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Peroxidase Activity of Peroxidasin Affects Endothelial Cell Growth

Kyung A Ham[1,2], Seong Bin Jo[1,2], Min Ju Lee[1,2] and Young Ae Joe[1,2]*

[1]*Department of Medical Life Sciences, College of Medicine, The Catholic University of Korea, Seoul 06591, Korea*
[2]*Department of Biomedicine & Health Sciences, College of Medicine, The Catholic University of Korea, Seoul 06591, Korea*

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Peroxidasin (PXDN), a multidomain heme peroxidase containing extracellular matrix (ECM) motifs, as well as a catalytic domain, catalyzes the sulfilimine crosslink of collagen IV (Col IV) to reinforce Col IV scaffolds. We previously reported that PXDN is required for endothelial cell (EC) survival and growth signaling through sulfilimine crosslink-dependent matrix assembly. In this study, we examined whether peroxidase activity is required for PXDN function in ECs. First, we constructed a mutant PXDN by point mutation of two highly conserved amino acids, Q823 and D826, which are present in the active site of the peroxidase domain. After isolation of HEK293 clones highly expressing the mutant protein, conditioned medium (CM) was obtained after incubating the cells in serum-free medium for 24 hours and then analyzed by Western blot analysis under nonreducing conditions. The results revealed that the mutant PXDN formed a trimer and that it was cleaved by proprotein convertase-like wild-type (WT) PXDN. However, peroxidase activity was not detected in the CM containing the mutant PXDN, in contrast to that of WT PXDN. In addition, the sulfilimine crosslink ability of the mutant PXDN was lost. Moreover, the CM containing the mutant PXDN failed to promote the growth of PXDN-depleted ECs, unlike the CM containing WT PXDN. These results suggest that the peroxidase activity of PXDN affects EC growth by forming a sulfilimine crosslink.

Key words : Collagen IV, endothelial cell, peroxidase, Peroxidasin, sulfilimine crosslink

Introduction

The extracellular matrix (ECM) provides structural support to organs or tissues and also elicits signaling cues that regulate cell behavior and function [8, 15]. Basement membrane (BM) is a specialized ECM that underlies a layer of polarized cells such as epithelial and endothelial cells (ECs) [25]. BM forms stabilizing extensions of the plasma membrane that provide cell adhesion and act as solid-phase agonists. During the sprouting phase of angiogenesis, ECM binding to integrin provides signaling support for EC proliferation, survival, and migration [21]. ECM also provides a binding scaffold for various cytokines that exert essential signaling during angiogenesis. A major component in BM is a collagen IV (Col IV) scaffold that is essential for tissue genesis.

Col IV incorporates other ECM components such as laminin, nidogen, and perlecan into the scaffold to form a highly organized macromolecular architecture [20]. The Col IV network is assembled by oligomerization of triple-helical protomers and is covalently crosslinked by peroxidasin (PXDN), contributing to the stability of the network [1]. The crosslink of Col IV is formed by crosslinking hydroxylysine 211 and methionine 93 of adjacent protomers with a sulfilimine bond [24]. This sulfilimine linkage is located at α1α2α1 of the Col IV network, and up to 6 sulfilimine bonds can crosslink the interface of the trimeric non-collagenous 1 (NC1) domain of two adjacent protomers to strengthen the quaternary structure of the network.

PXDN was first discovered as an ECM protein expressed in hemocytes of Drosophila during the early differentiation of the head mesoderm [16]. PXDN is one of the mammalian heme peroxidase family, and unlike other enzymes, it has domains with characteristics of ECM components in addition to the highly conserved peroxidase domain [6]. These domains consist of N-terminal leucine-rich repeats (LRR), immunoglobulin C2 (IgC2)-type domains, and a von Willebrand factor type C domain (vWFC) [12], suggesting a possibility that the ECM motifs of PXDN may be involved in protein-protein interactions [3]. Thyroid peroxidase (TPO),

