Peroxidasin-like protein: a novel peroxidase homologue in the human heart

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1. Introduction

Building and maintenance of the extracellular matrix (ECM) involves diverse synthetic and degradation pathways that are still incompletely understood. Excessive matrix deposition and abnormal cross-linking of components such as collagen leads to pathological fibrosis which acts as a substrate for arrhythmia and contributes to contractile dysfunction. On the other hand, abnormal degradation, involving enzymes such as matrix metalloproteinases, contributes to the chamber dilatation characteristic of chronic heart failure. In lower species including Caenorhabditis elegans and sea urchin, peroxidases use H2O2 to covalently cross-link proteins in the extracellular space, thus stabilizing the cuticle in the worm or the fertilization envelope of the sea urchin eggs. A potential link between peroxidase function and ECM modification is represented in the structure of peroxidasin (PXDN), a less-known member of the animal peroxidase family. While PXDN contains a peroxidase domain with enzymatic activity, the presence of other protein modules that are characteristic of ECM proteins makes this protein unique among animal peroxidases. PXDN was first described in Drosophila melanogaster as a constituent of the ECM. The C. elegans PXDN has important roles in the formation and maintenance of basal membranes. We have recently shown that the secretion of mammalian PXDN represents a previously unknown mechanism of extracellular matrix formation by myofibroblasts, that is up-regulated during tissue fibrosis. Very recently, PXDN was identified to be the peroxidase that catalyses the formation of a sulfilimine bond between collagen IV protomers during collagen biosynthesis through the synthesis of hypohalous acids.

In this paper, we describe and characterize peroxidasin-like protein (PXDNL), a protein that is highly homologous to PXDN and is specifically expressed in the heart. Our results suggest that the expression of PXDNL in the heart may represent a unique mechanism of ECM formation with the potential of antagonizing PXDN activity in the heart.

2. Methods

2.1 Materials

AlexaFluor488- and AlexaFluor568-labelled goat anti-rabbit and anti-mouse Fab- and rhodamine-falloidin were obtained from Life Technologies. β-Actin...
2.2 PXDN and PXDNL antibodies

We carried out intracutaneous injections of glutathione S-transferase (GST)-PXDN (amino acids 1329–1479) and GST-PXDNL (amino acids 1312–1463) to raise polyclonal antibodies against PXDN and PXDNL in rabbits. The animals were sacrificed, and antibodies were affinity purified from the sera using Affigel 10 or 15 beads (BioRad Laboratories) loaded with the antigens according to the manufacturer’s instructions. A polyclonal anti-PXDNL antibody, which was raised against a region within the peroxidase-like domain (amino acids 1078–1209), was purchased from Sigma.

2.3 Cloning of PXDNL

We have identified a human DNA sequence in GenBank (GenBank accession no. NM_144651), which encoded a partial protein sequence, showing substantial homology to both human and Drosophila PXDNs. We used BLAST search to identify a matching IMAGE clone (Genbank accession no. AA927870), which was subsequently obtained (Invitrogen) and sequenced. A fragment of this clone was used for the analysis of mRNA expression in several human tissues. The expression pattern of this previously unknown gene prompted us to name it cardiac peroxidase. While this study was performed, Genbank has assigned the name Peroxidasin-like (PXDNL) to this gene what we have adopted and use in this paper. The complete cDNA sequence of PXDNL was obtained by 5’ and 3’ RACE, using human heart poly(A) RNA. The cDNA sequence of PXDNL was submitted to Genbank (accession number AY877349). Cheng et al.2 also found the sequence in the Genbank and renamed it cardiovascular peroxidase 2 (VPO2), but did not further characterize it. A short isoform of PXDNL was described as human PMR1 endonuclease by Gu et al.,10 but we did not detect the corresponding endogenously expressed transcript and protein. To search for mouse and rat homologues of PXDNL, we performed BLAST searches in mouse and rat genomic databases at NCBI.

2.4 In situ hybridization

Fresh frozen tissue blocks were cut into 12 μm thick serial sections in a cryostat (Leica Microsystems). The sections were thaw-mounted and air-dried at 37°C onto positively charged SuperFrost Plus slides (Thermo Scientific), and kept at ~ 80°C until use. Hybridizations were performed overnight in humid chambers at 55°C with 106 cpm/slide of the [35S]UTP-labelled probes, as earlier described.11 The slides were dipped into Kodak NTB nuclear track emulsion (Carestream Health, Inc.) and stored at 4°C for 3 weeks. The emulsion-coated slides were developed using Kodak Dektol developer (Carestream Health, Inc.) and stored at 4°C until analyses.

