Osteoprotegerin in organ fibrosis: biomarker, actor, and target of therapy?

Putri, Kurnia Sari Setio

OSTEOPROTEGERIN IN ORGAN FIBROSIS: BIOMARKER, ACTOR AND TARGET OF THERAPY?

Kurnia Sari Setio Putri

Paranymphs

Dorenda Oosterhuis

Fransien van Dijk

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Kurnia Sari Setio Putri

born on 29 August 1985
in Jakarta, Indonesia

Supervisors
Prof. P. Olinga
Prof. B.N. Melgert

Co-supervisor
Dr. W.L.J. Hinrichs

Assessment Committee
Prof. J.K. Burgess
Prof. R. Gosens
Prof. S. Meiners

Bismillahirrahmanirrahim
In the name of Allah, the Most Gracious, the Most Merciful

مِنَ ٱلظُّلُمَٰتِ إِلَى ٱلنُّورِ

Minazh zhulumaati ilannuur (Al-Quran, Surah Al- Hadid (57), verse 9)
Door Duisternis tot Licht - Dari Kegelapan menuju Cahaya

CONTENTS

General Introduction

FIBROSIS

Fibrosis is associated with many diseases and is characterized by excessive deposition of extracellular matrix (ECM) in tissue. Production of ECM is actually part of a normal repair response after tissue damage and includes a clotting phase, an inflammatory phase, a remodelling phase and a resolution phase, which will ultimately lead to resolution of the damage and restoration of normal tissue architecture[1]. However, persistent injury and/or inflammation of a tissue may lead to an imbalanced regulation of ECM formation and resolution in this tissue.

Fibrosis can occur in many organs, including vital organs like liver[2], kidney[3], lung[4], intestine[5], heart[6,7], and bone marrow[8]. The development of fibrosis in each organ is associated with several persistent triggers and damage, as described in **Table 1**.

Table 1. Triggers of organ fibrosis

Triggers of fibrosis development		Organ affected
Viral and bacterial infections	Hepatitis B[9] and hepatitis C[10]	Liver
	Tuberculosis[11], diphtheria[12]	Lung
	Microbiota[13]	Liver & intestine
Environmental factors	Cigarette smoke[14], metal and silica dust[4]	Lung
	Fat and alcohol[15]	Liver
Chronic diseases	Ischemic/hypertensive and diabetic nephropathy[3]	Kidney
	Hypertension and cardiomyophaty[16,17]	Heart
Adverse effects of radiation and chemotherapy[4]		Lung

An advanced stage of fibrosis often leads to organ failure and thus malfunction of these vital organs and can ultimately cause the death of patients. To date there are no possibilities to stop or reverse fibrosis, besides transplantation, and only a few therapies are available that slow down the fibrotic process[7,18,19].

Late detection of fibrosis is one of the main reasons for the high mortality in patients. In most patients, fibrosis is only detected when the organ is already severely damaged and the fibrotic organ is not able to properly perform its normal functions anymore. Several diagnostic tools have been applied to assess the stage of fibrosis, including non-invasive imaging methods like magnetic resonance and transient elastography[9,20] and high-resolution computed tomography[21,22] to assess fibrosis stage

in liver and lung, respectively. However, it is still of paramount importance to have reliable and easy-to-assess diagnostic methods and markers to detect the slow and long-term progression of fibrosis in the earliest phase possible in order to prevent the incurable end-stage of the disease. Furthermore, the markers may also be applied to assess the response of fibrotic organs towards antifibrotic therapy, to determine therapeutic effectiveness of antifibrotic drug candidates, and eventually be used as surrogate endpoints in drug studies. In order to determine the reliability of candidate fibrosis markers that can accurately diagnose fibrosis and therapeutic effectiveness, it is necessary to understand how mechanisms, pathways, cell types, growth factors, and cytokines interact in fibrosis development and resolution.

FIBROSIS-ASSOCIATED CELLS

Fibrosis is a complex condition, which involves many different cell types, growth factors, and cytokines. Therefore, it is of utmost importance to understand intercellular mechanisms and signalling pathways of fibrosis, to identify specific targets for therapy and to establish reliable markers for detecting fibrosis and evaluating therapy effectiveness. Extensive studies on those subjects will give more insights on the progression of fibrosis, which will facilitate the development of effective antifibrotic drugs.