***Corresponding author**
Tel : +82-2-3147-8406, Fax : +82-2-593-2522
E-mail : youngjoe@catholic.ac.kr

lactoperoxidase (EPO), and myeloperoxidase (MPO) are found only in vertebrates and show a limited tissue-specific expression pattern, whereas PXDN is widely distributed in the BM of almost all tissues like the expression of Col IV [17]. EPO or MPO can also crosslink solubilized Col IV in vitro by generating hypohalous acid [22], but it is less effective than the crosslink by PXDN [14]. PXDN is known to catalyze sulfilimine crosslink formation mainly via bromide [14].

Previously, our group has reported the crucial importance of PXDN-dependent supramolecular network formation of ECM components and sulfilimine crosslinks of the Col IV network in cellular behavior and signaling of ECs [13]. In this paper, we also suggested that ECM motifs and peroxidase domain are required for PXDN function. However, we provided limited experimental evidence to support this idea, because we used mutant proteins in small size with large deletion. Among the multiple domains of PXDN, it has been reported that the N-terminal IgC2 domain and peroxidase domains are required for sulfilimine bond formation [5]. Thus, it will be interesting to ask whether loss of enzyme activity alone can lead to loss of PXDN function in ECs. In this study, we performed a mutagenesis study to avoid large alteration in protein structure and provided clear evidence showing that peroxidase enzyme activity of PXDN affects EC function.

Materials and Methods

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from human cords following previously described procedures [9]. The umbilical cord samples were collected according to procedures approved by the Institutional Review Board at The Catholic University of Korea, College of Medicine (approval No. CUMC09U157). HUVECs were cultured in M199 medium supplemented with 20% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 30 μg/ml endothelial cell growth supplements (ECGS, Corning, NY, USA), heparin 90 μg/ml (Sigma) and 1% antibiotics (Gibco). Basic fibroblast growth factor (bFGF) was purchased from Peprotech (Rocky Hill, NJ, USA). Human embryonic kidney (HEK 293) cells were purchased from KCLB (Korean Cell Line Bank, Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Korea) supplemented with 10% FBS and 1% antibiotics.

Construction of stable cell line overexpressing mutant PXDN

A stable cell line overexpressing wild type (WT) PXDN with Flag-tag was obtained previously [13]. The plasmid carrying WT PXDN cDNA was point mutated to change CAG (Q823) and GAC (D826) to TGG (W) and GAA (E) within the peroxidase domain (Cosmogenetech, Seoul, Korea). Then, HEK293 cells were transfected with the constructed pCMV-SC-CF/PXDN-mutant-Flag using Lipofectamine 2000 reagent (Invitrogen, Massachusetts, Waltham, MA, USA) according to the manufacturer's instructions. To establish a stable cell line consistently overexpressing recombinant mutant PXDN, the cells were treated with G418 sulfate (Sigma) up to 1,000 μg/ml for 4 weeks. Then, the G418-resistant clones were screened for mutant PXDN expression.

Preparation of CM

HEK293 cells overexpressing WT or mutant PXDN were washed with phosphate-buffered saline (PBS, WelGENE Inc.) and stimulated with fresh serum-containing media for 12 hr. Then, the cells were washed with PBS and cultured in serum-free medium for 24 hr. Then, the supernatant was collected and clarified by centrifugation at 4℃, 3,200 rpm for 15 min to obtain a conditioned medium (CM).

Primary antibodies

Polyclonal antibody recognizing the PXDN IgC2 (3-4) subdomain (amino acids 431-612) of human PXDN was generated previously [13]. Polyclonal anti-Flag-tag antibody was purchased from Genetex (Irvine, CA, USA). Rat monoclonal antibody against the NC1 domain of Col IV alpha 2 isoforms (Col IV α2) was purchased from Chondrex (Redmond, WA, USA). The following primary antibodies/dilutions were used: anti-PXDN (1:3,000), anti-Flag-tag (1:3,000) and anti-Col IV α2 (1:3,000).