2.5 Cell culture and treatments

COS-7 (ATCC) and FreeStyle 293 (Life Technologies) cells were grown in DMEM (Lonza) supplemented with 10% FCS, 50 μM penicillin and 50 μg/mL streptomycin (Sigma-Aldrich). iCell cardiomyocytes, differentiated from induced pluripotent stem (iPS) cells, were purchased from Cellular Dynamics and were grown according to the manufacturer’s instructions. Within 48 h after plating spontaneously beating cardiomyocytes were observed. PXDN or PXDNL encoding plasmids were transfected by using Fugene HD (Roche, Promega) or Lipofectamine LTX (Life Technologies). Stably transfected, PXDNL- or PXDNL-expressing FreeStyle 293 cell lines were selected in the presence of 1 mg/mL G418 (Life Technologies).

2.6 Peroxidase assay

COS-7 cells transfected with PXDN and/or PXDNL were lysed in PBS containing 1% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The Amplex Red peroxidase assay (Molecular Probes) was applied to measure the peroxidase activity of cell lysates. The lysates were incubated in the presence of 1 mM H2O2 and 50 μM Amplex Red for 30 min, and resorufin fluorescence was measured at 590 nm.

2.7 Immunofluorescent labelling and confocal laser microscopy

Cells were grown on coverslips were fixed in 4% paraformaldehyde PBS, and then washed two times in PBS, coverslips were permeabilized in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 20 min (in case of experiments on non-permeabilized cells, we excluded this step from the protocol) then blocked for 1 h in 3% BSA PBS. Subsequently, cells were incubated with the primary antibody in 3% BSA PBS for 1 h, washed in PBS and incubated with the secondary antibody for 1 h, and finally washed in PBS again. For mounting, we used Mowiol 4–88 antifade reagent [prepared from polyvinyl alcohol 4–88, glycerol, H2O, and Tris (pH 8.5)].

Immunostainings were analysed using an LSM 510 confocal laser-scanning microscope (Carl Zeiss) with a 63 × 1.4 numerical aperture plan Apochromat objective (Carl Zeiss). Usually images from optical slices of 1–2 μm thickness were acquired. Cross-talk of the fluorophores was negligible.

2.8 Gene expression studies

For the human PXDN mRNA detection, human multiple-tissue and heart panel [2 μg of poly(A)+ RNA] northern blot membranes (Clontech) were probed at 65°C with a randomly radiolabelled cDNA fragments corresponding the 3′-untranslated regions of PXDNL mRNA following standard hybridization methods. For the quantitative PCR experiments, RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg total RNA, using oligo(dT)18 primers and RevertAid M-MuLV Reverse Transcriptase in 20 μL reaction mix according to the manufacturer’s (Fermentas) recommendations.

Pre-designed TaqMan Gene Expression assays for target genes PXDNL, PXDNL, and reference gene RPL32 were obtained from Applied Biosystems. All cDNA samples were run in triplicate along with no template controls. The quantitative PCR was performed, using LightCycler 480 Probes Master mastermix on a Roche LightCycler 480 system with the following protocol: 95°C for 5 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. Gene expression values were then analysed by the LightCycler 480 SW 1.5 software, using the second derivative method.

2.9 Western blots and co-immunoprecipitation

For western Blot experiments, cells were lysed in 2 × Laemmli sample buffer (0.1 M Tris, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, pH 6.8). For coimmunoprecipitation studies, cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% Triton-X) and then were spun at 13 400 g for 5 min to remove cell debris. Pre-cleared supernatants were incubated with Anti-V5 Agarose Affinity Gel (Sigma-Aldrich) and bound proteins were eluted from the washed beads and analysed by western blotting. Non-transfected and lystate-free controls were routinely included to confirm specificity of the immunoprecipitated proteins. Protein samples were boiled and loaded on 7.5 or 10% SDS–polyacrylamide gels and after
electrophoresis were blotted onto nitrocellulose membranes. To check the effectiveness of transfer and similarity of protein amount, membranes were stained by Ponceau S. Blots were then blocked in PBS containing 5% milk powder and incubated with the primary antibody. The binding of the antibody was visualized by peroxidase-coupled goat anti-mouse or anti-rabbit IgG (GE Healthcare), using the enhanced chemiluminescence method according to the manufacturer’s instructions (Millipore).

2.10 Statistical analysis
Data are presented as representative blots or immunofluorescence pictures of at least three similar experiments or as the means ± SE of the number of experiments indicated (n). Statistical significance was calculated using Student’s t-test.

