Role of metabolic overload and metabolic inflammation in the development of Nonalcoholic Steatohepatitis (NASH)

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in modern societies and constitutes an important global health problem. NAFLD is a multifactorial disease and encompasses a spectrum of pathologies that range from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH) and fibrosis. Metabolic overload from excessive intake of calorie-dense diets and associated chronic inflammation ('metabolic inflammation') are thought play a critical role in the development NASH and fibrosis. A better understanding of the complex etiology of NAFLD, including the sequence of events over time, identification of NASH-promoting disease pathways and the inflammatory cross-talk between liver and adipose tissue, is needed to develop tools for studying the disease and to come up with first treatment regimens for patients.

This chapter discusses the major findings of this thesis in the following order: 1) Chronic inflammation exists in various forms and induction of metabolic inflammation depends on the nature and intensity of the inflammatory triggers employed; 2) Interrelated inflammatory responses in metabolically active tissues and their coordination; 3) Implications for human pathophysiology: human data versus data from experimental models.

Chronic inflammation exists in various forms and induction of metabolic inflammation depends on the nature and intensity of the inflammatory triggers employed

In 2006, Hotamisligil (1) studied chronic inflammation in metabolic diseases and proposed a new term for this type of inflammation, 'metaflammation'. Metaflammation refers to inflammation which is metabolically induced or amplified. Obesity is considered to be closely associated with sub-acute chronic inflammation, which develops systemically but also on tissue level, and this low-grade inflammation is thought to promote the development of metabolic diseases. There is strong link between excess calorie intake resulting in a condition of chronic energy surplus, and the induction of chronic inflammation. Therefore, diet-evoked chronic metaflammation may constitute a crucial factor in the progression of bland liver steatosis to NASH. The nature of this chronic inflammatory component that drives this transition towards NASH has long been unclear.

In ***Chapter 3*** we compared the effects of different inflammatory triggers, among which were metabolic inflammatory triggers (dietary carbohydrate, cholesterol) and non-metabolic (classical) inflammatory triggers (IL-1β, LPS). All these inflammatory triggers exhibited a comparable NFκB-activating effect but this property appeared to be insufficient to induce NASH, even when superimposed on HFD feeding. While HFD feeding alone merely induced bland liver steatosis, non-metabolic inflammatory triggers superimposed on HFD also did not induce a human-like NASH pathology, although additional inflammatory pathways (e.g. STAT3) were activated. Surprisingly, metabolic triggers of inflammation did promote the progression towards NASH. A common denominator the effect of metabolic triggers was the additional activation of the pro-inflammatory transcriptional master regulator AP-1 and the pronounced infiltration of neutrophils, both of which could be causative factors in the development of NASH. The

data of ***Chapter 3*** thus demonstrate that chronic liver inflammation exists in different forms and that the progression from steatosis to NASH depends on a specific type of inflammation, which can be evoked with calorie-dense diets (metabolically inflammatory triggers).