Western blot analysis

The CM obtained from WT- or mutant PXDN-overexpressing cells and collagenase-digested cell lysate were subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% skim milk. Blots were incubated with specific primary antibodies and then with horseradish peroxidase-conjugated anti-rabbit IgG (Genetex), or anti-rat IgG (Genetex) antibody [23]. Immunoreactive bands were visualized using a chemiluminescent substrate (ECL kit) (GE Healthcare, Piscataway, NJ, USA).

Analysis of PXDN oligomerization

An aliquot of CM was mixed with 5X sample buffer without β-mercaptoethanol (Sigma), and then the samples were boiled at 95℃ for 5 min. Non-reduced samples were separated on 6% polyacrylamide-SDS gels with HiMark Pre-Stained Protein Standard (Novex, Life Technologies, Carlsbad, CA, USA), followed by Western blot analysis. Then, oligomerization pattern of the secreted protein was evaluated based on the presence of PXDN-specific band at molecular masses corresponding to trimeric PXDN.

Peroxidase activity assays

After adjusting the pH of the CM to 4.5-5.0 by addition of acetic acid (Sigma), 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma) was added to the CM and the mixture was incubated for 30 min. Then, the absorbance of the reaction mixture was measured at 650 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Dot-blot analysis

The CM was blotted onto a nitrocellulose membrane using a 96-well Bio-dot device (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in 5% skim milk and incubated with the poly anti-PXDN antibody, followed by the horseradish peroxidase-conjugated secondary antibody. Immunoreactive dots were visualized using an ECL kit.

PFHR-9 matrix overlay assay to detect NC1 crosslinking

PFHR-9 matrix overlay assay was performed as reported before [5]. PFHR-9 cells were cultured in DMEM supplemented with 5% FBS and bFGF 10 ng/ml under 5% CO_2 for 5 days in the presence of 50 μM phloroglucinol (PHG, Sigma) to inhibit PXDN-mediated sulfilimine crosslink formation. Then, the cells were detached by detergent extractions using hypotonic buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM $CaCl_2$, and 0.1% bovine serum albumin) containing 1% Triton X-100 (Sigma). Cellular debris was further removed by washing twice with hypotonic buffer containing 0.1% sodium deoxycholate (Sigma). The matrix was further purified by extraction with 4 M guanidine hydrochloride (Sigma) in 50 mM Tris-Cl, pH 7.5, for 15 min and then washed with 1X PBS to remove any residual detergents. HEK293 cells overexpressing WT or mutant PXDN were seeded on the PFHR-9 matrix plates and then incubated for 72 hr in the presence of 5 μM hematin. After the culture medium was harvested, the cells were washed with 1X PBS and 300 mM HEPES buffer (Biosesang, Seongnam, Korea) and digested with collagenase (50 μg/ml: CLSPA grade, Worthington Biochemical Corp., Lakewood, NJ, USA) in hypotonic buffer at 37°C for 3-4 hr. The digested material was then subjected to SDS-PAGE under reducing conditions followed by Western blot analysis. Crosslinked dimeric and un-crosslinked monomeric NC1 domains were detected with anti-Col IV a2 antibody.

Complementation assay of PXDN-depleted HUVECs

Small interfering (si) RNAs targeting PXDN (siPXDN, 5'-GCAUCAAUGCUGGCAUCUUTT-3'), and control siRNA (siCTL, 5'- GUUCAGCGUGUCCGGCGAGTT-3') were obtained from Bioneer. Co. (Seoul, Korea). siCTL was used as a negative control. HUVECs were seeded at 1×10^3 in each well of a 96-well plate and cultured for 24 hr. Then, the cells were transfected with 50 nM siRNA using lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were cultured in fresh medium for 48 hr. Then, the transfected cells were cultured in each CM supplemented with bFGF 10 ng/ml for another 48 hr. Then, 20 μl MTS reagent (Promega, Madison, WI, USA) was added to each well. After 3 hr, the plates were read at 490 nm using an ELISA reader.

