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Osteoprotegerin, RANKL and extracellular matrix intersection in fibrosis

Habibie, Habibie

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CHAPTER NINE

GENERAL DISCUSSION

I. OSTEOPROTEGERIN EXPRESSION IN LIVER AND PULMONARY FIBROSIS

Osteoprotegerin (OPG) was initially recognized for its biological function in bone homeostasis as a decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL) (1). In this interaction, OPG prevents RANKL from binding to its receptor, i.e. receptor activator of nuclear factor kappa-B (RANK) on the surface of osteoclasts, resulting in inhibition of osteoclast activation, less degradation of ossein and maintenance of bone matrix integrity (2, 3). Recent studies indicated that OPG may also be involved in other physiological and pathological events, including organ fibrosis (4-6). Elevated levels of OPG were found in patients with chronic/alcoholic liver disease (7, 8) and therefore OPG was included into a panel of serum biomarkers to increase the accuracy of liver fibrosis diagnosis (9). However, before the start of the studies presented in this thesis, little was known about the source of OPG found in serum of patients with liver fibrosis, nor was anything known about its role in (liver) fibrosis development and progression.

In this thesis (**Chapter 3**) we demonstrated that a possible origin of the elevated levels of OPG detected in patients with liver fibrosis could be their fibrotic liver and more specifically hepatic stellate cells. Mouse fibrotic and human cirrhotic liver tissue produce more OPG as compared to non-diseased liver tissue. Using human and mouse liver slices, we confirmed that OPG was produced locally by liver tissue. Interestingly, the production of OPG was stimulated in the presence of TGF-β (the master regulator of fibrosis) and its expression was associated with other markers of fibrosis. In bone, TGF-β also regulates OPG and RANKL production in osteoblasts after being released from bone matrix upon degradation of this matrix (10-13). Fibroblasts had already been described to produce OPG especially in pathological conditions (14-16) and we now show that OPG is produced by hepatic stellate cells in human healthy liver and by scar tissue-associated (myo) fibroblasts in cirrhotic liver. Moreover, using a mouse model of spontaneous and drug-induced resolution of liver fibrosis, we found that OPG expression is not only associated with fibrosis progression but also associates with resolution of disease and this indicates that OPG could potentially be used as a biomarker for testing of novel drugs against liver fibrosis.

Relatively high levels of OPG were also found in mouse models of pulmonary fibrosis. Brass et al. found that OPG was elevated in lung tissue of mice exposed to bleomycin and silica (17, 18). Bleomycin administration resulted in higher OPG levels and collagen expression and both proteins decreased again over time with a similar trend (17), implying OPG levels are correlated with fibrotic processes and reduce upon resolution. As was the case for liver fibrosis, these data also suggest that OPG expression may potentially be used as a marker to diagnose, track or predict the progression of lung fibrosis. In **chapter 4** we have shown that OPG is expressed in lung tissue and that its expression is higher in fibrotic human lung tissue compared to control. Using mouse lung slices, we confirmed that OPG was produced locally by lung tissue and that the production of

OPG was stimulated in the presence of TGF-β. Even though OPG levels in serum did not directly discriminate patients with IPF from healthy individuals, high OPG levels in serum of patients with IPF at first presentation were linked with progression of disease. In our small-scale cohort, patients with serum OPG levels higher than 1234 pg/mL progressed faster as compared to patients with lower levels. Interestingly, a recent proteomic study also identified and validated OPG as a circulating plasma biomarker for progressive fibrosing interstitial lung diseases (ILD) in a combined cohort of patients with connective tissue disease-associated ILD, chronic hypersensitivity pneumonitis and unclassifiable ILD (19). Moreover, proteomic profiling of bronchoalveolar lavage fluid in interstitial lung diseases revealed higher levels of OPG in patients with IPF than with sarcoidosis (20). These data suggest that OPG has potential properties to be used as a marker for progressive fibrotic lung disease and further study with a larger cohort is warranted to validate the use of OPG in the clinic.

II. ROLE OF OSTEOPROTEGERIN IN LIVER AND PULMONARY FIBROSIS

OPG has been shown to be elevated in a multitude of fibrotic organs including liver and pulmonary fibrosis. In **chapter 3 and 4**, we found higher production of OPG in both healthy liver and lung tissues after only 48 hours stimulation with TGF-β1 indicating that OPG is upregulated early during the process of wound healing and fibrosis. Additionally, our immunohistochemistry analysis of OPG staining in both mouse and human fibrotic lung tissue showed that OPG expression was observed within areas of fibrosis development or fibrotic loci (**Chapter 4**). Moreover, treatment of liver slices with OPG resulted in increased gene expression of several fibrosis-related proteins such as procollagen 1α1 (Col1α1), α-smooth muscle actin (αSMA), fibronectin 2 (Fn2), transforming growth factor β1 (TGF β1) and therefore OPG seems to have a profibrotic capacity (**Chapter 3**). These data imply that OPG may be involved and play a role in the pathogenesis of fibrosis, however little is known about its molecular involvement in fibrosis development.