Fibroblasts, including tissue resident (like hepatic stellate cells in liver) types and circulating precursors i.e. fibrocytes, play key-role in fibrosis development. Injury/damage of tissue leads to activation of fibroblasts into myofibroblasts. These myofibroblasts secrete profibrotic cytokines and chemokines, initiate migration of circulating fibrocytes and other profibrotic cells into the injured tissue/organ, induce proliferation and differentiation of fibrosis-associated cells, promote tissue contraction, and produce ECM[1,2]. Due to the central role of myofibroblasts in the development of fibrosis, many studies aim to inhibit activation of fibroblasts into myofibroblasts, e.g. by inhibiting the transforming growth factor beta (TGFβ)-pathway[23,24], the Wnt-pathway[25] and the PI3K/Akt pathway[26].

Beside fibroblasts, smooth muscle cells exhibit similar properties as fibroblasts towards fibrosis stimulation[27,28]. Several studies have shown that smooth muscle cells

can differentiate into myofibroblasts and secrete TGFβ[29] and platelet-derived growth factor (PDGF) in lung[30], and PDGF in intestine[31].

Myofibroblasts, being the major producers of extracellular matrix, have been the focus of fibrosis research for many years. However, in recent years, there is increasing evidence that several other cell types also play an important role in fibrosis development and resolution. Other cells that are involved in the development of fibrosis, are described in **Table 2**.

Table 2. Cells involved in fibrosis

Fibrosis-associated cells	Role in fibrosis
Fibroblasts[1,32,33]	- produce profibrotic cytokines and chemokines - initiate migration of circulating fibrocytes and other profibrotic cells into injured tissues/organs - induce proliferation and differentiation of fibrosis-associated cells - promote tissue contraction and produce ECM
(Profibrotic) macrophages[34,35]	- produce profibrotic mediators like TGFβ and PDGF that induce proliferation and activation of fibroblasts - produce CC chemokines that can attract profibrotic cells
(Antifibrotic) macrophages[36–39]	- produce specific matrix metalloproteinases (MMPs) and other proteolytic enzymes like cathepsins to degrade ECM. - phagocytose pieces of degraded ECM - produce tissue inhibitor of metalloproteinases (TIMPs)
Fibrocytes [28,40,41]	- can develop into (myo)fibroblasts and produce connective tissue proteins such as vimentin and collagens I and III
Th2 (Type 2 T helper) cells [42]	- produce profibrotic cytokines including interleukin-4 (IL4), interleukin-10 (IL10), and interleukin-13 (IL13) - produce growth factors (TGFβ, PDGF)
B cells[43]	- produce IL-6, IL10, and TGFβ
Smooth muscle cells[29–31,44]	- produce TGFβ and PDGF - produce cytokines and chemokines - produce matrix proteins, MMPs and TIMPs - express integrins
Dendritic cells[45–47]	- produce MMPs, including MMP2 and MMP7, and produce IL10 and TGFβ

Studies showed that Th2 cells[42] and B cells[43] also play role in producing profibrotic cytokines (IL4, IL10 and IL13) and growth factors (TGFβ, PDGF) that can

activate fibroblasts to produce ECM. Dendritic cells contributes to remodeling of tissue by secreting MMPs, including MMP2 and MMP7[46] and producing IL10 and TGFβ[47].

Macrophages play an important role in controlling ECM homeostasis, which is dysregulated during fibrosis[42,48]. Several studies have shown that macrophages promote fibrosis by producing profibrotic mediators like TGFβ and PDGF that induce proliferation and activation of fibroblasts[35,42] . However, other studies have shown that macrophages also facilitate resolution of fibrosis by producing specific MMPs and other proteolytic enzymes like cathepsins that degrade fibrotic ECM[49,50]. In addition, macrophages have also been shown to express receptors that can phagocytose pieces of degraded ECM[48,51,52]. Macrophages can also express/ produce membrane-type MMP (MT-MMP) and TIMPs[39] which can activate proteolytic activity of MMPs via proteinase cleavage[53,54]. These studies thus reveal that macrophages behaviour is highly plastic.