Statistical analysis

Western blot data demonstrated in this study are representatives of at least three independent experiments. Data from peroxidase activity assay and cell proliferation assay are presented as means ± SEM of representative experiments. One-way ANOVAs with Tukey tests were used to compare results between the control and the samples using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) for most experiments. $^{**}p<0.01$, $^{***}p<0.001$; ns, not significant.

Results and Discussion

Expression and characterization of secreted mutant protein.

We hypothesized that peroxidase activity of PXDN could affect EC function. In order to examine this hypothesis, we constructed mutant PXDN by changing two amino acids Q823 and D826, which are involved in heme-binding and catalytic reaction and are highly conserved in various peroxidases [3, 7].

Previously, we constructed HEK293 cells overexpressing Flag-tagged WT PXDN [13]. Using this construct, we re-

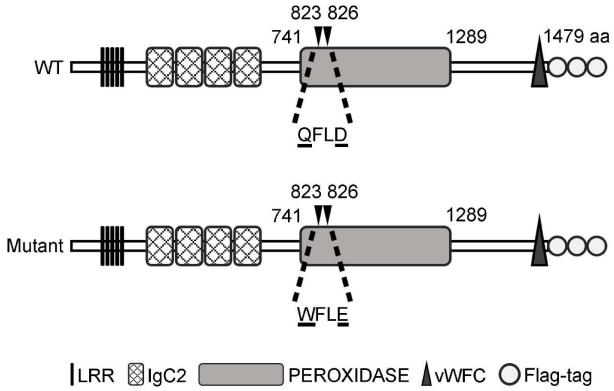

Fig. 1. Schematic depiction of recombinant WT and mutant PXDN constructs. PXDN consists of five LRR motifs, four IgC2 domains, one peroxidase domain, and one vWFC domain. WT-plasmid carrying a Flag-tag at the C-terminus was mutated to change amino acids Gln 823 (Q) and Asp826 (D) to Trp (W) and Glu (E) within the active site of peroxidase domain.

placed amino acids Q823 and D826 with Trp (W) and Glu (E) (Fig. 1). First, CM was obtained by incubating the cells overexpressing WT or mutant PXDN in serum-free medium for 24 hr, and then oligomerization pattern of the secreted recombinant protein in the CM was determined by Western blot analysis (Fig. 2A). As in WT PXDN, only trimeric form was detected in the mutant PXDN.

Proprotein convertase (PC) is involved in proteolytic processing that turns inactive precursor protein into a biologically active state and improves the enzymatic activity of PXDN by processing PXDN proteolytically at Arg1336 [4]. To investigate whether the mutant is processed by PC, anti-Flag-tag antibody was used in Western blot analysis of the CM (Fig. 2B). We identified a C-terminal fragment of the mutant PXDN with less than 30 kDa in size, which is identical to that of WT PXDN. Therefore, these results indicate that the structure of secreted protein mutated at peroxidase active site of PXDN is unchanged by forming trimers and being processed by PC.

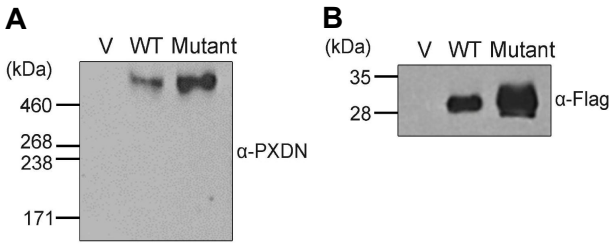

Fig. 2. Oligomerization pattern of mutant PXDN and its cleavage by PC. (A) Western blot analysis was performed using anti-PXDN antibody for the CM obtained from the HEK293 cells overexpressing WT or mutant PXDN under non-reducing condition. Both WT and mutant PXDN showed only trimeric form. (B) Western blot analysis was performed using anti-Flag-tag antibody for the CM obtained from HEK293 cells overexpressing WT or mutant PXDN under reducing condition. Both WT and mutant PXDN showed cleavage by the PC to generate a C-terminal fragment shorter than 30 kDa.

