observed after 72 hours, when PXDN-containing fibrils appeared in the cell culture.

PXDN Expression Is Increased in a Murine Model of Kidney Fibrosis

We have also studied the possible changes of PXDN expression in a well-characterized mouse model of kidney fibrosis, induced by unilateral ligation of the ureter. We chose this model because the rapid development of kidney fibrosis in this model is dependent on TGF- β 1 signaling²⁰ and it has been previously shown that myofibroblasts appear during the process and secrete large amount of ECM proteins.²⁰ Figure 8A shows, that after 7 days of ligation we have observed a more than threefold increase in PXDN expression. Intense blue staining of kidney tissue with Goldner's trichrome method (Figure 8, B and C) indicated that fibrotic remodeling of the kidney paralleled the increased expression of PXDN. We could also demonstrate the increase of PXDN expression in fibrotic kidneys (Figure 8, D–I). While PXDN was barely detectable in normal kidneys (Figure 8G), the protein becomes enriched in the peritubular space of fibrotic

Figure 5. PXDN-containing fibrils are frequently formed between neighboring cells and localize to the extracellular space. **A–B:** HDFs (**A**) and HPFs (**B**) were serum-deprived in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for 72 hours in the presence of 500 μ mol/L ALA. Paraformaldehyde-fixed, permeabilized cells were stained for PXDN (**A**) or PXDN (red) and Lamin (green) (**B**). **White arrows** indicate that PXDN-fibrils frequently dock to the proximity of nuclei. **C–F:** HDFs were serum-deprived in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for 72 hours in the presence of 500 μ mol/L ALA. Paraformaldehyde-fixed, non-permeabilized (**C,D**) or permeabilized (**E,F**) cells were stained for PXDN and SMA. Scale bar = 20 μ m.

Figure 6. PXDN partially colocalizes with fibronectin in PXDN-containing fibrils. HPFs (**A–C**) and HDFs (**D–F**) were serum-deprived in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for 72 hours in the presence of 500 μ mol/L ALA. Paraformaldehyde-fixed, permeabilized cells were stained for PXDN (**A,D**) and fibronectin (**B,E**). Note the intense staining of fibril-like structures around the cells, whereas the intracellular staining is essentially absent. Merge of red and green fluorescences are shown in (**C**) and (**F**). Note the colocalization of PXDN and fibronectin in large, cable-like structures. Scale bar = 20 μ m.

kidneys where it colocalized with fibronectin (Figure 8, D–I). This observation suggests that the stimulatory effect of TGF- β 1 on PXDN expression and secretion was not restricted to *in vitro* conditions.

Figure 7. Role of hydrogen peroxide and peroxidase activity in PXDN function. **A:** Effect of PXDN-specific siRNA treatment on TGF- β 1-induced H₂O₂ production. HPFs were transfected with PXDN sequence-specific siRNAs or “minimally” changed control siRNAs. siRNAs were transfected to the cells during serum deprivation for 24 hours and cells were treated with 5 ng/ml TGF- β 1 in the presence of 500 μ mol/L ALA for 24 hours. H₂O₂ production was measured by the Amplex Red assay. H₂O₂ production of uninduced cells is defined as 1. **B:** Kinetics of TGF- β 1 induced H₂O₂ production. HPFs were serum-deprived in the presence of 0.05% serum for 24 hours and were subsequently treated with 5 ng/ml TGF- β 1 for the indicated periods of time. H₂O₂ production of uninduced cells is defined as 1.

Discussion

Tissue fibrosis represents a leading cause of morbidity and mortality worldwide. Fibrosis can arise in nearly every organ and it is especially important in the development of kidney diseases, liver cirrhosis, heart disease, and interstitial lung disease.^{21–23} Emerging evidence suggests that regardless of the location, myofibroblasts seem to have a central role in the development of fibrosis. Myofibroblasts also have an important physiological role in wound healing and development. These fascinating cells can develop from fibroblasts and also from epithelial cells during the course of epithelial–mesenchymal transition. During their differentiation, myofibroblasts develop a contractile phenotype due to increased expression of SMA and they also show increased synthesis of ECM proteins, including fibronectin and different types of collagen.^{15,16} In this work we have found that myofibroblasts also synthesize and secrete PXDN, a mammalian peroxidase with previously unknown function. Our results suggest that PXDN deposited in the extracellular space provides a previously unprecedented pathway of ECM formation.