The interaction and roles of fibrosis-associated cells are schematically summarized in **Figure 1**. This scheme illustrates key players (including mechanisms, pathways, cell types, growth factors, and cytokines) in fibrosis development and resolution that may be studied in more detail for development of fibrosis markers and targets of therapy.

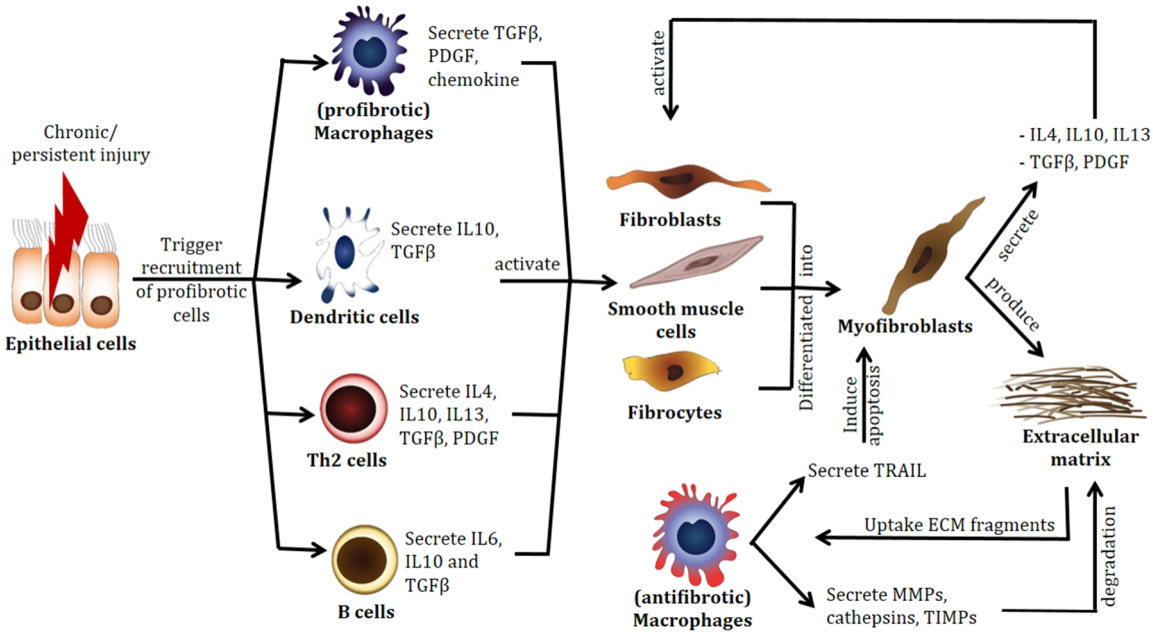

Figure 1. The interactions and roles of fibrosis-associated cells

FIBROSIS MARKERS

In several studies, possible fibrosis markers have been identified in various organs. These markers can be classified as follows:

1. Fibrosis-associated cells, including fibroblasts[32,33], fibrocytes[28,40], macrophages[34,37,38], monocytes[35], dendritic cells[45–47], Th2 cells[42], and B cells[43] as described in Table 2
2. Fibrogenesis-related cytokines, including TGFβ, connective-tissue growth factor (CTGF), PDGF , IL13, tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and their receptors[55]
3. Profibrotic chemokines, including ligand of CXC chemokine-13 (CXCL13)[56] and ligand of CC chemokine-8 (CCL18)[41,57,58]
4. Fibrosis-associated proteins, including galectin-3[59,60] and klotho[61]
5. Markers of myofibroblast activation/ differentiation: α-smooth muscle actin (α-SMA)[62]
6. Markers of ECM formation, including collagen, glycoprotein, hyaluronan, pro-peptide of collagen type III (PIIINP), pro-peptide of collagen type I (PINP), type IV collagen, hydroxyproline, fibronectin, and plasminogen activator inhibitor 1 (PAI-1)[63].
7. Collagen chaperones, including heat shock protein 47 (Hsp47)[64], FK506-binding protein 10 (FKBP10)[65]
8. Markers of fibrolytic processes, including MMP-2, MMP-9, MMP-13[63]
9. ECM degradation products, including collagen type III, VI, I fragments generated by MMP-2, 9, 13[55,66,67]
10. Epithelium-specific markers, including αvβ6 integrin[68], integrin alpha 11[69], surfactant protein A, C, and D[70], MMP-7[71–73] and MMP-3[74]

