Extracellular histone H3

Valorization

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The social and economic relevance of academic research is becoming increasingly important for its impact on current societies. Researchers are continuously requested to critically evaluate the validity and value of their work, keeping in mind the potential translation of scientific knowledge into novel products, processes and services. Although sometimes the direct utilization of research may not be that clear, unraveling fundamental principles of physiological and pathological processes is essential for the development of future clinical applications.

The work in this thesis describes the potential use of extracellular histones as both biomarkers and therapeutic targets in several conditions associated with tissue damage. The rationale was to not only verify the presence of histones in these conditions, but also test approaches aimed to reduce their potential pathological effects.

The initial discovery that nuclear histones can be released into the extracellular space and there serve different pathological functions, has since revealed a vast array of pathological conditions associated with these extracellular proteins [1]. One strategy to neutralize the toxicity of extracellular histones is the use of heparin. Heparin exerts both anticoagulant and nonanticoagulant effects, which are summarized in **Chapter 2**. The diversity of heparin's functions has led to its use in a wide range of clinical applications. The differential functions of heparin can be separated by sophisticated production methods to render heparin formulations that are specifically tailored for certain applications. Providing a detailed and up-to-date overview of heparin's functions may offer peers and those who are new to the study of heparin new insights for the use and optimization of this versatile molecule.

The number of people on the waiting list for kidney transplantation in the Netherlands at the end of 2017 was 673, with a median waiting time of 2.3 years since start of dialysis (annual report 2017 Nederlandse Transplantatie Stichting). To reduce these numbers, there is a need to make better use of the sparse donation pool and increase the number of transplantations with (suboptimal) donation after circulatory death (DCD) kidneys. As of January 2017, all kidneys from DCD donors in the Netherlands are preserved using hypothermic machine perfusion due to increased graft and patient survival rates as compared to cold storage in the absence of perfusion [2]. However, still 33% of all registered DCD kidneys get rejected for transplantation as compared to only 10% for donation after brain death (DBD) kidneys or kidneys from living donors (numbers Nederlandse Transplantatie Stichting). This difference can likely be explained by the fact

that DCD kidneys suffer from increased and inevitable periods of ischemia compared to the other kidney groups. Therefore, understanding and optimization of machine perfusion might increase both the use and transplant success of DCD kidneys.

A major advantage of machine perfusion is that it allows the direct measurement of circulating markers and assessment of organ quality from the perfusion liquid. Many different markers have been detected in machine perfusates, but none of the individual biomolecules was shown to accurately predict short-term graft function [4]. Given that ischemia is associated with processes of cell death, we investigated the presence of extracellular histones in machine perfused DCD kidneys (**Chapter 3**). Our initial goal was to determine its use as a pre-transplantation viability marker that could potentially aid in the decision to transplant DCD kidneys. We found that histone H3 levels circulating within the machine perfusate correlated negatively with graft function after transplantation. Also, survival rates of the transplanted grafts were higher for DCD kidneys with lower circulating histone levels during preservation. The predictive value of extracellular histone H3, however, was limited and it did not perform better than other perfusate markers. As we are the first to describe the presence of extracellular histones in ischemically damaged machine perfused kidneys, additional independent studies should investigate the (additive) value of measuring histones during machine preservation. In order to become a better biomarker candidate for both monitoring and diagnostic purposes, more rapid and accurate quantification of extracellular histones is desirable. Preferably, this would entail a real-time and easily performed measurement that would help guide current clinical practice. This test would not only be convenient in the context of organ donation, but any clinical condition associated with extracellular histones.

The presence of potentially cytotoxic histones in human machine perfused kidneys prompted us to investigate this in more detail (**Chapter 4**). For this, we first set up an *ex vivo* porcine kidney perfusion model in which the extent of ischemic damage was varied between both kidneys from the same animal. The use of porcine kidneys serves a great alternative to human kidneys, do to their comparable size and function and ease of obtaining such kidneys from slaughterhouses [5]. We observed that the release of extracellular histones into the perfusate over time was both time and ischemia dependent. This confirms the results of our human study and stresses the importance of minimizing the length of ischemia in terms of histone release.

