

Novel molecules and signal transduction pathways in fibrotic processes

PhD thesis

Zalán Péterfi M.D.

Semmelweis University
Molecular Medicine School of PhD Studies



Supervisor:

Miklos Geiszt M.D., PhD.

Official reviewers:

András Kiss M.D., PhD.

Attila Bácsi, PhD.

Chairman of the examination board:

András Falus M.D., PhD., D.Sc.

member of the Hungarian Academy of Sciences

Members of the examination board:

Tamás Arányi M.D., PhD.

Gábor Bánhegyi M.D., PhD., D.Sc.

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1. Introduction

Fibrosis is usually defined as a wound-healing process that has gone out of control. Repair of damaged tissues is a fundamental biological process that allows the ordered replacement of dead or damaged cells after injury, a mechanism that is critically important for the survival of multicellular organisms. There are many known acute and chronic sources of injury, including infections, autoimmune reactions, and mechanical and chemical injury. There are two distinct ways for the repair process: the first one is called regenerative way, where injured cells are replaced by cells of the same type, leaving no lasting evidence of damage. The second way is known as fibroplasia, or fibrosis, which occurs when the first way is not possible for some reason. In this case the connective tissue replaces normal parenchymal tissue. Although initially beneficial, the healing process becomes pathogenic if it continues unchecked, resulting in substantial remodeling of the extracellular matrix (ECM) and formation of permanent scar tissue. In some cases, it might ultimately lead to organ failure and death. According to the literature nearly 45% of all deaths in the western world can be attributed to some form of tissue or organ fibrosis.

The key feature of the fibrotic process is the tissue accumulation of contractile and potentially invasive ECM producing cells, the myofibroblasts, hallmarked by the expression of a smooth muscle actin (SMA). There are many known local and circulating progenitor cells for these cells. For example in addition to resident

mesenchymal fibroblasts, myofibroblasts can be derived from epithelial cells in a process known as epithelial-mesenchymal transition (EMT). EMT has a key role in development, carcinogenesis and fibrotic processes. Previous findings show that this process has a central role in the tubulointerstitial fibrosis, a common pathology in chronic kidney disease. In a transgenic mouse model of TIF, nearly 40% of fibroblasts have been shown to originate from the tubular epithelium that underwent EMT. During this process tubular cells lose their polygonal shape and epithelial markers (e.g., E-cadherin), acquire fibroblast-specific proteins (e.g., FSP1), increasingly synthesize extracellular matrix (e.g., fibronectin), and ultimately differentiate into myofibroblasts. Both *in vivo* and *in vitro*, transforming growth factor- β 1 (TGF- β 1) is the main inducer of EMT and fibrogenesis. However, our previous studies revealed that in intact, confluent monolayers of tubular (LLC-PK1) cells, TGF- β 1 alone is insufficient to induce SMA synthesis and thus myofibroblast formation. The additional prerequisite is a partial loss or injury of intercellular contacts. In this work we investigated the signal transduction pathway between the contact injury and SMA promoter. It is known that in smooth muscle cells the activity of the SMA promoter is primarily controlled by serum response factor (SRF) and its coactivators the myocardin related transcription factors (MRTF-A, B). These coactivators are mainly regulated by changing their intracellular localization. Under resting conditions the MRTF is bound to G-actin in cytosol which prevents

its nuclear accumulation. If a stimulus activates the small GTP-ase protein Rho, it will lead the polymerization of G-actin to F-actin fibers. After MRTF dissociates from G-actin, it translocates to the nucleus and forms a heterodimer with SRF and this complex is able to enhance the serum response element mediated gene expression. Our experiments focused on the question whether these isoforms are able to regulate SMA promoter activity in epithelial cells.

During my PhD years I worked predominantly on the characterization of two extracellular matrix located peroxidases, the peroxidasin (PXDN) and the peroxidasin-like (PXDNL) protein. The human peroxidasin proteins (PXDN or VPO1 (vascular peroxidase 1) and PXDNL or VPO2 (vascular peroxidase 2)) have unique structural features among the peroxidases because besides their peroxidase homology domain they possess motifs characteristic for extracellular matrix proteins. 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. *Drosophila* PXDN was found to be expressed in several stages of development, but the exact function remained unknown. 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. 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. 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.

