Effect of alkaline phosphatase administration to mice with sepsis
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Summary, conclusions and perspectives

In this Ph.D. project we have explored the possibilities of using alkaline phosphatase to treat LPS-induced sepsis, by detoxifying this initiator of the disease. Sepsis is still a major problem worldwide, for which currently no effective treatment has been described that is applicable for most of the patients. LPS is a molecule from the outer membrane of Gram-negative bacteria, and it induces the production of many different inflammatory mediators after entering the body. The production of these mediators may in principle be beneficial to deal with this 'non-self' antigen in the human body, but an excess of multiple mediators in the systemic circulation may in fact abrogate this process. The pathophysiology of LPS-induced sepsis is described in chapter 1 as well as various therapeutic agents for sepsis that have been developed in recent years. They are mainly aimed at counteracting one of the LPS-induced pro-inflammatory mediators involved in the disease process. We chose a different approach of detoxification of LPS in vivo using the enzyme alkaline phosphatase (AP). We showed that AP is able to dephosphorylate the lipid A moiety of LPS, and administration of AP to mice is associated with less bioactivity of LPS.
Chapter 2 describes the *in vitro* and the *in vivo* effects of LPS and monophosphoryl lipid A (MPLA), the dephosphorylated form of the LPS. These studies were conducted in order to explore the potential toxicity of this dephosphorylated LPS-form. MPLA appeared completely non-toxic in our models. Furthermore, we showed the LPS-dephosphorylating abilities of endogenous alkaline phosphatase (AP) in different tissues in the mouse and established that MPLA is no longer a substrate for this enzyme. To examine the therapeutic activity of exogenous AP in LPS-induced sepsis, we used the placental isoform of AP (plAP) for a survival study. This study showed a clearly increased survival rate of plAP-treated mice as compared to mice that received only LPS.

To examine the effects of plAP *in vivo*, we performed further studies that are described in chapter 3. We used the placental as well as the intestinal isoform of AP, and characterised both isoforms. PlAP and ciAP were obtained as highly pure preparations. We showed that after plAP administration, plAP could be detected for a relatively long period of time in plasma (>24 hours), whereas ciAP was completely removed from the circulation within 2 hours. The mice received plAP intravenously, simultaneously with LPS/galactosamine that was injected intraperitoneally. At different time points, samples of the blood were taken and several tissues were harvested for further analysis. We showed that administration of plAP strongly reduced the LPS-induced TNFα peak levels as well as the NO levels as compared to the control mice that received no treatment. The administration of plAP also had a significant effect on the body-temperature of the septic mice: in contrast to control animals receiving only LPS, the AP-treated mice showed quite normal body temperatures. In addition, plAP also reduced the amount of ROS-producing cells in the lungs and reduced the expression of the cell adhesion molecules VCAM and PECAM in the liver. To exclude whether the observed effect of plAP could be described solely to this isoform, we also tested calf intestinal alkaline phosphatase (ciAP). We focussed on peak TNFα levels 2 hours after the injections, as this cytokine is a well-known early marker for sepsis. Using this alternative isoform (ciAP) similar results were obtained as described for plAP, despite the profound difference in plasma half-life. This indicates that the protective effects seen with plAP are not isoform specific.

Since treatment of sepsis in the clinic generally starts after the onset of the disease, we investigated the effects of plAP in another model, which resembles the clinical
situation more closely. In this study, which is described in chapter 4, the mice were injected intraperitoneally with whole *Escherichia coli* bacteria while plAP was administered after the detection of bacteria in the blood circulation. The overall findings of this study were quite similar to the studies performed using the LPS-challenge model. PlAP appeared to have a protective effect in reducing the NO levels 24 hours after the plAP injections, plAP also improved the body temperatures of the mice at t=7 hours. Furthermore, a major effect was observed on survival in this mouse model. Treatment of the mice with plAP increased the survival up to 100%, whereas the survival rate in the non-treated group was ± 60%. To study the dephosphorylating capacities of plAP with LPS as a substrate, we performed a phosphate release assay and showed that plAP was able to enzymatically release approximately 33% of the LPS-bound phosphate. These results clearly showed the *in vitro* activity of AP. Unfortunately, currently the LPS-dephosphorylating efficacy of AP *in vivo* cannot be directly measured. This represents a major challenge for this line of research in the future.