Among the above-mentioned markers, fibrosis markers that can be detected in serum/plasma (blood-based biomarkers) offer advantages in diagnosing fibrosis due to their easier sampling procedure. Therefore, several blood-based (serum) biomarkers, have been further developed to be applied in the clinical field. Maher et al.[70] for instance have shown that four serum biomarkers (surfactant protein D,

MMP-7, carbohydrate antigen 19-9, and carbohydrate antigen 125) predict disease progression in a cohort of patients with idiopathic pulmonary fibrosis (IPF).

In the case of liver fibrosis, several serum tests have been developed to diagnose liver fibrosis, i.e. the European Liver Fibrosis test (ELF)™ using hyaluronic acid, procollagen III N-terminal peptide and TIMP1[20], the FibroTest/Fibrosure using α-2-macroglobulin, apolipoprotein A1, haptoglobin, L-glutamyltranspeptidase, and bilirubin[75], and the Coopscore using α-2-macroglobulin, apolipoprotein A1, AST, collagen IV and osteoprotegerin. For the latter one, osteoprotegerin (OPG) was included as an additional biomarker to increase the accuracy of liver fibrosis diagnosis[76].

A few other studies have shown that higher OPG serum levels are associated with having liver fibrosis [77,78]. However, it is unclear whether higher OPG levels are only associated with fibrosis of the liver or also of other organs. Moreover, the role of OPG in fibrotic processes and how OPG is regulated during fibrosis are still unclear. Therefore, the aim of this thesis is to elucidate the role of OPG in fibrosis and investigate whether it is a general phenomenon or only associated with liver fibrosis.

OSTEOPROTEGERIN IN FIBROSIS

OPG is a secretory protein that belongs to the tumor necrosis factor (TNF) receptor superfamily. It functions as a decoy receptor for several ligands including receptor of nuclear factor κB ligand (RANKL), TNF-related apoptosis-inducing ligand (TRAIL) and glycosaminoglycan[79,80]. OPG is best known for its regulation of bone tissue ECM by binding RANKL and blocking RANKL-RANK interactions, thus inhibiting osteoclast activation and preventing bone ECM degradation[81]. However, recent studies have shown that higher OPG levels were not only detected in bone but also in fibrotic lung[82], heart[83], and vasculature[84]of murine models as well as in fibrotic liver[77,78], epidural fibrosis[85], and chronic kidney disease due to vascular damage[86], and inflammatory bowel disease[87] in humans.

Unlike markers that can only be detected in fibrotic organs, OPG is a soluble protein and can be detected in blood and urine. Several studies have shown that higher OPG serum levels correlate with fibrosis of the liver[76,78], kidney[88,89] and colon[90]. These

results suggest that OPG could be a biomarker to assess organ fibrosis and may possibly also be used to assess the effectiveness of antifibrotic therapy. Furthermore, having a better understanding of the role of OPG in fibrogenesis in specific cells/tissues/organs may even lead to new opportunities for OPG as a target for antifibrotic therapy.

OPG was first discovered to be produced by osteoblasts[81] but recent studies have shown that OPG is also produced by fibroblasts[91], smooth muscle cells[92] and epithelial cells[93]. OPG production was found to be stimulated by TGFβ, IL4, and IL17 and inhibited by interferon-γ (IFNγ)[94]. OPG itself could also induce the expression of fibronectin, collagen type I, III, IV, and TGFβ1[84]. The association with several types of fibrosis and its production by key cells in fibrogenesis indicate that OPG may play role in fibrosis, however, how OPG contributes to the development of organ fibrosis needs to be further studied.

