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

*Osteoprotegerin as marker of fibrosis*

Fibrosis is a chronic disease, which develops slowly for years and can eventually cause death of patients\(^1\). Therefore, it is highly important to have reliable biomarkers to detect fibrosis in the earliest phase possible in order to prevent an incurable stage of the disease. An easily measurable and sensitive biomarker, which can monitor fibrosis development is crucial to efficiently track antifibrotic efficacy of candidate drugs in clinical trials\(^2\). In this thesis, we have investigated osteoprotegerin (OPG) as a potential marker for fibrosis in different organs and for assessing antifibrotic treatment efficacy.

As OPG is already used clinically to diagnoses and stage liver fibrosis, we investigated in chapter 2 and chapter 3 whether OPG also plays a role in pulmonary fibrosis and compared fibrotic responses in lung, liver, kidney and intestine in chapter 4. We found higher OPG levels in lung tissue of IPF patients (chapter 2) as compared to the control lung tissue. We also showed that pulmonary fibrosis induced by silica in mice was associated with higher OPG mRNA expression and protein production in the lung tissue. In addition, we demonstrated that OPG mRNA expression was higher after transforming growth factor β1 (TGFβ1) stimulation and significantly correlated with other fibrosis markers, including collagen1α1 (Col1α1), fibronectin (Fn1), α-smooth muscle actin (αSMA), and plasminogen activator inhibitor-1 (PAI-1), *in vitro* in 3T3 fibroblasts and *ex vivo* in murine liver (chapter 4 and 5), lung (chapter 2, 3 and 4), and kidney slices (chapter 4), thus showing that OPG is strongly associated with fibrosis development in those organs. As OPG was strongly correlated with other fibrosis markers, it should be investigated as a biomarker of fibrosis. Furthermore, higher production of OPG after only 48 hours of TGFβ1 stimulation of healthy tissues indicate that OPG is upregulated early in the process of wound healing and fibrosis. This idea was reinforced in chapter 3, in which we found that OPG production by murine lung slices was also higher after stimulation with profibrotic cytokine interleukin-13 (IL13), while other regular fibrosis-associated markers (Col1α1, αSMA, Fn1 and PAI1 mRNA) did not yet respond.
The fact that OPG is a soluble protein, which is secreted into in serum/plasma and urine of animals and patients, and can therefore be easily detected, may make it an attractive biomarker. In this thesis, we demonstrated that OPG can be detected in medium of studies with primary lung fibroblasts and 3T3 fibroblasts (chapter 2 and chapter 5), medium of precision-cut tissue slices (chapter 2, 3, 4 and 5), as well as in serum of mice in various models of fibrosis (chapter 4). We also showed that TGFβ1 can induce OPG protein secretion from murine liver (chapter 4 and 5), lung (chapter 2, 3 and 4), and kidney slices (chapter 4). Our studies are in line with other studies showing higher levels of OPG protein in blood and urine of patients with cirrhotic livers\(^3\) and chronic kidney disease\(^4,5\). We also investigated expression of OPG in intestines. Previously, OPG was found to be overexpressed in the colon of patients with inflammatory bowel disease (IBD)\(^6\), ulcerative colitis (UC) and Crohn’s disease (CD)\(^7\). However, we could not demonstrate OPG was associated with the induction of fibrosis in intestine, nor did we see OPG change with antifibrotic treatment of murine colon or human ileum slices (chapter 4). This may be due to the absence of infiltrating peripheral immune cells (e.g. monocytes, T cells, neutrophils) in this study with murine colon slices\(^8\) and/or or due to absence of OPG\(^+\)-cells in muscle layer of human ileum slices\(^9\).

In addition to being a possible marker of fibrosis progression, our study also showed that OPG may be useful to assess treatment efficacy of antifibrotic drugs. Lower OPG mRNA expression and protein secretion was associated with decreased mRNA expression of other fibrosis-associated markers in murine lung slices after treatment with two FDA-approved antifibrotic drugs: pirfenidone, and nintedanib (Chapter 3). These result is in line with other studies which showed that pirfenidone and nintedanib can inhibit production of extracellular matrix (ECM) in vitro\(^10-12\), in vivo\(^13,14\) and in clinical studies\(^15\). Moreover, lower OPG mRNA and protein levels were also accompanied with lower mRNA level of other fibrosis-associated markers in murine lung, liver and kidney slices after treatment with galunisertib, a TGFβ receptor type I kinase inhibitor which was previously applied as an anticancer drug (chapter 4).
General Discussion

**Osteoprotegerin role in fibrosis development**

It is an underestimation to think that OPG is only a biomarker of fibrosis. In a recent study by us *(manuscript in preparation)*\(^{16}\) we demonstrated that OPG itself can induce TGFβ1 mRNA expression in murine liver slices and thereby also other fibrosis-associated markers (Col1α1, αSMA, Fn1 and PAI1 mRNA). Therefore, OPG in liver appears to be involved in a positive feed-forward loop with TGFβ. In lung slices stimulated with OPG, however, we did not find significant induction of TGFβ mRNA expression *(results not shown)* but there seemed to be a trend towards induction that we need to investigate in more detail. These results indicate that OPG may have a role in fibrosis development by stimulating TGFβ1 production.