A next question is related to the dynamics of diet-inducible metabolic inflammation, i.e. whether metainflammation is induced gradually up to at a stable, elevated level or whether it has a more dynamic patter. In ***Chapter 3, Chapter 4 and Chapter 6***, studies with different time periods of HFD+cholesterol feeding were performed. There is a positive association between the severity of steatosis and fibrosis and the time of treatment with these cholesterol-containing diets. However, the extent of lobular inflammation as reflected by the number of inflammatory cells aggregates in liver lobes (including neutrophils) showed a more complex pattern over time. In the early phase of the pathogenesis (e.g. t=8 w or t=12 w of diet-feeding the number of inflammatory cell aggregates strongly increased with time. However, the number of these inflammatory foci decreased once fibrosis occurred at the more late stages of the disease, that is after t=20w of diet feeding. This counter-intuitive finding could be explained by the phenomenon of inflammation resolution (reviewed in (2)) which is frequently associated with the process of wound healing. In the early phases of inflammation, infiltrating neutrophils reach peak levels and these cells are gradually replaced by other types of inflammatory cells (from the macrophages/monocytes lineage) (2). In the late phase of tissue inflammation, macrophages or monocytes take a dominant role and the numbers neutrophils may decline. Finally, tissue repair mechanisms are mounted including the deposition of collagen to stabilize damaged areas within an organ. Consistent with this, we observed a decrease of inflammatory cells aggregates and neutrophils alongside with an increase of fibrosis in those studies with prolonged HFD+cholesterol feeding, e.g. in ***Chapter 6***. Although the studies which are part of this thesis may suggest to some readers that inflammation and fibrosis develop independently during the pathogenesis of NASH, it is more likely that, during the resolution phase of inflammation, inflammatory processes and pro-fibrotic processes are tightly interconnected. Studies of this thesis as well as those reported in literature indicate that the interrelationship between diet-induced inflammation and fibrosis is very complex and that the dose of the trigger of the inflammatory insult in liver (e.g. cholesterol) is critical for processes showing high dynamics such as lobular cellular inflammation. For instance, in ***Chapter 5***, we used a much lower dose of cholesterol (0.1% w/w) than in ***Chapter 6*** (1% cholesterol, w/w), while the length of both studies was comparable (20 weeks). We found that a higher dose of cholesterol (1% w/w) did not affect the induction of microvesicular steatosis (±10%) but led to two times more macrovesicular steatosis (20% vs 10%), thus a more severe form of human-like hepatic steatosis. Furthermore, the higher dose of cholesterol decreased the level of hepatic lobular inflammation, which may be unexpected when the concept of inflammation resolution is ignored, and increased the level of hepatic fibrosis.

Although the mechanism underlying the relationship of hepatic inflammation and fibrosis during the progressive stages of NASH is unclear, our studies have demonstrated that induction of metabolic inflammation is very dynamical process and that it largely depends on the quality and quantity of inflammatory triggers employed. Since these metabolic inflammatory triggers are employed for very long periods (up to 40 weeks), the

interpretation of the results of such studies should consider that an organism mounts counter-regulatory processes to resolve inflammation and to restore organ functions. Therefore, the outcomes of such studies (and interventions) should not only be interpreted and discussed in the light of a pro-inflammatory metabolic condition but should also consider that anti-inflammatory processes and tissue-repair pathways can take place.

Interrelated inflammatory responses in metabolically active tissues and their coordination

After investigating how inflammation develops in the liver in the context of diet-induced obesity, it is intriguing to explore the effects of metabolic overload on other metabolically active organs, the more so because it has been unclear in which tissue inflammation does start and whether organ-organ cross-talk may contribute to the pathogenesis of NASH.

Tracing back the development history of biology, interestingly, in the lower organisms, such as *Drosophila melanogaster*, the metabolic and immune responses are controlled by a single organ, the fat body (reviewed in (1)). In higher organisms, this association (and hence molecular interaction) between metabolism and inflammation is preserved and also can be found in the major metabolic tissues. For example, the liver and white adipose tissue (WAT) share the similar immune effector cells, e.g. Kupffer cells and macrophages which constitutively reside in these tissues, as well as metabolic cells, such as hepatocytes and adipocytes. Many gene expression studies and microarray analyses demonstrated that the expression of genes involved in (lipid) metabolism and inflammation is adjusted extensively after long periods of HFD feeding (3, 4). However, significant effects on gene expression are likely to start early and may change over time, i.e. may be much more dynamical than assumed.