2. Objectives

The main aims of our work were as follows:

- 1. Characterization of the MRTF transcription factors in LLC-PK1 cells.*
- 2. In vitro and in vivo investigation of PXDN.*
- 3. Description of PXDNL, a human peroxidasin homolog.*

3. Methods

Anti-PXDN and anti-PXDNL antibodies

PXDN and PXDNL polyclonal antibodies were purified from rabbit serum following intracutaneous injections of glutathione S-transferase-PXDN (amino acids 1329 to 1479) or -PXDNL (amino acids 1312-1463). The antibodies were affinity purified using Affigel 10 beads (BioRad Laboratories, Richmond, CA) loaded with the antigens.

Cell culture

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 µg/ml streptomycin (Sigma). Human pulmonary and dermal fibroblasts (PromoCell, Heidelberg, Germany) were grown in fibroblast basal medium supplemented with 2% fetal calf serum, 5 µg/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-β1 treatment, primary fibroblasts were serum-deprived in the presence of 0.05% serum. Cells were treated with TGF-β1 (R&D Systems, Minneapolis, MN) for 24 to 72 hours in the absence of serum. In some experiments the medium was supplemented with 100 µmol/L δ-aminolevulonic acid (Sigma). LLC-PK1 (CL4) proximal tubular

cells were cultured in DMEM (Invitrogen) and Chinese hamster ovary (CHO) cells in α -minimal essential medium (α -MEM, Invitrogen), supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin at 37°C under humidified atmosphere of air/CO₂ (19:1). For chronic Ca²⁺ deprivation, the cells were washed four times with phosphate-buffered saline (PBS, Invitrogen), and once with serum- and Ca²⁺-free DMEM followed by incubation in the latter solution. Control samples were incubated with serum-free DMEM containing Ca²⁺. Mammalian expression plasmids were transfected by using Fugene6 or FugeneHD (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).

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 antirabbit 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 4x sample buffer then boiled for 10 minutes before they were loaded onto polyacrylamide gels.

Measurement of peroxidase activity

COS-7 cells expressing PXDN or PXDNL 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.

Immunofluorescent Labeling

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 antibody for 1 hour and finally washed six times in PBS again. Coverslips were mounted using Mowiol 4-88 antifadereagent (prepared from polyvinyl alcohol 4-88, glycerol, H₂O, and Tris pH 8.5).

Quantification of Nuclear/Cytoplasmic Distribution of Proteins

Fluorescence intensities were determined at three random nuclear and cytoplasmic points along a line, or in three equal rectangular areas within the nucleus or the cytoplasm. An average of three determinations per cell was used, and the nuclear/cytoplasmic ratio was calculated. Ratios measured along lines or within rectangular areas were identical. Nuclei were independently visualized by DAPI staining. MRTF distribution was categorized as cytosolic or nuclear when the nucleus was clearly demarcated either by exclusion or accumulation of the label. Otherwise the distribution was regarded as even (or pancellular). To make these categories exact, distribution data were verified using the nuclear/cytoplasmic ratios as <0.75 (cytosolic), 0.75–1.25 (even), and >1.25 (nuclear).

4. Results and conclusion

Because the distribution of MRTF, and its regulation has not been characterized in epithelial cells, initially we sought to compare the localization of MRTF isoforms in LLC-PK1 cells and fibroblast-like CHO cells. As expected, in CHO cells both MRTF-A and -B exhibited predominantly cytosolic staining (in 67 and 82% of the cells, respectively). In contrast, in LLC-PK1 cells MRTF-A was mostly nuclear (>70%), whereas MRTF-B was mainly cytosolic (>70%). This finding indicates that there are significant cell type-specific differences in MRTF distribution between fibroblasts and LLC-PK1 epithelial cells. Next we investigated whether the distribution of MRTF-B, which under resting conditions partitioned mostly in the cytosol, was responsive to Rho signaling and cytoskeletal changes in epithelial cells. Coexpression of constitutively active GFP-Rho resulted in a large (\approx 8-fold) increase in nuclear MRTF-B accumulation. Next we investigated the effect of contact disassembly on MRTF-B distribution. Ca^{2+} removal caused a more than 2.5-fold increase in nuclear localization of MRTF-B. Moreover, inhibition of Rho signaling by the overexpression of Rho-GAP suppressed the contact disruption-promoted nuclear translocation of MRTF-B. These results suggest that MRTF transcription factors might have an important role in the contact

injury induced regulation of the SMA promoter, and are involved in the complex regulation of the EMT.