Mutation of peroxidase active site leads to loss of peroxidase activity

Since we mutated peroxidase active site of PXDN, we examined whether the mutant PXDN retains peroxidase activity. As shown in Fig. 3, the CM containing the mutant PXDN showed very low TMB oxidation level similar to the oxidation level of empty vector-derived CM. In contrast, the CM containing WT PXDN showed high level of TMB oxidation. Similar amounts of secreted PXDN (WT/mutant) were detected in the CM by dot blot analysis. Thus, we confirmed that mutation of the active site residues of peroxidase domain abolishes peroxidase activity of PXDN. This result is consistent with the previous observation that PXDN-like protein has 58% identity and 72% similarity to PXDN in amino acid sequence but lacks peroxidase activity due to differences in two highly conserved amino acids (W808 and E811) of peroxidase domain [19].

Sulfilimine crosslink ability of PXDN is abolished by mutation of peroxidase active site

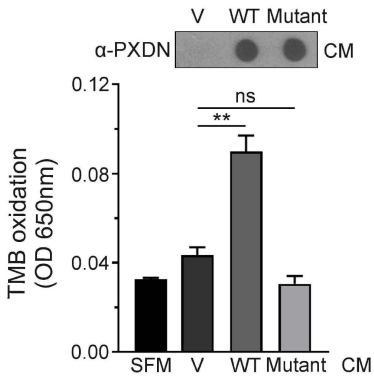

Fig. 3. Mutation of peroxidase active site results in loss of peroxidase activity. The peroxidase activity of the CM obtained from the HEK293 cells overexpressing WT or mutant PXDN was measured by a TMB oxidation reaction. Serum-free DMEM medium (SFM) or the CM derived from vector-transfected cells (V) was used as a control. An inset shows the secretion level of WT or mutant PXDN in each CM by dot-blot analysis. ** $p<0.01$; ns, not significant.

PXDN covalently catalyzes crosslinks between the NC1 domains of triple-helical protomers-adjacent to each other to form sulfilimine bond and contribute to the integrity of Col IV [24].

To determine whether the mutant PXDN with defective peroxidase activity is able to form sulfilimine bond in Col IV, an overlay assay was performed using PFHR-9 mouse endodermal cell line. PFHR-9 cells produce a high level of Col IV among other BM components and accumulate Col IV matrix [1]. PFHR-9 cells were treated with PHG to inhibit PXDN-mediated NC1 crosslinking, which resulted in the accumulation of uncrosslinked Col IV matrix on the plates. Then, the cells stably overexpressing WT or mutant PXDN were seeded onto the uncrosslinked Col IV matrix, and cultured for 72 hr, followed by evaluation of NC1 crosslinking (Fig. 4A). In contrast to WT PXDN-expressing cells, the mutant PXDN-expressing cells exhibited very weak intensity of NC1 dimer band due to their failure to adequately form sulfilimine crosslinks. Both samples showed similar PXDN (WT/mutant) levels (Fig. 4B). Therefore, these results suggest that two amino acids predicted to be involved in heme-binding and catalysis were essential for physiologic sulfilimine bond formation [3, 7].

Unlike other mammalian heme-peroxidases, PXDN has several domains characteristic of ECM proteins in addition to peroxidase domain, and most of them are involved in protein-protein interaction [18]. To find the correlation between these various domains and peroxidase activity of PXDN, many studies used the PXDN mutant constructs generated by deleting the ECM motifs. Besides peroxidase domain, IgC2 domains are also required for sulfilimine crosslinking among the non-catalytic domains of PXDN [5]. It was also revealed that the IgC2 domains and peroxidase domain are evolutionarily conserved in several animal peroxidases [6], suggesting an important role of these domains in PXDN function.

