Molecular mechanisms of platelet-mediated liver regeneration after partial hepatectomy

Kirschbaum, Marc

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SUMMARY AND DISCUSSION

Summary

Chapter 1 is a general introduction to this thesis, including the aims of each chapter. We provide a brief overview on liver resection, regeneration, and the role of platelets in this process. The subsequent **Chapter 2** is a letter in response to a review article published in the Journal of Hepatology, which summarized the current knowledge on the role of platelets in liver regeneration. We pointed out that platelet-mediated liver regeneration has to be investigated more intensively and described what we know and don´t know about platelet-mediated liver regeneration.

In **Chapter 3** we provide *in vitro* evidence for a potential novel mechanism of platelet-mediated liver regeneration. In our model we used HepG2 cells and freshly isolated human blood platelets. We demonstrated that platelet-mediated stimulation of HepG2 cell proliferation requires platelet internalization by hepatocytes. Following this internalization, platelets transfer their RNA to the hepatocyte and we demonstrated protein synthesis from platelet-derived mRNA by the hepatocyte. Importantly, platelet RNA contributed substantially to platelet-mediated hepatocyte proliferation. These findings suggest that transfer of platelet RNA to the hepatocyte with subsequent protein synthesis by the recipient cell is key in this process. In addition we also demonstrated *in vivo* platelet internalization by hepatocytes following a partial hepatectomy in mice. It appears plausible that functional platelet RNA transfer is also relevant for platelet-mediated liver regeneration.

In **Chapter 4** we investigated the mechanism of platelet recruitment into the liver parenchyma after partial liver resection. We observed immediately after partial liver resection a temporary platelet infiltration and platelet aggregate formation in the remnant liver. By inducing thrombocytopenia 2 hours pre or 2 hours post partial liver resection, we were able to show that the presence of platelets in the very first hours after partial liver resection is essential for the progression of liver regeneration. In mice that were rendered thrombocytopenic 2hours prior to partial liver resection liver regeneration was significantly reduced compared to mice in which platelets were present until two hours after partial liver resection. In addition we provided evidence that the temporary platelet influx into the liver parenchyma depends on VWF expression. Importantly, deficiency in VWF resulted in impaired in liver regeneration. These results indicate that the need for platelet to stimulate liver regeneration is within the first hours after partial liver resection. Furthermore, the expression of VWF by liver sinusoidal endothelial cells is key in the temporary platelet influx after partial liver resection.

Chapter 5 aimed to investigate in a prospective study, the levels of platelet related growth factors in patients undergoing a partial liver resection. Although platelet growth factors were subject of several *in vitro* studies in animal experiments, it has never been proven

that growth factors stored in platelets are actually responsible for platelet mediated liver regeneration *in vivo*, for example in patients undergoing partial hepatectomy (1). Our hypothesis was that platelet-derived growth factors are consumed during and after partial liver resection. We examined the levels of various growth factors (VEGF, HGF, FGF, PDGF, PF4, TSPI and endostatin) in plasma and in platelets and compared the levels obtained in patients undergoing partial hepatectomy to patients undergoing a Pylorus-preserving Pancreaticoduodenectomy (PPPD) and healthy controls. Our results showed no substantial differences between platelet and plasma levels of growth factors which were measured during surgery and at several post-operative days in patients undergoing partial hepatectomy, compared to patients undergoing a PPPD. These results suggest that platelet-derived growth factors are less important than generally assumed for platelet-mediated liver regeneration in humans.

In **Chapter 6** we investigated whether partial liver resection accelerates the progression of NAFLD in mice and whether treatment with vitamin E reduces this progression. We fed mice with choline-deficient L-amino acid-defined diet to induce mild steatosis and performed partial liver resection. In our studies, mild liver steatosis did not impair liver regeneration in mice. However, partial hepatectomy substantially accelerated the progression of NAFLD. In addition, we provided evidence that enhanced oxidative stress following partial liver resection contributes to the progression of NAFLD and showed that antioxidant therapy with vitamin E attenuates the progression of NAFLD. These findings may have clinical relevance as increasingly partial hepatectomies are performed in patients with steatosis.