As OPG is a soluble receptor we tried to identify how a receptor can have an effect on fibrosis development. The most logical explanation being by inhibiting the effects of its ligands, the most important being RANKL and TRAIL. It is well known that OPG binding to RANKL prevents RANKL to bind to RANK-expressed macrophages\(^ {17,18}\), and OPG binding to TRAIL (TNF-related apoptosis-inducing ligand) prevents TRAIL to bind to DR4 and DR5 on myofibroblasts\(^ {19,20}\). We hypothesized that the higher concentration of OPG in fibrotic lung tissue inhibits activation of ECM-degrading (antifibrotic) macrophages\(^ {21}\) and/or inhibits apoptosis of myofibroblasts\(^ {20,22}\), which thus eventually leads to fibrosis progression. In **chapter 2**, we therefore tried to neutralize excess levels of OPG by treating with soluble RANKL (sRANKL) to ameliorate fibrosis. By treating with sRANKL, we expected this excessive RANKL to bind to macrophages and thus activate an antifibrotic phenotype of macrophages. From this study, we expected RANKL treatment could be considered as a novel potential therapeutic for lung fibrosis.

Unfortunately, RANKL-treatment did not affect collagen-1 content and Col1α1 and Fn1 mRNA expressions in lung tissue of mice with silica-induced pulmonary fibrosis. RANKL-treatment also did not activate antifibrotic macrophages and did not induce ECM-degrading proteins, as assessed by MMP-9 mRNA expression, in lung tissue of mice with silica-induced pulmonary fibrosis (**chapter 2**). The explanation may have been that RANK expression on interstitial and alveolar macrophages was lower in fibrotic lung tissue than in healthy lungs. Therefore, due to lower RANK expression on macrophages, additional RANKL may not have had the opportunity to
induce effects on macrophages. These results prompted us to do a literature study to get better ideas about how to induce antifibrotic macrophages and the results of this endeavour are described in chapter 1.

The results in chapter 2 did show a tight balance between RANKL and OPG in pulmonary fibrosis. Instead of activating antifibrotic macrophages, administration of sRANKL to mice with pulmonary fibrosis even stimulated OPG production and surprisingly resulted in higher numbers of epithelial cells. This suggests that the role of RANKL may be related to epithelial proliferation and that OPG is regulating this process. Interestingly, in non-small cell lung carcinoma, which develops from epithelial cells, higher RANKL expression and addition of RANKL were associated with higher metastatic potential and this could be inhibited by adding OPG. This suggests that OPG and RANKL may have a role in turnover of epithelial cells in lung.

Transforming growth factor β (TGFβ) is a cytokine, which not only plays an important role in wound healing processes, but is also widely known as key factor in fibrosis. In chapter 4, we show that TGFβ1 stimulation induced mRNA expression of several fibrosis-associated markers like Col1α1, αSMA, Fn1, and PAI-1 in murine lung, liver, kidney and colon slices. In this study we also showed that galunisertib treatment, a TGFβ-receptor type I kinase inhibitor that acts via the SMAD1/SMAD2 phosphorylation pathway, of murine lung, liver and kidney slices successfully inhibited expression of those fibrosis-associated mRNA markers. In addition, we showed that OPG mRNA and protein expression in lung, liver and kidney slices after TGFβ1 stimulation went up and that it could almost be completely inhibited by Galunisertib, thus indicating that OPG production is mainly regulated by TGFβ1 via the SMAD pathway.

TGFβ1, however, was not the only cytokine being able to induce expression of OPG. Our study in chapter 5 revealed that IL13 also stimulated OPG mRNA and protein expression in murine liver slices and stimulated TGFβ1 mRNA expression as well. Moreover, the effect of IL13 stimulation on murine liver slices could also be completely inhibited by galunisertib. This suggests that IL13-induced production of OPG is predominantly dependent on TGFβ1. Mechanistically, we showed that IL13-induced OPG production was dependent on IL13Rα1-signalling through STAT6 and subsequent increased expression of IL13Rα2 and signalling through AP1 as inhibiting both
pathways also completely abrogated OPG production. These results indicate that, IL13-induced OPG production is completely dependent on TGFβ through a pathway involving both IL13Rα1/STAT6 and IL13Rα2/AP1.

As a soluble protein, OPG in serum or even in the organ itself, may originate from multiple sources, not limited to the fibrotic organ itself. Therefore, it is important to have suitable 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 specific organs without interference of other organs in more details.