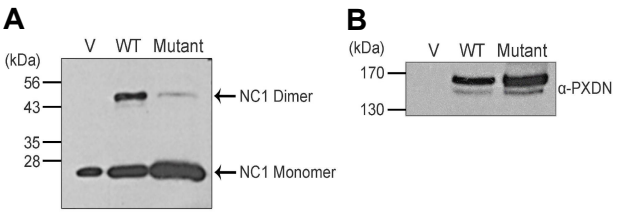

Fig. 4. Mutation of peroxidase active site results in loss of sulfilimine crosslinking ability. To examine the ability of mutant PXDN to form sulfilimine crosslinks in Col IV, PFHR-9 matrix overlay assay was performed. After digestion of matrix and cell lysate with collagenase, NC1 domains of Col IV (A) and PXDN (B) were detected by Western blot analysis. Empty vector-derived sample (V) was used as a control.

Mutant PXDN with defective peroxidase activity failed to restore proliferation of PXDN-depleted cells

As PXDN was endogenously expressed in primary EC [2], we treated HUVECs with PXDN siRNAs to inhibit its expression and observed reduced survival and proliferation of the cells upon PXDN depletion in the previous paper [13]. When the CM containing WT PXDN was added to the PXDN-depleted cells, cell proliferation was restored. Therefore, we performed a complementation assay to determine whether the CM containing the mutant PXDN could restore proliferation of PXDN-depleted ECs. While the CM containing WT PXDN could restore proliferation of PXDN-depleted cells, the CM containing the mutant PXDN failed to restore it, as in the CM derived from the empty vector-transfected cells (Fig. 5). We also confirmed the similar levels of PXDN (WT/mutant) in the CM. Therefore, we concluded that peroxidase activity of PXDN is required for EC proliferation.

The analysis of mutant PXDN showed a trimeric form as examined in Drosophila PXDN [16]. Multimerization is a shared feature of heme peroxidases such as LPO and MPO. LPO forms dimers crosslinked by dityrosine bonds, the for-

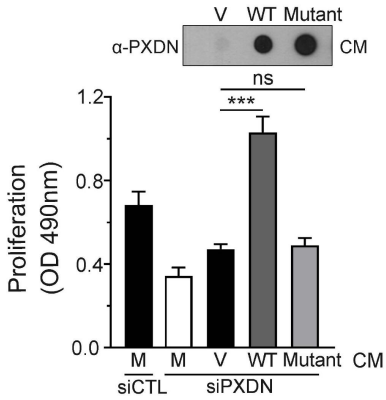

Fig. 5. Complementation assay of PXDN-depleted HUVEC proliferation in the CM containing WT or mutant PXDN. The CM was collected from the WT or mutant PXDN-expressing HEK293 cells and then added to siPXDN-transfected HUVECs (48 hr) together with 10 ng/ml bFGF followed by further incubation for 48 hr. For controls, serum-free M199 (M) or the CM derived from vector-transfected cells (V) was used as a control. Cell proliferation was measured by MTS assay. An inset shows the secretion level of WT or mutant PXDN in each CM by dot-blot analysis. *** $p<0.001$; ns, not significant.

mation of which depends on the peroxidase activity of the enzyme [11]. MPO also forms dimers, but its structure is stabilized by intermolecular disulfide bonds [7]. WT PXDN also exists in trimers by intermolecular disulfide bond formation between two cysteines, C736 and C1315 that are highly conserved across the animal kingdom [12]. Thus, the PXDN mutated at the active site of peroxidase domain of PXDN has no change in structural integrity such as oligomerization to pursue cellular function.