3. Results
We used a PXDNL cDNA fragment for the analysis of mRNA expression in several human tissues and found that PXDNL mRNA was expressed only in the heart (Figure 1A). We, therefore, cloned the full-length PXDNL cDNA from the human heart (details of the cloning procedure are provided in the Methods section). The open-reading frame of PXDNL encodes a protein, which contains 1463 amino acids and its calculated molecular weight is 164 kDa. PXDNL shows 58% identity and 72% similarity to PXDN. The peroxidase domain of PXDNL spans between amino acids 726 and 1273. The protein also contains domains, which are present in PXDN in a highly similar manner (Figure 1B). These include an N-terminal signal sequence (1-24) followed by leucine-rich repeats (55-182) and four immunoglobulin-like C2 domains (240-584). The C-terminal part of the protein contains a vWFC-type domain (1400-1450). The TargetP 1.1 protein localization software predicted that PXDNL contains a signal sequence and localizes to the extracellular space. The peroxidase domain of PXDNL has strong homology to other mammalian peroxidases; however, important differences were observed between PXDNL and other members of the peroxidase family. Highly conserved amino acids, which have an instrumental role in the catalytic activity of animal peroxidases, are conserved in PXDNL. Figure 3A shows that the expression of PXDNL in the human heart, where the protein is naturally expressed. In immunostaining, experiments performed on human heart sections, we detected PXDNL at the intercalated discs and also on the lateral surface of cardiomyocytes. Since PXDNL is also expressed in cardiomyocytes (data not shown) and the two proteins are highly homologous to each other, we were interested in determining whether the two can form a complex. To study this, we co-expressed PXDNL and a V5-tagged version of PXDN in Cos7 cells and immunoprecipitated PXDN with an anti-V5 antibody. As shown in Figure 3A, PXDNL was also detected in the immunoprecipitate, indicating that PXDNL formed a complex with PXDN. Next, we wanted to examine whether the complex formation has any effect on the peroxidase activity of PXDNL. Cos7 cells were transfected either with PXDN alone or in combination with PXDN. Figure 3B shows that the expression of PXDNL inhibited the peroxidase activity of PXDN, while the amount of PXDNL was not decreased in the presence of PXDNL (Figure 3A).

Animal peroxidases often exist in multimeric form and a trimeric quaternary structure was suggested for the Drosophila PXDN. Since PXDNL also expressed in cardiomyocytes (data not shown) and the two proteins are highly homologous to each other, we were interested in determining whether the two can form a complex. To study this, we co-expressed PXDNL and a V5-tagged version of PXDN in Cos7 cells and immunoprecipitated PXDN with an anti-V5 antibody. As shown in Figure 3A, PXDNL was also detected in the immunoprecipitate, indicating that PXDNL formed a complex with PXDN. Next, we wanted to examine whether the complex formation has any effect on the peroxidase activity of PXDNL. Cos7 cells were transfected either with PXDN alone or in combination with PXDN. Figure 3B shows that the expression of PXDNL inhibited the peroxidase activity of PXDN, while the amount of PXDNL was not decreased in the presence of PXDNL (Figure 3A).

Since an altered synthesis of ECM is characteristic for several cardiac diseases, we sought to examine whether the expression of PXDNL changes in congestive heart failure. We used quantitative PCR to study the expression of PXDNL mRNA in tissue samples from normal and failing human hearts. As shown in Figure 4A, the expression of PXDNL was increased in the diseased samples. The results of in situ hybridization experiments (Figure 4B and C) supported the qPCR findings and showed that PXDNL mRNA localized to cardiomyocytes, similar to that observed in the healthy heart tissue (Figure 1D). Since angiotensin II (Ang II) has a central role during the development of heart failure, we tested if Ang II affects the expression of PXDNL. We treated human iPSC-derived cardiomyocytes with Ang II and analysed the PXDNL mRNA expression by quantitative PCR. Figure 4E demonstrates that Ang II increased the expression of the PXDNL mRNA.
4. Discussion

The heart is a functional syncytium, where efficient electrical and mechanical coupling between neighbouring cells, involving the ECM, is essential for proper functioning of the organ. In this work, we propose a previously unrecognized form of ECM modification in the heart that seems to be a specific feature of cardiomyocytes. According to our results, cardiomyocytes express PXDNL that localizes to cell–cell junctions. PXDNL is highly homologous to PXDN, a multi-domain protein that was originally described as a Drosophila ECM-constituent with peroxidase activity and subsequently found in other species as well. Similar to its close homologue, PXDNL is also a hybrid protein composed of a peroxidase-like part and domains that are characteristic for constituents of the ECM. The unique combination of leucine-rich repeats and immunoglobulin-like domains together with the ability to form multimers suggests that PXDNL is ideally suited for an adhesive function to establish cell–cell and cell–ECM contacts. Although we could show that heterologously expressed PXDNL reaches the extracellular space, further studies should determine whether PXDNL is a constituent of ECM in the heart, where it is naturally expressed. The localization of PXDNL to the intercalated discs indeed supports the idea that the protein has adhesive function in the heart. This feature can be especially...
important in a tissue that is normally exposed to high mechanical stress and may explain the unique expression pattern of PXDNL.