In the chapters 5 and 6 we describe the studies performed with modified plAP-preparations. As described in the introduction, the main cells involved in sepsis are the macrophages and endothelial cells that produce an excess amount of inflammatory mediators. We considered the possibility to achieve an enhanced distribution of AP to these two cell types, as at these sites LPS will rapidly accumulate *in vivo*. In these studies we showed that alkaline phosphatase, with a complex folding structure and many glycosylation sites, can be chemically modified with regard to the overall charge. We obtained a polyanionic succinylated enzyme (succ-plAP) that still retained at least some enzymatic activity, although the net phosphatase activity after succinylation appeared to be significantly attenuated. *In vitro* studies revealed an increased binding of succ-plAP to endothelial cells in the presence of LPS. In accordance with these *in vitro* observations, *in vivo* studies showed a reduced half-life of succ-plAP in plasma as compared to non-modified plAP. However, no beneficial effect was obtained using succ-plAP over non-modified plAP in the LPS/galactosamine mouse model. In contrast to the study described in chapter 3, where administration of native plAP exerted a protective effect in mice challenged with LPS and reduced plasma levels of TNFα profoundly, administration of succ-plAP in this mouse model even enhanced the
TNFα response strongly. We inferred from these observations that either succ-plAP binds LPS and presents it to the CD14/toll like receptor or that LPS binds to succ-plAP and is internalized by the scavenger receptor, thereby causing activation of the target cells.

In chapter 6 we describe that plAP can also be mannosylated. This mannosylated plAP (mann-plAP) was found to bind to macrophages in vitro and showed a reduced half-life in vivo as compared to non-modified plAP. However, similar to succ-plAP, mann-plAP potentiated the TNFα-level in vivo significantly. No enhanced TNFα expression was observed when the mice received the modified preparations succ- or mann-plAP without LPS, indicating that this is not a direct effect of the modified enzymes. Using another modified form of plAP, fucose-plAP, to deliver the LPS-dephosphorylating enzyme mainly to macrophages, the potentiation of the LPS-response by the charge- and sugar-modified AP could be confirmed.

Conclusions

Taken together, these studies describe a potential role of AP in fighting sepsis or systemic inflammatory responses initiated by LPS. PIAP was shown to dephosphorylate the substrate LPS in vitro using a phosphate-release assay as well as by employing histochemical techniques.

PIAP clearly enhanced the survival of E. coli bacteria-injected mice and LPS-challenged mice. The enhanced survival was accompanied with lower plasma levels of various markers for sepsis. In the experiments described in this thesis, nitric oxide (NO) was chosen as a late marker and TNFα as an early marker of sepsis. Other markers included tissue infiltration of reactive oxygen species (ROS), a decrease in the body-temperature and the expression of cell adhesion molecules such as VCAM and PECAM. Based on these parameters a major protective effect was found for the non-modified AP.

PIAP appeared suitable for chemical modification, as observed after succinylation, mannosylation and fucosylation of the enzyme. These chemical modifications profoundly affected the plasma disappearance rate of the enzyme. However, none
of these modifications did enhance the therapeutic efficacy of the enzyme: on the contrary, they rather worsened the condition when administered to the mice in the acute LPS-induced sepsis model.

Based on the efficacy studies in vivo, using different isoforms of AP as well as chemically modified forms of AP, all with a different organ and cellular distribution pattern, the following conclusions can be drawn.

The unmodified, naturally occurring forms of AP (placental and intestinal AP) are both quite effective in reducing the LPS response, despite their profound difference in plasma half life and organ distribution. Placental AP remains in the plasma for many hours due to the terminal sialic acid groups, which prevent uptake by various sugar recognizing receptors. In contrast, intestinal AP is rapidly cleared from the blood ($t^{1/2} \approx 8$ min.) by hepatocytes, via the asialoglycoprotein receptor that recognizes the terminal galactose moieties as present in this form of AP $^{12}$.

Modified forms of AP, targeted at either Kupffer cells (KC) or liver endothelial cells, strongly potentiated the response upon LPS, but did not elicit a response without LPS. Our studies indicate that these modified forms are able to bind LPS. This phenomenon may explain the above mentioned potentiating effect because the increased targeting of AP to sinusoidal endothelial cells (SEC) and KC at the same time may lead to an increased exposure of LPS to these cell types. This obviously will lead to a further activation of these effector cells of sepsis.

It can be concluded that the preferred site of action of AP is blood plasma (placental AP) or the surface of the hepatocytes (intestinal AP). After LPS administration, endogenous AP activity rapidly declines in plasma (chapter 2) and subsequently increases above baseline values $^3$. Simultaneously, after LPS administration, endogenous AP expression in the liver is highly upregulated on the plasma membrane of hepatocytes $^4$. The accumulation of effective preparations of AP at these very same sites may therefore mimic the normal physiological response. Exogenous AP may therefore serve to support the endogenous response to LPS in patients suffering either from an increased LPS influx from the gut or exhibit a deficient endogenous AP response.
Further perspectives

To date, treatment of sepsis is still a major problem in intensive care units. A successful pharmacological intervention is hampered by the heterogeneity of patients that develop sepsis. In many cases, sepsis is a secondary condition to different underlying diseases. Also differences between the animal models of sepsis that do not clearly reflect the complicated situation seen in humans, make it difficult to simply extrapolate results from the therapies tested in laboratory animals to the clinical situation. All of these factors have led to a situation where there is no efficient therapy available for this disease.