We present in **Chapter 7** an intermezzo, investigating a novel mechanism which may partly explain the prophylactic properties of once-daily rFVIIa injections in patients with inhibitor-complicated hemophilia. First we showed that rFVIIa was dose-dependently taken up by the megakaryoblastic cell line MEG-01. Secondly, we were able to demonstrate that MEG-01 cells selectively transfer endocytosed rFVIIa to PLPs. Importantly, rFVIIa in PLPs was hemostatically active. Finally we demonstrated that rFVIIa uptake by megakaryocytes is dependent on the EPCR receptor. Taken together, rFVIIa-containing platelets appear a plausible mechanism to partly explain the efficacy of once-daily rFVIIa prophylaxis in inhibitor-complicated hemophilia.

Discussion

The notion that blood platelets are involved in the stimulation of liver regeneration has been firmly established in the last years (1-4). The therapeutic options are very limited in patients with acute liver insufficiency and the mortality and morbidity rates are high. Therefore, platelets may be an exciting new target for the development of strategies to prevent liver damage and accelerate liver regeneration in patients with liver insufficiency. Nevertheless, we do not understand the molecular mechanisms of platelet-mediated liver regeneration very well. For example, the role of platelet-derived growth factors in the process of liver regeneration has only been poorly investigated and it has still to be demonstrated that delivery of platelet-derived growth factors to the regenerating liver indeed occurs in the process of liver regeneration (1). Furthermore, platelet communication with the liver environment after partial hepatectomy has also been insufficient investigated. The role of platelet RNA in liver regeneration has yet to be investigated. By yet unexplored mechanisms, platelets contribute to the repair of damaged liver tissue by stimulating liver cell proliferation. In this thesis, multiple aspects of platelet-mediated liver regeneration have been investigated in vitro and *in vivo* as well as in a clinical study with patients undergoing a partial liver resection.

Chapter 3: The role of platelet RNA in liver regeneration

We have described a novel mechanism of platelet-mediated stimulation of liver regeneration *in vitro*, in which platelets transfer their RNA content to the recipient hepatocyte. We show in **Chapter 3**, that following platelet uptake by the hepatocytes, platelet-derived mRNA is translated and protein is synthesized by the hepatocyte itself. We have shown in a set of various experiments that both the uptake of platelets by the hepatocyte and the platelet RNA content is essential for the proliferative response of hepatocytes to platelets.

In the last few years, there is growing evidence that platelets have a complex RNA signature, including a rich ensemble of mRNAs and miRNAs which contribute to various biological and pathological processes, such as inflammation and the progression of cancer (5-9). When compared to leukocytes or other cells, the quantity of mRNA in platelets is considerably less (10, 11). Therefore platelets have developed unique mechanisms to translate mRNAs more efficiently (12, 13). Numerous platelet mRNAs have a significant extended poly(A)-tail compared to mRNA from nucleated cells, leading to a prolonged half-life time of the platelet mRNA molecule and a higher translation efficiency (12). Also the 3'-UTRs of platelet mRNAs tend to be longer and more complex when compared to mRNAs of nucleated cells (13). As a result, more protein can be synthesized from platelet mRNA than from "nucleated mRNA" and a transferred platelet mRNA molecule would be much more efficient in stimulating protein synthesis than the "host" mRNA of a nucleated cell. From this point of view it seems

logical for both parties, the platelet as well as the hepatocyte, to transfer platelet mRNA to the hepatocyte.