There are several hypotheses that could explain the role of OPG in fibrosis. Firstly, OPG may bind TRAIL and avert TRAIL-induced apoptosis of myofibroblasts and therefore myofibroblasts will continue to produce ECM[84,95,96]. Secondly, OPG may bind RANKL thereby preventing interaction of RANKL with RANK-expressing macrophages, which may lead to inactivation of MMP-producing macrophages and consequently to inhibition of ECM degradation[97]. Key to these hypotheses is the assumption OPG is produced locally in the fibrotic organ of study. However, in patients and animal models of fibrosis OPG protein in serum or even the organ itself may originate from multiple sources. Therefore, beside using patient material or whole animals, we need additional methods to be able to study OPG regulation on the organ level. One such method is the *ex vivo* method of precision-cut tissue slices. This technique offers advantages to investigate OPG production and regulation in in specific organs without interference of other organs in more details.

PRECISION-CUT TISSUE SLICES

To study fibrosis, many different models are being used, including *in vitro* and *in vivo* models[98,99]. *In vitro* studies on fibrosis focus on how specific cell types respond to fibrotic stimulation. However, fibrosis is a complex disease involving interactions

between various types of cells and *in vitro* studies with only a single cell type cannot comprehensively explain these complex mechanisms. On the other hand, *in vivo* studies with animals provide better insight into the complex mechanisms between cells in the fibrotic organ, but are sometimes overly complex and often require large numbers of animals. In addition, animal models often do not accurately mimic human disease.

Over the years our lab has specialized precision-cut tissue slices as an in between, alternative model to study chronic diseases such as fibrosis in lung, liver, intestine and kidney[100–104]. Intercellular and cell-matrix interactions remain intact in these tissue slices[102] and therefore tissue slices allow study of multicellular processes as the contain all the different cells in their original environment and their tissue architecture. Precision-cut human tissue slices can also provide better prediction of therapy success in clinical research since precision-cut human tissue slices enable more accurate translation of preclinical studies into clinical studies because there are no species differences. Therefore, an important part of this thesis has been generated using the model of precision-cut tissue slices to study different aspects of fibrogenesis.

SCOPE OF THE THESIS

Several studies have shown that macrophages exhibit a "dual role" in fibrosis [35,38,42,105–107]. Therefore, in **Chapter 1**, we discuss this elusive behaviour of macrophages during the development of fibrosis in various organs and identify pro- and antifibrotic characteristics of macrophages to design strategies to suppress their fibrotic nature and to stimulate the antifibrotic nature of macrophages.

We further continued our studies in **Chapter 2,** investigating whether OPG plays a role in pulmonary fibrosis and to test our hypothesis that OPG has an effect on fibrosis development through interactions with RANKL. To test this we treated mice with silica-induced pulmonary fibrosis with RANKL to possibly activate antifibrotic macrophages and we assessed fibrosis development after RANKL treatment.

In **Chapter 3**, we used precision-cut lung slices to study the regulation of OPG in lung tissue during fibrogenesis in more detail using TGFβ-stimulated murine lung slices and slices from human fibrotic lung tissue. In this chapter, we also investigated whether pirfenidone and nintedanib, currently the only approved treatments for IPF,

affect OPG production to assess whether OPG could potentially be used as a marker for treatment effects.

One of the challenges of using precision cut tissue slices is that they can only be used for short-term (2 days) experiments, while fibrosis is a chronic disease that develops over several months/years. Therefore, it is important to have a marker that can detect development of fibrosis in early stages as well as detect early changes after antifibrotic therapy. In **Chapter 4**, we further explore OPG as marker of both early- and end-stage fibrosis in lung, liver, kidney and intestine using murine and human tissue slices. We further evaluated OPG as marker to assess treatment effect using a new drug candidate: galunisertib, a TGFβ-receptor type I kinase inhibitor, which was previously applied as an anticancer drug[108–110].

To gain deeper understanding of OPG regulation in fibrotic organs, in **Chapter 5**, we further studied regulation of OPG in liver tissue after TGFβ- and IL13-stimulation.

Finally, in the **General Discussion**, we summarize our findings and discuss the perspectives of the use of OPG as biomarker to detect fibrosis in early stages, to assess therapy effectiveness of new drug candidates, and as target for antifibrotic therapy.

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