Therefore, in ***Chapter 7***, we performed a refined HFD induced time course study in which we investigated *the early dynamical events* of metabolic adaptation in a) the expanding WAT and b) the liver. This allowed us to demonstrate that the adjustment of lipid metabolism genes is related to the onset of inflammatory gene expression during diet-induced obesity, and that a relationship exists between the processes in WAT and those occurring later in time in the liver. By means of gene expression analysis and histology, we found that lipid metabolism genes in WAT were adjusted in the first place. Later in time, when the storage capacity of WAT reached its limit, inflammatory genes were expressed in WAT. When WAT inflammation started, genes involved in lipid metabolism and inflammation in the liver were adjusted, as demonstrated by analysis of gene expression time profiles of WAT and corresponding livers. We showed that the transcriptional regulators Jun, Fos, Rarα, Pparα, Stat1, Stat5, Sp1 controlled lipid metabolism and the inflammatory responses to HFD feeding in both liver and WAT, which is in line with the evolutionary relationship of both tissues described above and the view that transcriptional control mechanisms of the metabolic-inflammatory network are highly preserved (1). Because WAT inflammation precedes NAFLD, our studies support the view that inflammatory factors released by WAT contribute to the pathogenesis of NAFLD. Experimental evidence for a causal role of WAT in the development of NASH has not been provided so far and studies on the role of inflamed WAT are subject to future investigations of our group. Because inflammatory factors secreted by WAT and liver may

affect other tissues, and because chronic HFD-induced metabolic overload is unlikely to be restricted to the expanding WAT and the liver only, we investigated whether other organs (in particular those with a high blood flow and high density of capillaries) may be effected affected as well. As shown in ***Chapter 8***, kidney function deteriorated significantly after long-term (40 weeks) HFD feeding and this deterioration appears to be caused, at least partly, by chronic inflammation.

Hence, metabolic overload evoked inflammation starts locally in WAT and affects, as time proceeds, more and more tissues. This concept is visualized in the figure below (***see* Figure 1**): metabolic overload (from overnutrition, HFD) leads to expansion of adipocytes in WAT. Once further expansion is not possible anymore, low-grade inflammation develops which is characterized by local expression of inflammatory mediators (e.g. TNF-α, IL-1β, CCL2) and related inflammatory pathways, such as IKK and JNK. Factors released by WAT and/or spill-over of lipids now stored in other organs such as liver and kidney subsequently triggers a pro-inflammatory response in these tissues. Over time, this local chronic inflammation (in WAT, liver and other organs) may intensify and further induce recruitment and activation of inflammatory cells. This metaflammation, which is in first place a consequence of metabolic overload of the entire organism, is typically not noticed for decades and, without resolution, may lead to chronic metabolic disease, such as NAFLD/NASH and chronic kidney disease.

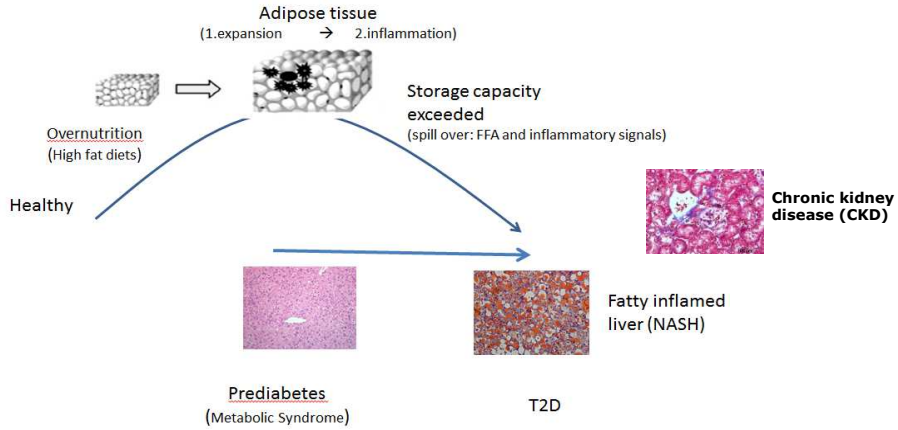

Figure 1: How different tissues are affected by metabolic overload and metabolic inflammation.