II.1 Potential role of OPG and its ligands in liver and pulmonary fibrosis

Several hypotheses exist about the role of OPG in fibrosis but none of them have been extensively studied yet. OPG is well known as a decoy receptor for RANKL and TRAIL preventing binding of these proteins to their receptor (21, 22). OPG could be involved in fibrosis through these ligands since RANKL and TRAIL have been documented to have activities associated with fibrosis (23-26). We previously speculated that RANKL could contribute to degradation of extracellular matrix (ECM) and resolve fibrosis via activation of tissue macrophages to produce extracellular matrix-degrading enzymes such as cathepsins and metalloproteinases in a similar way as it does in osteoclasts (27-29). Therefore, high levels of OPG may prevent RANKL-induced macrophage-assisted degradation of matrix and potentially abrogate fibrosis resolution. However, we found no evidence to support

this hypothesis. Another possibility of OPG modulating fibrosis development could be inhibition of TRAIL-induced apoptosis of myofibroblasts. Fibroblasts have been reported to express death receptor 4 and 5 (TRAIL receptors), During fibrogenesis, fibroblasts can transform to myofibroblasts and they may start producing high levels of OPG to escape TRAIL-induced apoptosis. In **chapter 3 and 4**, we confirmed that human primary lung (myo)fibroblasts secrete high levels of OPG. However, using neutralizing antibodies against RANKL and TRAIL we could not inhibit the profibrotic effects of OPG suggesting other mechanisms may be at play. An interesting study from our collaborators found that RANKL is produced by primary lung fibroblasts (30) and a Japanese group showed that its expression is elevated in silica-induced pulmonary fibrosis (31). RANKL is also reported to have a crucial role in epithelial cell regeneration in breast and thymus (32-34) and therefore we hypothesized that elevated levels of RANKL in diseased lung tissue may reflect an attempt at epithelial repair to overcome lung tissue damage. In **chapter 5**, we show that RANKL stimulates proliferation of mouse and human alveolar type II cells in a model of lung organoids: we found more alveolar organoids after RANKL treatment. Interestingly, the presence of OPG in lung organoid cultures completely inhibited RANKL-induced growth of both mouse and human alveolar organoids. These data imply that OPG may contribute to the development of fibrosis and other lung diseases via inhibition of epithelial repair resulting chronic injury.

II.2 Potential interactions between OPG/RANKL axis with ECM components in pulmonary fibrosis

Pulmonary fibrosis is defined by excess accumulation of ECM in lung tissue resulting in irreversible remodeling of normal lung architecture and loss of function (35). During fibrogenesis ECM is continuously remodeled by tissue-residing cell such as fibroblasts. Fibroblast secrete and produce most of the components of the ECM including the structural proteins, adhesive proteins and space-filling ground materials (36). These components do not passively constitute the development of fibrosis, but rather they interact and promote cellular and extracellular processes central in the pathogenesis (37). OPG was shown to bind to ECM-associated growth factor proteins (CTGF) (38) and cell surface molecules (syndecan-1 and αV integrins) (39, 40). In **chapter 6**, we found that OPG has a close interaction with fibulin-1, a secreted glycoprotein that also interacts with other ECM proteins (41) and functions as a bridge of ECM structures to maintain the integrity of the ECM (42). In patients with IPF, elevated levels of fibulin-1 in serum have been linked with the severity of disease and shown to be a promising novel biomarker for IPF (43). We now found that OPG levels and expression positively correlate with fibulin-1 in both serum and lung tissue of patients with IPF. Moreover, our data show that these proteins colocalize in lung tissue and fibroblasts derived from patients with IPF. In addition, using fibulin-1c$^{-/-}$ mice with bleomycin-induced fibrosis, we found that fibulin-1c deficiency inhibits OPG deposition in lung tissue accompanied with less fibrosis development (41).

Fibulin-1 has a crucial role in collagen deposition and in regulation of ECM integrity with its interaction with other ECM proteins like fibronectin, tenascin-c and periostin. Interestingly, the expression of these proteins was also altered in the absence of fibulin-1c in mice (41). These data imply that OPG may also be involved in this mix of ECM molecules during the pathogenesis of fibrosis. However, further study is warranted to investigate how they interact.