In our experiments investigating PXDN, we demonstrated that the protein is expressed by human primary cells, including fibroblasts of different origin, where the protein is localized to the endoplasmic reticulum. On stimulation by 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 did not detect the peroxidase activity of the endogenously expressed PXDN. We also show that this pathway of ECM formation is probably not mediated by the peroxidase activity of the protein. 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. In fibrotic kidneys PXDN becomes enriched in the peritubular space where it colocalized with fibronectin. This observation suggests that the stimulatory effect of TGF- β 1 on PXDN expression and secretion was not restricted to *in vitro* conditions. 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.

We identified and cloned a novel human gene product, PXDNL, which is highly homologous to the human peroxidase. According to its expression pattern it can be found only in heart. Our Northern Blot experiments showed that its expression is evenly

distributed within the organ. This is consistent with our *in situ* hybridization experiments where we saw that *pxdnl* is expressed by the cardiomyocytes. The gene's expression is significantly increased in dilatative cardiomyopathy, which calls the attention to its possible role in this pathological process. According to the sequence analysis of the peroxidase domain, some of the conserved amino acids essential for the hem binding are missing. We showed that in contrast to PXDN, PXDNL does not have a peroxidase activity. This finding might be related to the lack of the conserved amino acids in the peroxidase domain of the protein. We carried out immunostainings on PXDNL transfected cells and we saw the colocalization of the protein with the ER marker PDI. Besides the ER staining, we detected the protein at the cell membrane partially localized to the Eberth lines in human heart and in cultured cardiomyocytes. We also found that PXDNL is able to associate with other protein through disulfide bonds. According to the molecular weight of these complexes it is possible that the protein forms oligomers. We carried out immunoprecipitation studies where we found that PXDNL and PXDN are able to attach to each other. The PXDNL's organ-specific expression and its domain structure along with our immunostainings raise the possibility that it is a previously unidentified way to strengthen the extracellular matrix of the heart.

5. List of publications

The PhD thesis is based on the following publications and manuscript:

Peterfi Z, Donko A, Orient A, Sum A, Prokai A, Molnar B, Vereb Z, Rajnavolgyi E, Kovacs KJ, Muller V, Szabo AJ, Geiszt M

Peroxidasin is secreted and incorporated into the extracellular matrix of myofibroblasts and fibrotic kidney

***AMERICAN JOURNAL OF PATHOLOGY* 175:(2) pp. 725-735. (2009)**

IF: 5.673

Fan LZ, Sebe A, Peterfi Z, Masszi A, Thirone ACP, Rotstein OD, Nakano H, McCulloch CA, Szaszi K, Mucsi I, Kapus A
Cell contact-dependent regulation of epithelial-myofibroblast transition via the Rho-Rho kinase-phospho-myosin pathway
***MOLECULAR BIOLOGY OF THE CELL* 18:(3) pp. 1083-1097. (2007)**

IF: 6.028

Zalán Péterfi, Zsuzsanna E. Tóth, Adrienn Sum, Hajnal A. Kovács, Ágnes Donkó, Ajay M. Shah and Miklós Geiszt
Identification of a cardiac specific form of cell adhesion in the human heart

2012 (in preparation)

Other publications

Lanyi A, Barath M, Peterfi Z, Bogel G, Orient A, Simon T, Petrovszki E, Kis-Toth K, Sirokmany G, Rajnavolgyi E, Terhorst C, Buday L, Geiszt M
The Homolog of the Five SH3-Domain Protein (HOFI/SH3PXD2B) Regulates Lamellipodia Formation and Cell Spreading
***PLOS ONE* 6:(8) Paper e23653.** (2011)
IF: 4.411

Donko A, Ruisanchez E, Orient A, Enyedi B, Kapui R, Peterfi Z, de Deken X, Benyo Z, Geiszt M
Urothelial cells produce hydrogen peroxide through the activation of Duox1
***FREE RADICAL BIOLOGY AND MEDICINE* 49:(12) pp. 2040-2048.** (2010)
IF: 5.707

Donko A, Peterfi Z, Sum A, Leto T, Geiszt M.
Dual oxidases
***PHILOSOPHICAL TRANSACTIONS B BIOLOGICAL SCIENCES* 360:(1464) pp. 2301-2308.** (2005)
IF: 6,325