Previously, it has been shown that normal processing by PC occurred in secreted WT PXDN [4]. It has been also found that a mutant in which the RGRR sequence recognized by PC is mutated to RGAA shows reduced peroxidase activity and less efficient formation of sulfilimine crosslinks in Col IV. In our results, the PXDN mutated at the active site of PXDN peroxidase shows normal processing by PC. Thus, in the mutant protein, PC processing is not a factor to affect formation of sulfilimine crosslink in Col IV, and cellular function.

In the previous study, we investigated the enzymatic activity of C-terminal peroxidase domain-deleted mutant (ΔP) and confirmed that ΔP mutant (with no peroxidase activity) failed to restore the proliferation of PXDN-depleted ECs [13]. These results provide a clue that there are critical residues at amino acids between Leu698 and Phe1106 that affect the role of PXDN for EC function. However, since ΔP mutant has a large deletion of amino acid residues (Leu698 to Phe1106), defects by the mutation could be reflected from other aspects besides loss of enzymatic activity. Thus, the mutant constructed in this study is a more proper model to study precisely the impact of peroxidase activity of PXDN on EC function.

From our results, we can emphasize the importance of peroxidase activity of PXDN for sulfilimine crosslinking of Col IV and cellular function. In the future, the mutant with loss of peroxidase activity can be generated in genetically engineered mice to examine the impact of peroxidase activity of PXDN *in vivo*. Since whole-body deletion of PXDN gene results in anophthalmia and severe eye malformation [10], the function of mutant PXDN can be easily evaluated by apparent phenotype.

In summary, our results suggest that intact peroxidase domain of PXDN including active site residues Q823 and D826 is required for EC growth. Our studies also provide evidence that sulfilimine bond formation by peroxidase activity of PXDN may be a prerequisite for EC function.

Acknowledgment

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 내피 세포 성장에 영향을 미치는 PXDN의 peroxidase 활성

함경아[1,2] · 조성빈[1,2] · 이민주[1,2] · 조영애[1,2*]
([1]가톨릭대학 의과대학 의생명과학교실, [2]가톨릭대학 의과대학 의생명건강과학과)

Peroxidasin (PXDN)은 촉매 도메인 외에도 세포외기질 모티프를 포함한 다양한 도메인을 가진 heme peroxidase로서, collagen IV (Col IV)에서 sulfilimine 가교를 형성하여 Col IV의 스캐폴드를 강화한다. 우리는 이전 논문에서 PXDN이 sulfilimine 가교 의존적인 기질 assembly를 통하여 내피세포 생존 및 성장 신호전달에 필요하다고 보고하였다. 이 연구에서는 내피세포에서의 PXDN 기능에 있어 peroxidase 활성의 필요성을 조사하였다. 첫번째로 peroxidase 도메인의 활성 부위에 존재하며 고도로 보존된 Q823과 D826을 각각 W823, E826으로 치환한 돌연변이체를 제작하였다. 이러한 돌연변이 단백질을 높게 발현하는 HEK293 클론을 분리하였고, 이들 세포를 무혈청 배지에서 24시간 배양하여 조건 배지를 확보하여 평가하였다. 조건 배지에 대해 비환원 조건으로 Western blot 분석을 실시하였을 때, 돌연변이 단백질은 삼량체를 형성하는 것으로 관찰되었고, proprotein convertase에 의해 야생형 PXDN처럼 절단되는 것을 확인하였다. 그러나, peroxidase 활성은 돌연변이 PXDN이 포함된 조건 배지에서 야생형 PXDN과는 대조적으로 관찰되지 않았다. 또한, sulfilimine 가교 형성 능력도 돌연변이 PXDN에서 소실되었음이 확인되었다. 이에 더하여, PXDN이 depletion된 내피세포에 돌연변이 PXDN이 포함된 조건배지를 가하였을 때, 야생형 PXDN 조건배지와 달리 증식을 촉진시키지 못함을 관찰하였다. 이러한 결과들은 PXDN의 peroxidase 활성이 sulfilimine 가교를 형성하여 내피세포 성장에 영향을 미침을 제안한다.