Each matrix component within ECM has its own function in order to maintain homeostasis, for instance contributing to the fibrillar structure or providing elasticity (44). In fibrogenesis, the balance of synthesis, modification and degradation is disturbed leading to abundant ECM production and aberrant ECM turnover. These events could lead to many physiological conditions including cellular senescence. Cellular senescence has been linked to fibrotic diseases across many organs (45). In IPF, senescent fibroblasts show unnatural activation, mitochondrial dysfunction, metabolic reprogramming, apoptosis resistance and a senescence-associated secretory phenotype (SASP) (46). However, the impact of ECM itself on cellular senescence, fibrosis and their intersection with the OPG/RANKL axis remain unexplored. In **chapter 7**, we investigated the impact of pathological stiffness, as observed in lung fibrosis, on the induction of cellular senescence and a profibrotic phenotype in fibroblasts. We found that increased stiffness resulted in an increase of fibroblast proliferation (47). We did not observe an increase in the main markers of senescence, $p16^{Ink4A}$ and $p21^{Waf1/Cip1}$ in fibroblasts in response to increased matrix stiffness but we did find upregulation of several fibrosis-associated genes such as ACTA2, COL1A1, OPG and fibulin-1 indicating fibroblasts are activated and show a profibrotic activity in response to the stiff environment. These findings strengthen our hypothesis about the involvement of OPG in fibrosis development and progression.

Surprisingly, we also found that RANKL was elevated in response to a stiff environment. Recently RANKL was reported to be a component of the SASP and elevated in senescence fibroblasts derived from patient with COPD (30). This is an interesting finding since RANKL is a ligand for OPG that is also upregulated in response to stiff matrices. We demonstrated in chapter 5 that RANKL promotes proliferation of alveolar type II cells, suggesting that this SASP may be involved in stimulating tissue repair. However, our data illustrated that all RANKL produced by fibroblasts was bound to OPG and there was no free RANKL detected in the medium of fibroblasts culture. This suggests that OPG may function as a negative regulator of this function of RANKL. These data also reinforce our hypothesis that OPG may contribute to the progression of fibrosis and other lung diseases via inhibition of RANKL-induced epithelial repair.

In IPF, both altered ECM deposition and cellular senescence are identified as main drivers of fibrosis. To dig deeper into the effects of ECM composition on fibrotic responses and cellular senescence, particularly in relation with OPG/RANKL axis, we studied in **chapter 8** whether ECM derived from fibrotic and senescent fibroblasts can modulate fibroblasts senescence. We found that ECM isolated from fibrotic and senescent fibroblasts did not induce $p16^{Ink4a}$ and $p21^{Waf1/Cip1}$ expression or production of secreted proteins

associated with senescence and fibrotic responses in fibroblasts. However, we did find upregulation of IL-6, CXCL8, TGF-β1 production and an increase of α-SMA and DCN gene expression in fibroblasts, but not of OPG and RANKL. Therefore, our data imply that ECM stiffness has more impact on cell function as compared to ECM composition.

III. SUMMARY OF KEY FINDING

As described in this thesis, in addition to the current knowledge about the role of OPG in fibrosis, we introduce that OPG is produced locally by lung myo(fibroblasts) and is elevated in a response to the fibrotic stimuli (TGF-β) or change of the ECM stiffness due to fibrosis development (**chapter 4 and 7**). We also found that RANKL is upregulated in response to the increase of the ECM stiffness (**chapter 7**). interestingly, RANKL may contribute to the lung recovery after injury via promotion of alveolar type II cell proliferation and this effect is completely abrogated in the presence of OPG (**chapter 5**). Moreover, aberrant production of OPG could be detected in the serum of patients with IPF, and OPG levels are linked with the progression of disease (**chapter 4**).