**PRECISION-CUT TISSUE SLICES: A MODEL TO STUDY FIBROSIS**

Fibrosis is a pathological process with multiple pathways and cell types involved. It is important to have an appropriate model to mimic this complexity. In vitro studies with cells, only provide information about these specific cells involved in a process. Therefore, due to the lack of complexity, in vitro studies cannot comprehensively explain complex mechanisms occurring during fibrosis, and thus extrapolation of in vitro drug effects to the patient is still a big challenge\textsuperscript{25}. In vivo studies with animal models provide models that have the complexity. However, several pathologic animal models cannot perfectly mimic the pathology and characteristics of the disease in humans, for example: unilateral ureteral obstruction (UUO) kidney model vs human kidney fibrosis\textsuperscript{26,27} and bleomycin-induced lung fibrosis vs IPF\textsuperscript{28}.

In order to overcome these above challenges, the ex vivo model of precision-cut tissue slices provides a model in which all different cells remain in their original environment, thus serve as an adequate model to study complex mechanisms between cells and cell-matrix during fibrogenesis\textsuperscript{29}. 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.

The model of tissue slices, however, probably cannot mimic in vivo crosstalk between organs\textsuperscript{29}, yet this feature offered us the advantage of studying fibrosis regulation in specific organs without interference of other organs. In chapter 2, we
combined *in vitro* and *in vivo* experiments with a study using murine precision-cut lung slices to gain detailed insight into OPG regulation. For example, even though RANKL-treatment led to higher OPG production in fibrotic murine lung tissue *in vivo*, RANKL-treatment of TGFβ-treated murine lung slices did not induce OPG mRNA expression. This result implies that OPG was probably produced by infiltrating (immune) cells rather than tissue-resident cells or was produced in lung tissue under the influence of a factor produced elsewhere in the body. This result also suggests that the model of murine precision-cut tissue slices is beneficial to deliver additional information that cannot be provided by other models.

**PERSPECTIVE**

OPG is used clinically as serum marker to diagnose liver fibrosis and in this thesis we have shown that OPG is clearly associated with other types of fibrosis too (lung, kidney) and that TGFβ1 is its main inducer. Moreover, OPG responded towards antifibrotic therapies of pirfenidone, nintedanib (chapter 3), and galunisertib (chapter 4). Therefore, OPG may be a potential marker to diagnose fibrosis and assess treatment efficacy in patients with lung or kidney fibrosis in clinical practice and it will be important to validate using serum OPG in relevant patient groups. This also holds true for patients with intestinal fibrosis. Even though we could not convincingly show OPG is associated with intestinal fibrosis, previous publications and the fact that in clinical practice intestinal tissue is studied in completeness with infiltrating immune cells present indicate that OPG levels in serum of patients with IBD, UC and CD may still be worth to be investigated.

OPG production is higher in fibrotic organs in experimental and human fibrosis. Our lung data and a concomitant study by us (*not in this thesis, manuscript in preparation*)\(^\text{12}\) show that OPG is highly produced by (myo)fibroblasts and in liver stimulates fibrosis by inducing TGFβ1 mRNA expression, the key player in fibrogenesis. In chapter 3 and chapter 5 we further showed that OPG production in liver and lung can be induced by two profibrotic cytokines, TGFβ and IL13, but both are completely TGFβ-dependent. These features of OPG regulation offer new insights indicating that OPG is more than merely a biomarker, OPG may also play role in fibrosis.
General Discussion

Our results thus provide evidence for further investigation of OPG as a novel target for the treatment liver fibrosis. It is important to explore more of how OPG is regulated and plays a role during fibrosis in various organs to determine whether strategies to inhibit OPG production would be a valid approach.

Despite the severity of fibrosis, there is currently no optimal treatment in particular at the end-stage of the disease. Considering the mechanism of fibrosis development, there are currently two approaches for improving fibrosis therapy: 1) slowing down fibrosis progression by inhibiting ECM production; 2) reverse fibrosis or promote resolution by inducing ECM degradation. In the context of OPG, we tried the latter approach in chapter 2 by treating with sRANKL to induce antifibrotic macrophages, but this unexpectedly taught us another possible function of RANKL in lung, namely epithelial regeneration: a mechanism that could potentially be inhibited by excessive OPG in fibrosis adding further to dysregulated repair. In addition, several studies \(^{30-32}\) have suggested that inhibiting excessive OPG production during fibrosis may facilitate apoptosis of myofibroblasts. This hypothesis, however, needs further investigation.

Several issues should be considered when using OPG as a target of therapy. Firstly, ECM production and degradation are part of the normal physiological wound healing process, thus interfering with this process can cause adverse effect \(^{33}\). Secondly, OPG is not only produced by (healing) tissues but also by healthy bone, thus inhibition of OPG production or OPG function could affect ECM regulation in bone leading to osteoporosis. Therefore, it may be also be important to design carriers that can specifically target OPG and deliver antifibrotic drugs to the affected organs or cells, thereby avoiding severe adverse effects.

Considering all results presented in this thesis, we suggest to further investigate OPG in the serum of patients with lung, kidney and intestinal fibrosis to investigate its use as a biomarker of fibrosis stage, disease progression and therapy success. Further studies should be performed to gain deeper understanding about the role of OPG in fibrosis and how OPG is regulated, in order to verify its possibility as a target for antifibrotic therapy.
REFERENCES