In addition to these unique platelet mRNA modifications, also the repertoire of platelet mRNAs and miRNAs is unique compared to that of nucleated cells (14). It seems plausible that platelets transfer platelet-specific mRNAs and miRNAs to the hepatocytes which are absent in the RNA repertoire of the recipient hepatocyte. Subsequently "foreign proteins" can be synthesized by the recipient cell from platelet mRNA. Platelet miRNAs, 19 to 24-nucleotide non-coding RNAs, can regulate several targets at once and thus can have a profound effect on the recipient cell with minimal material. The transfer of platelet miRNA to recipient cells presents a unique opportunity for platelets to have a large and broad effect on other cells. Nevertheless, the physiological relevance of platelet RNA transfer has only been poorly investigated. In the last years several different labs have investigated platelet mRNA and miRNA transfer to vascular/endothelial cells, resulting in 3 separate publications on the topic (8, 15, 16). Gidlöf et al. investigated platelet miRNA transfer and demonstrated that miRNA released from platelets during myocardial infarction can regulate ICAM1 expression in endothelial cells (15). The study by Risitano et al. has demonstrated that also platelets are capable of transferring mRNAs. They showed that labeled RNA from platelet-like particles is transferred to monocytes and endothelial cells, and this transfer alters gene expression patterns in the target cells (8). However, to our knowledge we were the first to demonstrate a functional change in the recipient cells caused by platelet RNA transfer. We demonstrated translation of platelet-derived mRNA into protein by the recipient hepatocyte, using an actin-GFP encoding RNA as a model gene. Future experiments will need to investigate which coding or regulatory RNA species are responsible for the stimulation of hepatocyte proliferation. At this moment we can only just speculate. Given the complexity and the abundance of around 8500 mRNA and 500 miRNA transcripts in platelets the pool of candidates is large (14). Nevertheless there are several indications that platelet-derived miRNAs are involved in the stimulation of liver regeneration. For example miRNA-21 is present in platelets and it has been demonstrated that increased levels of miRNA-21 stimulates the onset of liver regeneration and the cell-cycle progression of proliferating hepatocytes (17-21). Whether platelet derived miRNA-21 is the source for the elevated levels after partial liver resection or other sources are involved has to be the scope of future studies.

Until now, platelet internalization and transfer of platelet RNA to a recipient nucleated cell has been described for hepatocytes, monocytes, and endothelial cells (8, 15, 22). I think that it is plausible that this communication mechanism between platelets and nucleated cells is not limited to those three cell types and more recipient cells will be discovered. We are just beginning to understand the importance of RNAs in platelets and how the expression of mRNAs and miRNAs in platelets influence platelet functions and the processes in which platelets are involved. The limited studies performed have focused on specific miRNAs or mRNAs and used highly manipulated *in vitro* systems to investigate platelet RNA

transfer. It is likely that many transcripts are transferred and will alter the recipient cell in unclear and unknown ways. Also unclear is, whether platelets can transfer a specific group of RNAs or only their entire RNA to the recipient cell. It has been previously demonstrated that megakaryocytes selectively transfer genetic material to platelets, and it is therefore plausible that also platelets are able to selectively transfer RNA species (23). In addition, the mechanism of RNA transfer and the efficiency of platelet-derived mRNA translation into proteins remain unclear and may be variable in different recipient cells. All these question will be subject of future studies to fully understand the mechanism of platelet RNA transfer and the impact on the recipient cells.

In the last decades, also the pharmaceutical industry has become interested in RNA as a novel therapeutic target and drug. Several barriers had to be broken, because RNA is inherently unstable, potentially immunogenic, and requires in most cases a delivery vehicle for efficient transport to the target cell (24, 25). However a number of RNA-based therapeutics were investigated successfully in the last years and are currently under clinical investigation for diseases ranging from genetic disorders to HIV infection to thrombosis and bleeding disorders (25-28). It is conceivable that RNA-based therapies represent an option to develop novel strategies to prevent liver damage and accelerate liver regeneration. Therefore the platelet transcriptome and the specific role of individual platelet RNAs, including mRNAs and miRNAs, in stimulating liver regeneration has to be investigated more intensively in the next years.

Chapter 4: Platelet infiltration after liver resection or the begin of an inflammatory response?!