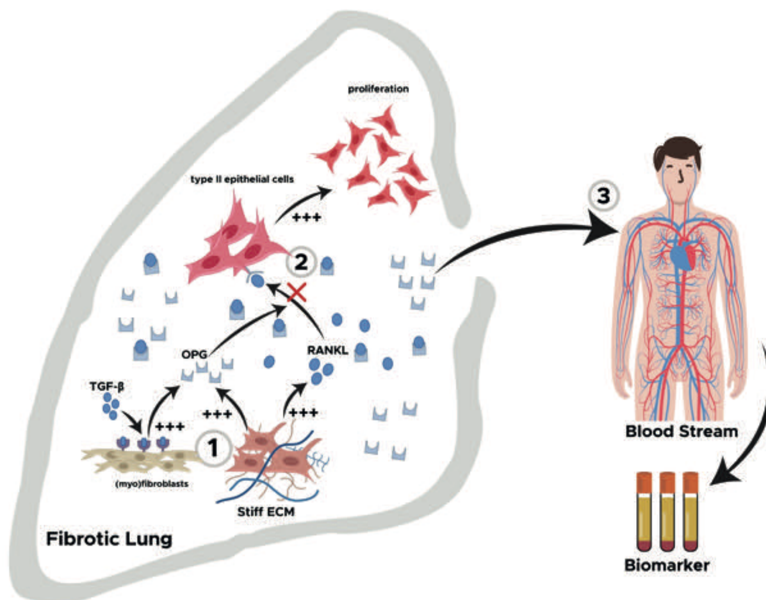

Figure 1. Elevated production of OPG in fibrotic lung affect the proliferation of type II epithelial cells and potentially used as a prognostic biomarker for patients with pulmonary fibrosis. (1) Release of profibrotic cytokine (TGF-β) and increase of the ECM stiffness will stimulate (myo)fibroblasts to produce aberrant amounts of OPG. (2) Increase of the ECM stiffness will also stimulate the secretion of RANKL by (myo)fibroblasts. RANKL will bind to its receptor on type II epithelial cells to promote their proliferation and thereby contributes to the lung recovery after injury. However, this effect is abrogated in the presence of OPG. (3) High production of OPG in the fibrotic lung is also reflected in its circulation in the blood stream and this potentially could be used as a prognostic biomarker for patients with pulmonary fibrosis.

IV. FUTURE PERSPECTIVES

IV.1 OPG as a biomarker for fibrosis

With the growing and ageing population, the prevalence of fibrosis will continue to rise and it will be followed by an increase in financial burden. As IPF is often diagnosed in a late stage and keeps progressing, the only effective current treatment remains lung transplantation. This highlights the urgent medical need to develop novel biomarkers as well as novel therapies for the treatment of IPF. Therefore, basic research should investigate the key pathogenic factors that drive progression of fibrosis. This may lead to the identification of novel biomarkers to diagnose or track the development of fibrosis as well as novel targets for therapeutics that may halt or reverse the disease. In this thesis we provide evidence that OPG has potential to be used as a blood-based biomarker for fibrosis. Future studies should use validation cohorts from multi center/population to warrant the use of OPG as a prognostic biomarker. Since multiple factors, which vary between diseases and patients, have been reported to initiate and be involved in fibrosis development, it is unlikely one single biomarker can distinguish and predict the progression of fibrosis. Therefore, the use of a panel consisting of several biomarkers will strengthen the accuracy of fibrosis diagnosis and addition of OPG could be beneficial in this regard as observed in liver fibrosis (9). Future studies could use this approach more widely in pulmonary fibrosis. Several ECM-related proteins have been reported to play a role in fibrosis and could be used as additional biomarkers in combination with OPG such as periostin (48, 49), fibulin-1 (43) and extra-domain-A fibronectin (50, 51).

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Cohort studies of covid-19 survivors show the follow-up CT scan obtained within 6 months of the onset of disease showed that in more than third of patients experienced lung fibrotic-like changes (ground glass opacities and interstitial thickening) (52) Moreover, persistent histopathological findings and chest imaging abnormalities of lung fibrosis were also found in a majority of survivors of the SARS-CoV-1 2003 infection (53) indicating that covid-19 survivors may have a risk to develop lung fibrosis in the future (54). In **chapter 3 and 4**, we found that OPG is upregulated early during the process of wound healing and fibrosis making OPG a potential candidate to be used as a biomarker to predict the development of early fibrosis in covid-19 survivors. Future studies could use cohorts of covid-19 survivors and follow their OPG levels in serum and correlate it with development of lung fibrosis.

IV.2 Role of OPG in fibrosis

OPG can bind to cell surface molecules such as αV integrins and syndecan-1 (55, 56). Interestingly, integrins can activate latent TGFβ1 which is a crucial event in the progression of fibrosis (57), suggesting interactions between αV integrins and OPG may also influence TGFβ1 activity. As the role of OPG in fibrosis is unclear, we postulated that these interactions OPG has with other matrix proteins may be part of its function in modulating development of fibrosis in the lung. One such component may be fibulin-1. A recent study

found that fibulin-1 can release and thereby activate TGF-β by binding to latent TGF-β-binding protein 1 (LTBP1). OPG may interfere with this interaction in yet unknown ways. Further studies could investigate the role of OPG in the activation of TGF-β with respect to interactions with ECM molecules.

The work presented in this thesis has strengthened the evidence that OPG may represent a novel biomarker and therapeutic target for fibrosis. However, validation cohorts and further in-depth mechanistic studies are warranted to validate the clinical relevance of OPG levels as a marker in fibrotic diseases.

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