PXDN is a unique member of the peroxidase family because its catalytic domain is surrounded by domains characteristic for proteins of the ECM. These parts include leucine-rich repeats and C2-type immunoglobulin domains, which localize to the N-terminus of the protein, while a vWF C-type domain is localized to the C-terminal part of the protein. PXDN was first described in *Drosophila melanogaster*.⁷ Kc cells of *Drosophila* origin secrete peroxidase to the extracellular medium, where the protein exists in trimers. Based on the expression pattern of its mRNA, *Drosophila* PXDN is present in hemocytes and thought to be involved in ECM synthesis during fly development. There is scant amount of information about the human PXDN. Horikoshi et al isolated cDNAs from cells where apoptosis was induced in a p53-dependent way.⁹ Among the induced cDNAs, they found an alternatively

Figure 8. PXDN expression is increased in a TGF- β 1-dependent model of kidney fibrosis. **A:** Induction of PXDN mRNA expression by unilateral ureteral obstruction. Unilateral ureteral obstruction was applied for 7 days after that kidneys were removed and their PXDN expression was measured by quantitative PCR analysis performed with the SybrGreen method. Relative expression levels of PXDN are shown using GAPDH as internal control. The PXDN expression level in control, non-obstructed kidneys is defined as 1. Values are the mean $\pm$ SEM. **B, C:** Detection of unilateral ureteral obstruction-induced kidney fibrosis by Goldner trichrome staining. After 7 days of unilateral ureteral obstruction, kidneys were removed and fixed in 4% phosphate-buffered paraformaldehyde. After paraffin embedding, sections were made and stained by Goldner's trichrome method. Blue-staining, marked by **black arrows** indicates the development of fibrosis in the peritubular space of obstructed kidneys (**B**). Untreated kidneys from the same animals served as controls (**C**). **D–I:** Detection of PXDN and fibronectin in control and obstructed kidneys. Acetone fixed, frozen sections were stained for PXDN (**D,G**) and fibronectin (**E,H**). Merge of red and green fluorescences are shown in (**F**) and (**I**). Note the peritubular co-localization of PXDN and fibronectin. Scale bar = 20 μ m.

spliced product of the human PXDN gene. The authors have hypothesized that the induction of PXDN might be coupled to the altered ROS metabolism in apoptosis, but they did not elaborate on this idea. According to their study PXDN is expressed in almost all human tissues. In an independent study PXDN was identified as a novel melanoma gene (MG50) based on its expression in melanoma samples and relative absence in normal tissue samples.¹⁰ Our results are in agreement with the report of Horikoshi et al since we have detected PXDN mRNA expression in several tissues, with particularly high expression level in heart, skeletal muscle, small intestine and placenta (Figure 1).⁹ PXDN functions as a peroxidase when expressed in COS-7 cells (Figure 1). To further characterize the peroxidase activity of a purified protein we have also expressed an epitope-tagged form of PXDN in Sf9 insect cells and attempted to purify it. Unfortunately, the protein remained in the insoluble fraction during purification and we have been unable to recover functional protein from that fraction (data not shown).

In our experiments TGF- β 1 increased the expression of PXDN and its secretion to the cell culture medium (Figure 3). Myofibroblastic differentiation is usually induced in serum-deprived cells, since responsiveness to TGF- β 1 is increased under those conditions. We have hypothesized that in the absence of serum, the cellular production of heme might not be sufficient for the synthesis of heme-containing peroxidases. This assumption was also originated from the observation that TGF- β 1

increases the expression of another heme-containing protein, the NADPH oxidase Nox4.¹⁷ In our experiments we observed a more than 100-fold increase in Nox4 expression at mRNA level (data not shown) proving a markedly increased demand for heme, during the myofibroblastic differentiation. When we have supplemented the medium with ALA, a precursor of heme biosynthesis, we could observe the localization of PXDN into fibril-like structures (Figure 4). Although heterologously expressed PXDN exhibited peroxidase activity we could not detect the enzymatic activity of PXDN in fibroblasts and differentiated myofibroblasts. The lack of measurable peroxidase activity might be explained by the fact that PXDN has very low enzymatic activity when compared with other peroxidases. For example COS7 cells, that heterologously express lactoperoxidase, show 100-fold higher peroxidase activity than PXDN-expressing COS7 cells (data not shown). Nelson et al has also shown, that compared with other peroxidases, *Drosophila* PXDN had lower affinity for classical peroxidase substrates.⁷ Alternatively, it is also possible that endogenously expressed PXDN is in complex with another protein that inhibits the enzymatic activity of PXDN. Incorporation of PXDN into larger complexes was also suggested by Nelson et al who could not recover active PXDN from *Drosophila* tissues showing high level expression of the protein.⁷ Nevertheless we cannot exclude the possibility that PXDN still functions as a peroxidase in the endoplasmic reticulum or in the extracellular matrix of myofibroblasts. Since we