Implications for human pathophysiology: human data versus data from experimental models

'Translational research' has been discussed and debated in academia, industry and government for many decades. In 2008, a report 'Translational research: Crossing the valley of death' was published in *Nature* (5) and the author, Declan Butler, addressed the chasm that exists between biomedical research and the needs of patients. Due to the ethical issues and the inaccessibility of many tissues in humans, experimental studies are performed in models of disease in biomedical research. The most important question

related to these models is 'Does the model reflect the human situation?'. Experimental models are frequently performed in rodents which differ from humans with respect to metabolic activity as well as drug metabolism, immunological properties etc. However, when it comes to underlying mechanisms of disease, models can be very similar to human pathology, provided that the inducers of the pathogenic pathways are comparable and physiologically relevant. We and others have demonstrated that diet-inducible models of NAFLD/NASH share defining characteristics of the human pathology, and that there are many commonalities between humans and experimental models in animals. In this thesis, APOE*3 mice, APOE*3Leiden.huCETP mice or human CRP transgenic mice were used. The APOE*3 background allows to mimic human lipid metabolism and animals develop a more human-like lipoprotein profile as well as the risk factors that promote the development of NAFLD in the context of visceral obesity and dyslipidemia (6).

Furthermore, important hallmarks of human NASH (as defined in human biopsies) should be validated in experimental NAFLD/NASH models. It is remarkable that a direct comparison between the human NAFLD pathology and the experimentally induced NAFLD pathology has not been made, and that there was no generally accepted well-documented grading system for experimental NAFLD/NASH. That is why we first characterized the steatotic and inflammatory components of NAFLD/NASH in human biopsies (in ***Chapter 3***), and evaluated the same components in our experimental NASH model. This resulted in a general scoring system for experimental NAFLD/NASH for rodent models of the disease which is generic and applicable for a broad spectrum of models (***Chapter 2***). More specifically, the critical histopathological criteria for grading and scoring of macrovesicular and microvesicular steatosis, inflammatory cells aggregates, ballooning cells and fibrosis were defined by us. Application of the scoring system for examination the longitudinal development of disease stages and the efficacy of interventions, allowed us to further validate and implement the grading system.

In ***Chapter 4***, we, furthermore, tried to establish a link between specific features of histopathology and biomedical parameters relevant for the progression of NASH. We showed that a correlation exists between macrovesicular steatosis and cellular lobular inflammation, and that there is a correlation between microvesicular steatosis and NFκB activation in liver. Our findings shows that the specific forms of steatosis (macrovesicular and microvesicular) develop have a distinct developmental pattern over time and that they are associated with different cellular and molecular inflammatory events, which probably involve different pathways.

Conclusions and future perspectives

The studies presented in this thesis have generated more insight in the pathogenic mechanisms which contribute to obesity-induced NAFLD, in particular the transition from NAFL to NASH, and the development of fibrosis. The complex interactions between metabolic and inflammatory pathways in key metabolic organs were investigated in detail and, overall, our analyses support an involvement of the inflamed WAT in the pathogenesis of NAFLD/NASH which adds another layer of complexity. As outlined in detail, there are clear differences between NAFLD in humans and the pathology in experimental models and experimental models cannot mimic the full spectrum of diversity and variation that is typically observed in human patients. A part of the

complexity in humans arises from genetic and ethnic differences, but different dietary habits and different preference to nutritional components such as dietary fat (e.g. consumer oils) both in quality and quantity, as well as differences in organ-crosstalk between humans, all of which can hardly be mimicked in a single model of disease. In NASH research, the most fundamental questions, which are related to the lipid component of the disease and to the complex etiology of the disease still needs to be answered. For example, what is the actual potential of steatosis to induce toxic effects in liver?, what is the actual cascade of pathologic processes leading to inflammation and fibrosis?, and how are these processes related to the events of steatosis? Furthermore, observed phenotypic difference in human NASH patients raise the fundamental question whether the different phenotypic features that exist in humans are appropriately reflected (with respect to the specific molecular events and pathways) in the models?

Research can answers questions, but, if well conducted, will lead to more detailed questions which will also open new opportunities for better understanding and/or treatment of diseases.

Let us keep on thinking, as the honored 19th century scientists, trying to find out the explanation of the phenomenon in order to truly understand Nature we live in!

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