Several independent studies have demonstrated impaired regeneration in mice that were thrombocytopenic or treated with platelet inhibitors (29-31). The recruitment of platelets in the liver after partial liver resection seems crucial for the onset of liver regeneration. It is, however, unclear how platelets infiltrate the liver remnant. Therefore, we aimed to investigate in **Chapter 4** the mechanism of platelet recruitment into the liver parenchyma after partial liver resection in mice. In this study we show, to our knowledge for the first time, that VWF is an essential mediator in the onset of liver regeneration after partial liver resection in mice. Although our study is limited to the investigation of the mechanism behind platelet recruitment into the liver remnant after partial liver resection, we provide clear evidence that platelets infiltrating the liver remnant in the early phase after liver regeneration are responsible for platelet-mediated stimulation of hepatic regeneration. Future experiments have to investigate the molecular mechanism underlying the stimulating effect of platelets in the early phase after liver resection.
We described in chapter 3 that direct contact between platelets and hepatocytes as well as platelet internalization is essential for platelet-mediated hepatocyte proliferation. In

addition, we showed in mice immediately after performing partial liver resection, platelet translocation from the sinusoidal space to the space of Disse and uptake by the hepatocyte. Whether this observation is part of a communication mechanism between platelets and hepatocytes *in vivo* is unknown. It is also unsure if this mechanism/observation is responsible for subsequent induction of the initiation of hepatocyte proliferation and the onset of liver regeneration. *In vitro* we demonstrated that platelet uptake and transfer of platelet RNA to the hepatocyte is in part responsible for platelet-mediated hepatocyte proliferation, but it is still the question whether this mechanism also takes place *in vivo*. Similarly, it has never been conclusively demonstrated that platelet-derived growth factors stimulate liver regeneration *in vivo* (1). Perhaps both mechanisms, platelet RNA transfer and the delivery of platelet-derived growth factors to the hepatic vasculature are irrelevant *in vivo* and have only indirect effects on liver regeneration.

We have shown in this chapter that impaired liver regeneration, such as in VWF deficient mice, is associated with reduced platelet infiltration and aggregation in the vasculature of the liver remnant. Multiple studies have investigated the interaction between platelets and (liver) endothelial cells (2, 32, 33). A study by Lalor et al. has demonstrated *in vitro* that platelets stimulate liver endothelial cell activation which induces the secretion of cytokines and chemokines form the endothelial cells (33). Subsequently immune cells such as neutrophils and leukocytes are attracted and bind to the liver endothelial cells. Lalor and co-workers were able to demonstrate the functional relevance of platelet binding by experiments that show a markedly reduced amount of attracted neutrophils and leukocytes in the absence of platelets. A direct association between leukocyte recruitment and the outcome of liver regeneration is described by Selzner and coworkers. They have shown in mice, deficient in the intercellular adhesion molecule 1 (ICAM-1), reduced leukocyte recruitment after partial liver resection and also a decreased hepatocyte proliferation activity (32). Moreover, several studies have demonstrated that pro-inflammatory cytokines, secreted from neutrophils and leukocytes, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF) are essential in the onset of hepatocyte proliferation (34-36).
Based on our observations and the results from the before mentioned studies, **I postulate an alternative hypothesis to explain the role of platelets in stimulation of liver regeneration. Perhaps platelets do not stimulate liver regeneration by direct mitogenic effects but by initiating the recruitment of immune cells into the hepatic vasculature after partial liver resection**. Liver regeneration is a highly orchestrated process and is part of a local and systemic inflammatory response. I would like to take a look at the evolutionary of platelets to support my alternative hypothesis on platelet-mediated liver regeneration.