In this thesis we have described a role for alkaline phosphatase (AP) in providing protection from LPS-induced sepsis. We used two different mouse-models, using the same Balb/c mouse-strain. In the first model, AP treatment was initiated simultaneous with the LPS-challenge, whereas in the second model AP was administered after sepsis had been induced by E. coli bacteria. The latter situation more closely mimics the clinical condition.

The series of experiments described in this thesis strongly support a role for AP in detoxifying LPS by dephosphorylation. The significance of this enzyme in vivo as an intrinsic part of the first line of host defence against bacterial infections remains to be elucidated. Our studies did not address this issue. We rather studied the effects of exogenous AP. However, our studies may shed some light upon the biological function of this enzyme, a function that remained quite obscure despite its presence in nearly all tissues of the body. The specific localization of this enzyme in the body fits with the proposed role as a protective enzyme. AP is located as an GPI-anchored enzyme at the outside of plasma membranes of epithelial cells in the intestinal lumen, along endothelial cells in all organs, including the adrenals. It is also present within macrophages and in the CD14-positive granula of neutrophils. At all these particular sites LPS accumulates in vivo. Moreover, a clearly enhanced expression of the enzyme is found in plasma and in the liver after LPS administration.

LPS detoxification may not be the only function of the enzyme since for example its localization in bone-tissue seems not relevant for an LPS-detoxifying enzyme. Yet a specific role of AP in bone formation has already been demonstrated.
As demonstrated here, exogenous AP exerts protective effects _in vivo_ in two different experimental models of sepsis in mice. _In vitro_ we demonstrated that AP might directly attack LPS itself. This can be seen as an advantage since AP thereby affects the causative agent of sepsis rather than secondary mediators. Yet, this may also be a disadvantage, because LPS has a very short half-life in plasma and is opsonized rapidly by several proteins, lipids and membrane receptors. This causative agent of sepsis therefore is probably gone from plasma when the patients are hospitalised. AP may therefore be especially effective at the onset of sepsis or it should be used as a prophylactic therapy when the anticipated risk of sepsis is high. Patients with high risks for sepsis include: patients with a severe trauma induced by burns, patients with liver diseases or patients undergoing extensive surgical procedures, like organ transplantations. However, even when the causative agent is gone from plasma during sepsis, an LPS-detoxifying agent may still be very useful. Sepsis is often associated with malperfusion of the intestinal wall and restoration of the blood circulation may subsequently lead to reperfusion ischemia with secondary damage to the vascular wall. This may again lead to leakage of LPS or even bacterial translocation from the intestinal lumen into the blood circulation, again leading to secondary sepsis. In addition, systemic inflammatory response syndrome (SIRS) is associated with high levels of circulating cytokines and some of them (TNFα, IL-2, PAF and NO) 11-14 induce an enhanced vascular permeability, which again may lead to LPS leakage or bacterial translocation from the intestinal lumen. Many recent reports indicate that the latter process plays an important role during the progression of liver diseases associated with high levels of circulating TNFα 15. Via this mechanism AP may also be beneficial for patients with sepsis induced by Gram-positive bacteria, fungi or other pathogens, in spite of the fact that AP does not detoxify the initial causative agent during these forms of diseases. Before AP can be tested in the clinic it should be tested in different large animals that allow clinical monitoring. AP should be tested in animals that resemble humans more closely in endotoxin susceptibility and pathophysiology. The pig makes an excellent model for endotoxin research as it is relatively endotoxin sensitive and has a cardiovascular physiology that is remarkably similar to humans. As a primate model, the chimpanzee is the perfect model for sepsis-research as its endotoxin-sensitivity and immunology closely resemble that of humans. However, the limitations of the readily usage of such animals are obvious.
The route towards an effective drug against Gram-negative sepsis may still be very long and many problems will be encountered. This is particularly true for proteinaceous agents such as AP. AP may be used as part of such a therapy. Alternatively it could be used together with drugs that inhibit the effects of the most prominent cytokines. It is not expected that a complicated and a rapidly progressing disease like sepsis can be cured by one drug. However, a drug that directly affects the causative agent of sepsis could well be incorporated in a therapeutic combination. The fact that LPS is rapidly cleared from the circulation calls for a search for drugs with a similar distribution pattern as LPS. Detailed investigations on the in vivo distribution of LPS together with an evaluation of the pharmacokinetic profile of anti-LPS agents are therefore crucial. The present studies touched upon that aspect. Although the modified forms of AP were not curative, they indirectly provided some information about the “ideal” body localisation of effective LPS-detoxifying agents: the bloodstream or on the surface of the hepatic parenchymal cells.