Machine perfusion also provides a platform for testing both diagnostics and therapeutics. Our research group has experience with a nonanticoagulant heparin fraction, the use of which is accompanied by a reduced risk of bleeding but a similar cytoprotective effect as unfractionated heparin [6]. This heparin formulation has already been successful in reducing histone-mediated organ damage in pre-clinical inflammation models [6], and is

now in clinical development. Therefore, we aimed to use this nonanticoagulant heparin to alter extracellular histone kinetics by reducing their levels in machine perfused kidneys.

It is important to note that in our study we perfused both the heparin treated and untreated kidney at 28°C. Our goal was therefore to ascertain tissue damage and thus, presence of perfusate histones. This gave us the opportunity to study the potential effects of the administration of heparin on histone perfusate levels. Overall levels of perfusate histone H3 were found to be similar in both groups. Interestingly, the addition of heparin at the start of perfusion did result in the gradual appearance of an alternative histone product. This product originated earlier in time for the heparin-treated kidneys as compared to the untreated counter kidneys. The exact implications of these findings remain to be investigated, as this is not the current practice in organ preservation. Perfusion at higher temperatures are often performed using autologous blood or under oxygenic conditions [7]. The consideration could therefore be made to test whether histones are also present in these different conditional setups and might be a target for neutralization by heparin.

As for now, the results from both studies seem to indicate that perfusion at low temperatures is beneficial in terms of ischemia and histone release. Both these parameters are linked to adverse outcomes in terms of short-term graft function after transplantation. Hence, careful consideration for both the choice of organ preservation method and transplantable ischemic kidneys is paramount.

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [8]. Sepsis is estimated to globally affect over 31 million people every year, leading to over 5 million deaths worldwide [9]. Both extracellular histones and microcirculatory dysfunction have been linked to disease severity and mortality in patients with sepsis [10,11]. Our initial aim in **Chapter 5** was therefore to study the potential relationship between extracellular histones and microcirculatory markers in sepsis. Due to yet undefined reasons, we did not detect plasma histones in the majority of our sepsis patients. This has been observed in other cohorts as well, which revealed up to 23% of the patients having no plasma histone H3 [10]. This observation displays the challenges in determining extracellular histones as well as the complexity and diversity of disease in sepsis. On the other hand, we did find that microcirculatory dysfunction was increased in patients who died of sepsis, as reflected by an increased degradation of the glycocalyx. Both the perfused boundary region (PBR), as an inverse indicator of glycocalyx thickness, and the plasma glycocalyx constituent syndecan-1 could discriminate between survivors and non-survivors. The assessment of PBR occurs through direct non-invasive imaging of the sublingual microcirculation. This could serve as an alternative for the use of plasma markers, which requires a longer, more invasive analysis method. The additional value of PBR for clinical monitoring the microcirculation should be investigated in larger

populations of sepsis patients or patients with any other pathological state associated with alterations in the microcirculation. Future studies should also focus on assessing the potential relationship between both histones and glycocalyx, and its role in pathological conditions.

APC is an important protein involved in the protein C anticoagulant pathway. It also possesses cytoprotective effects that include the proteolytic cleavage of extracellular histones [12], such as present in sepsis [10]. For sepsis, mortality rates remain as high as 30% since there is no adequate treatment strategy besides providing supportive therapy [9]. This number underlines that improvement of treatment of sepsis could have a great impact on society. The use of APC in sepsis, however, is complicated by its associated bleeding risk and the resulting discontinuation of the marketed Xigris-APC (an Eli-Lilly product) in 2011 [13].

For this reason, we designed novel APC variants with limited anticoagulant properties but retained or increased histone cleaving properties (**Chapter 6**). These APC variants (termed 5D-APC and 5D2A-APC) were indeed less anticoagulant than wildtype APC, but did not show an improved proteolysis of extracellular histones *in vitro*. As APC and extracellular histones can be associated with many other molecules [14], we believe that additional studies should unravel important determinants of histone cleavage by APC. A more complex *in vivo* environment could provide such factors that are important for APC's proteolytic functions, with mutations within APC possibly affecting the rate of substrate proteolysis. Our current study should thus be considered as a series of pilot experiments, providing the basis for further characterization of novel APC variant(s). Our APC variants are therefore candidate molecules for the development of an effective and safe treatment strategy in sepsis and other histone-mediated diseases.

References

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