The hemocyte: platelets ancestor

Limulidae polyphemus, the horseshoe crab, is the last surviving member of the class merostomata, which includes marine spiders (37). Horseshoe crabs have only one circulating blood cell in their circulation (hemolymph) which is called the Limulus amebocyte or hemocyte (38). Probably the Limulus hemocyte has been the most intensively investigated invertebrate blood cell involved in thrombosis and hemostasis. In contrast to mammalian platelets, the hemocytes are nucleated and their size range between 10 and 20 µm (38). Like platelets, after activation the discoid shaped hemocytes change their shape, form pseudopodia, and release the content of their granules (39). Large hemocyte aggregates are generated by cross-linking of soluble proteins with receptors on the surface of hemocytes, which is comparable to the formation of a platelet thrombus (40).
It is obvious that hemocytes, as the only circulating cell in the hemolymph of invertebrates, have to be involved in more processes than only thrombosis and hemostasis. Like mammalian blood cells, hemocytes are also responsible for innate and adaptive immune responses and for the defense against pathogens (41). They can recognize pattern recognition receptors and scavenger receptors, which are crucial for host defense (42, 43). If hemocytes become activated, a subset of hemocytes are able to degranulate very rapidly, thereby releasing a battery of antimicrobial peptides, such as defensin, tachyplesins, and anti-LPS factor into the hemolymph (39, 44-46). Consequently, pathogens are killed and become phagocytosed by the hemocyte which is comparable to the function of macrophages in higher vertebrates. Hemocyte biology represents us the evolutionary link between platelet functions in thrombosis, hemostasis, and immunity. In general, coagulation is initiated by tissue damage and the exposure of collagen to the blood. In invertebrates, pathogen associated molecules, including LPS are also able to trigger coagulation. Hemocytes detect those pathogen associated molecules by their surface receptors and start aggregating with other hemocytes (44, 47). The “hemocyte clot” functions as a trap and kills the pathogens. This process clearly demonstrates functional overlap between hemostasis and immunity. It seems that both processes share the same evolutionary roots and despite more than 400 million years of evolution, mammalian platelets retain many of the immune functions which are still required in a variety of biological processes, such as sepsis, cancer and also liver regeneration (9, 48, 49).

In summary, platelets are more than cellular fragments needed for blood clotting. Platelets are immune cells! I propose that this immune cell function for platelets is also vital for liver regeneration. Our results have demonstrated that the need for platelets after partial liver resection is limited in time. Platelets have to be present in the liver remnant merely in the first two hours after liver resection in order to initiate the full regenerative response. I propose that during this period platelets initiate the pro-inflammatory reaction cascade which is needed for successful liver regeneration. Immediately after liver resection, platelets

infiltrate the hepatic vasculature of the liver remnant, bind to the liver endothelial cells and facilitate the recruitment of neutrophils and leukocytes. Neutrophils and leukocytes and associated cytokines/chemokines are crucial for the successful progression of liver regeneration (32, 35). Without this platelet-mediated attraction of neutrophils and leucocytes, liver regeneration is impaired due to the absence of an inflammatory reaction (32). This alternative explanation for the mechanism behind platelet-mediated liver regeneration deserves attention in future studies.

Chapter 5: Platelet-derived growth factors in liver regeneration: not as important as generally assumed?

In **Chapter 5** we investigated the levels of growth factors stored within platelet alpha granules in patients undergoing a partial hepatectomy or PPPD. We showed in this clinical study that growth factors levels in platelets and in plasma were comparable between the two procedures, and found no evidence for immediate or delayed consumption of platelet-derived growth factors by the regenerating liver. We thus did not find evidence for a role of platelet-derived growth factors in stimulation of liver regeneration in humans.