could not measure the enzymatic activity of PXDN in myofibroblast cultures we cannot state that ALA indeed increased the maturation of PXDN. However without ALA supplementation TGF- β 1 did not induce the appearance of PXDN in extracellular, fibril-like structures. Since inclusion of ALA during myofibroblast differentiation did not change the amount of precipitable PXDN in the medium, it is possible that formation of PXDN-containing fibrils is a result of a different secretory pathway that is uncovered by assisted heme synthesis. Importantly *in vivo*, during kidney fibrosis PXDN appears in the peritubular space providing further evidence for its secretion (Figure 8). The presence of PXDN-containing fibrils in the extracellular space, suggested the multimerization of the secreted protein and its association to other constituents of the ECM. In fact we could detect partial colocalization of PXDN and fibronectin around the cells (Figure 6). Colocalization of PXDN and fibronectin was also observed in fibrotic kidneys. The domain organization of PXDN suggests that the protein is ideally suited for the stabilization of the ECM through protein-protein interactions. *Drosophila* PXDN was described to exist in a trimeric form, a complex that is thought to be organized through its C-terminal vWF C-type domain.⁷ The same domain is also recognized in mammalian PXDN. This domain is also found in other ECM proteins including the thrombospondin type I and II group of vertebrate matrix proteins, which also exist in the form of trimers.²⁴ The presence of leucine-rich repeats and immunoglobulin loops in PXDN also suggests that this protein readily associates with other ECM proteins. Leucine rich repeats are short sequence motifs present in a number of proteins with diverse functions.¹³ These protein modules are usually involved in homophilic and heterophilic protein-protein interactions. Immunoglobulin loop motifs are also frequently found in neural cell adhesion molecules such as fascilin and neuroglian.¹⁴ The combination leucine-rich repeats and immunoglobulin C2 type domains is not frequently observed in proteins. In fact the only other known mammalian example for such combination is the LRIG (leucine-rich repeats and immunoglobulin-like domains) family of proteins, members of which are transmembrane proteins and were previously shown to negatively regulate the ErbB family of receptor tyrosine kinases.^{25,26}

Peroxidases in lower species are involved in stabilization of the ECM through tyrosine-tyrosine crosslinks.⁵ Oxidative formation of dityrosines contributes to the cuticle synthesis in *C. elegans* and hardening of the fertilization envelope in sea urchin eggs.^{7,27} The ability to form dityrosine crosslinks is a general feature of mammalian peroxidases as well, but the physiological significance of such activity remains to be established. It was reasonable to assume that crosslinking peroxidase activity was involved in the formation of PXDN-containing fibrils, since Thannickal et al described increased H₂O₂ production by TGF- β 1-stimulated lung fibroblasts.^{19,28} In subsequent studies they have also found that H₂O₂ produced by fibroblasts indeed supports dityrosine formation by an exogenous peroxidase.²⁸ In our experiments however, it seems unlikely, since we could not detect the peroxidase

activity of PXDN in myofibroblasts and no increase in dityrosine level was observed during the course of myofibroblast differentiation (data not shown). On the other hand, since the peroxidase domain of PXDN is intact we were interested if reactive oxygen species, more specifically H₂O₂ has any role in the formation of PXDN-containing extracellular structures. Our results, however, suggest that TGF- β 1-stimulated H₂O₂ production unlikely has a role in the formation of PXDN-containing fibrils. First, suppression of PXDN expression did not affect ROS release from the cells, suggesting that H₂O₂ was not used in peroxidase-catalyzed reaction. Second, the kinetics of TGF- β 1-stimulated H₂O₂ production did not parallel the formation of PXDN-containing fibrils, since maximal H₂O₂ production was observed at 24 hours after TGF- β 1-stimulation, when fibroblasts have not differentiated yet into myofibroblasts. The kinetics of TGF- β 1-stimulated ROS production rather suggests that H₂O₂ has a role in the differentiation process itself. Experiments by Cucoranu et al suggest that induction of the ROS-producing enzyme Nox4 would be responsible for oxidative changes during myofibroblastic differentiation.¹⁷

At least three members of the mammalian peroxidase family have host defense function, therefore it will be interesting to explore if secreted PXDN has any antimicrobial activity. This possibility would certainly be important during wound healing when myofibroblasts are normally activated. Activated neutrophil granulocytes have been recently described to form fibril-like structures, neutrophil extracellular traps, which contained elastase, DNA, myeloperoxidase, and showed antimicrobial activity.²⁹

In summary, we conclude that the synthesis and secretion of PXDN by myofibroblasts is a previously unrecognized pathway in the formation of the ECM. Future studies should identify the function of PXDN in myofibroblasts and explore the importance of this pathway in wound healing and in the development of diseases where fibrogenic response seems to be an important part of the underlying pathology.

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