An intriguing feature of PXDNL is that the protein lacks any detectable peroxidase activity due to substitutions of conserved amino acid residues that are normally involved in haeme binding to animal peroxidases.\textsuperscript{5,12} It is possible that the loss of peroxidase function along with keeping the adhesive properties was advantageous in an organ where, due to the intense metabolic rate, ROS production is generally high. Gu et al.\textsuperscript{10} has recently reported that an enzymatically active mRNA endonuclease is also encoded by the \textit{pxdnl} gene, however, using the siRNA technique and PXDNL-specific antibodies we could not confirm the existence of this gene product (data not shown).

On the other hand, we and others have shown that the \textit{Drosophila} and mammalian PXDNs do have peroxidase function.\textsuperscript{4,7,9} The physiological

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**Figure 2** Characterization of PXDNL activity and localization. (A and B) Cos-7 cells were transfected with either PXDN or PXDNL encoding plasmids (+) and the cell lysates were tested for protein expression (A) and peroxidase activity as indicated by resorufin fluorescence. (B, \(n = 4\)). (C–E) Cos-7 cells were co-transfected with PXDNL and an endoplasmic reticulum-targeted mCherry construct and stained for PXDNL. Inserts show images of COS-7 cells that were transfected only with endoplasmic reticulum-targeted mCherry. (F–H) Immunocytochemistry was carried out on iPS-derived cardiomyocytes for PXDNL and F-actin. Note the peripheral PXDNL labelling (arrows). (I and J) Staining for PXDNL in human heart section. Note the intense signal at the intercalated discs and on the lateral sides of cardiomyocytes (arrows), the bar indicates 10 \(\mu\)m. (K) Western blot analysis of the whole heart (lane 1) and the heart atrium (lane 2) lysates using a polyclonal antibody, anti-PXDNL (1078 – 1209), that was raised against a region within the peroxidase-like domain of PXDNL.
role of this activity was recently discovered by Bhave et al.,8 who have demonstrated that PXDN mediates the formation of sulfilimine bonds in collagen IV, catalysing an oligomerization mechanism that puzzled researchers for a long time. We demonstrated that PXDNL can form a complex with PXDN and inhibits its peroxidase activity. Our results suggest that hetero-oligomerization of PXDNL and PXDN represents a cardiac-specific regulatory mechanism for PXDN. Interestingly, a functional antagonism between two PXDN isoforms in basal membrane assembly was recently also described in C. elegans.6

To our knowledge, the cardiac-specific expression of PXDNL is unprecedented among other constituents of the ECM. This unique expression pattern warrants an analysis of the pxdnl gene promoter with a special emphasis on cardiac-specific transcription factors that are responsible for heart-specific gene expression.14 One might speculate that identification of the pxdnl promoter region can be useful in the design of cardiac-specific expression vectors. Furthermore, although the exact localization of PXDNL requires further studies, our results raise the possibility that anti-PXDNL antibodies might be used to target drugs to the heart.

Our findings may be of clinical significance, since we found a higher expression of PXDNL in the failing heart. It is possible that an increased expression of PXDNL might represent a compensatory mechanism assisting adaptation to elevated mechanical stress in the diseased organ. Since Ang II stimulated PXDNL expression in cardiomyocytes...
of iPSC cell origin, it is likely that Ang II is responsible for elevated PXDNL expression in the failing heart. Ang II has a key role in cardiac remodelling with direct effects on both cardiomyocytes and cardiac fibroblasts. While the expression of PXDNL was stimulated by Ang II, the level of PXDN was not affected (data not shown). If an antagonism between PXDNL and PXDN also exists in the human heart, then increased PXDNL expression may inhibit collagen IV biosynthesis leading to altered composition of basement membrane around cardiomyocytes.

Since PXDNL is not expressed in rodents, it will be challenging to explore the exact role of PXDNL in congestive heart disease. Recently described gene manipulation techniques, however, may provide an option to study the function of PXDNL in non-rodent animals.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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