A single animal study has suggested a role for a specific platelet derived growth factor, serotonin, in platelet-mediated liver regeneration (2, 30, 50). However, the results of this study may also be explained by the platelet stimulating effect of serotonin instead of its mitogenic effect (Lisman, Kirschbaum 2015). Starlinger et al. published in 2014 that serotonin is a relevant inducer of liver regeneration after major partial liver resection in humans, a finding which was not confirmed by a study from our group (51, 52). Importantly, Starlinger and colleagues compared post-operative serotonin levels only to pre-operative levels and not to control patients, and were not able to conclude whether changes in platelet serotonin content related to liver regeneration or to effects of major surgery. We decided to compare growth factor levels of patients undergoing partial liver resection to a group of patients undergoing a PPPD, a major abdominal surgery of similar extent and in a patient group with cancer but without the requirement for liver regeneration.
The findings from this clinical study indicate that platelet-derived growth factors and also serotonin may be less relevant in platelet-mediated liver regeneration in humans than previously assumed. Alternative options including RNA transfer and platelet-mediated initiation of inflammation should be explored also in the human setting. We are currently performing RNA sequencing of platelets isolated from the patients described in this chapter to investigate whether hepatectomy-specific changes in the platelet transcriptome can be detected.

Chapter 6: Partial liver resection in mice and the progression of non-alcoholic fatty liver disease

The previous chapters discussed partial liver resection with focus on the mechanisms of platelet-mediated liver regeneration. In **Chapter 6** we studied liver regeneration in mice with mildly steatotic livers and unexpectedly detected that liver resection markedly accelerated the progression of non-alcoholic fatty liver disease (NAFLD). We found that mild steatosis in mice does not impair liver regeneration after performing partial liver resection but accelerates the progression of a mild steatosis towards steatohepatitis and to a more progressive inflammatory phenotype of NAFLD. In addition we were able to show that antioxidant therapy with vitamin E attenuates the progression of NAFLD.

A number of studies have suggested that oxidative stress and cytokines play an important role in the pathogenesis of NAFLD (53-55). The stimulation of inflammatory responses is one mechanism by which oxidative stress triggers the progression of mild steatosis towards NAFLD (56). Here we demonstrated that partial liver resection and consequently liver regeneration is such a trigger to stimulate the progression of NAFLD. Interestingly, and perhaps an alternative explanation for our findings, platelets have been previously shown to accelerate the progression of NAFLD (57). It would therefore be of great interest if thrombocytopenic mice show similar progression patterns of a mild hepatic steatosis towards NAFLD after performing partial liver resection. It is conceivable, that a reduced platelet count decelerates the inflammatory response after partial liver resection, which consequently lowers the level of oxidative stress and the progression of NAFLD. Several studies have indicated in patients with chronic diseases, that anti-platelet therapy such as clopidogrel, results in a significant reduction of inflammatory and oxidative stress markers (58-60).

The results in chapter 6 show that the progression of a mild liver steatosis towards NAFLD is accelerated by major partial liver resection in mice. In Western countries, NAFLD is the most common hepatic parenchymal disorder, affecting 6% to 11% of individuals in the general population (61). In the last decades partial liver resection has become a common surgical procedure and is still the only potentially curative therapy for primary and metastatic tumors of the liver and biliary tract (62-64). While extensive partial liver resection (up to 70%) can be safely performed on patients with healthy livers, the risk of such resections in patients with underlying hepatic disease, such as NAFLD are elevated (65, 66). Whether progression of NAFLD also occurs in humans is currently unknown, but if this would be the case, the clinical implications are substantial. Given the success of vitamin E in delaying NAFLD progression, administration of vitamin E during and after a liver resection to avoid resection-induced acceleration of disease would be a realistic clinical scenario.

Taken together, the studies described in this thesis give new insights on the mechanism underlying platelet-mediated stimulation of liver regeneration. Some of the concepts outlined may have direct clinical relevance, as it may result in therapeutic strategies aimed at supporting liver regeneration, for example in patients at risk for the small for size syndrome. Nevertheless, future studies aimed at validating some of the concepts in animal models or humans are required. These studies include animal model and human studies aimed at testing the relevance of platelet RNA transfer in liver regeneration, exploration of alternative strategies to promote liver regeneration by enhancing the local inflammatory response required for liver regeneration, and assessment of the effect of liver resection in humans with NAFLD on disease progression.

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