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The design of a liver-selective form of interleukin-10

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Chapter - 8

Summary, conclusion, and future perspectives

Summary

In this thesis we have explored a hepatic targeting system for a therapeutic cytokine, IL-10, for the treatment of liver fibrosis. To date, no pharmacotherapy for this disease is available and removing the causative agent is the only effective therapy to stop or reverse liver fibrosis. Therefore, the development of effective antifibrotic therapies represents a challenge for modern hepatology.

Liver fibrosis, a chronic inflammatory disease in the liver, is a worldwide health problem. This disease is characterized by an excessive amount of matrix deposition in the liver as a net result of an imbalance between matrix synthesis and degradation. In the past decade, great advances have been made in the understanding of the cellular and molecular mechanisms underlying liver fibrogenesis. The identification of activated hepatic stellate cells (HSCs) as the major fibrogenic cell type in the injured liver,[1-6] as well as the recognition of key cytokines involved in this process,[7-12] have facilitated the design of promising new antifibrotic therapies. Most of these therapies aim at inhibiting the accumulation of activated HSCs at the sites of liver injury and preventing the deposition of extracellular matrix. Although many of these approaches are effective in experimental models of liver fibrosis, their efficacy and safety in humans is generally low.[13]

IL-10 is in recent years recognized as an antifibrotic cytokine, which downregulates HSC activities and attenuates matrix deposition, two hallmarks of fibrosis.[14-16] A potential antifibrotic activity of this cytokine has been reported in patients with HCV-associated liver fibrosis.[17,18] However, an undesired immunosuppressive effect during a prolonged therapy with this cytokine was also observed, leading to a flare-up of the viral hepatitis.[18] This is a logical consequence of pleiotropic activities of IL-10. An enhancement the delivery of IL-10 to the target organ i.e. the liver is required to increase the effectiveness of therapy and to eliminate undesired side effects. This can be achieved by designing a liver-selective delivery system for IL-10.

In this study we described the successful preparation of a modified form of IL-10. IL-10 modified with mannose 6-phosphate efficiently reached the target organ i.e the liver, in contrast to unmodified IL-10 which highly distributed to the

kidney. The conjugate was pharmacologically active *in vitro* and was also effective *in vivo*. It reduced liver fibrosis induced by bile duct ligation in rats.

Before designing a targeting system for IL-10, an essential factor to know is the pharmacokinetic profile of IL-10 during liver fibrosis. As described in **chapter 3**, the pharmacokinetic and biodistribution profile of IL-10 was studied in fibrotic rats induced by bile duct ligation and compared with normal rats. The results show that within 2 minutes, IL-10 was rapidly cleared from the circulation by the kidney. The kidney was the most predominant tissue for uptake of IL-10; 10 minutes after iv administration of IL-10 in normal rats, 30% of the injected dose accumulated in this organ. The liver was the second organ contributed to the plasma clearance of IL-10 (11% uptake after 10 min). Impairment of liver function induced by bile duct ligation highly influences several pharmacokinetic parameters and distribution of IL-10 (**chapter 3**). The area under the plasma concentration-time curve (AUC), plasma clearance (Clp), and distribution volume (Vd) of IL-10 all significantly changed compared to normal rats. In particular hepatic uptake of IL-10 in rats with liver fibrosis increased by approximately 2-fold as compared to normal rats, while kidney uptake did not change. A study of IL-10 receptor expression revealed an enhancement of this receptor in fibrotic livers both at the protein and at the mRNA levels as compared to normal livers, indicating an involvement of this receptor in the hepatic uptake of IL-10. However, although the liver contributed to the uptake of IL-10, the kidney remained a major target organ for this cytokine. Expression of IL-10 receptor in normal glomeruli may account for the biological activities of exogenous IL-10, but most likely this uptake reflects tubular uptake after glomerular filtration of this low molecular weight protein.

Since tubular uptake of IL-10 is not expected to yield biological effects, we tested beneficial effects of IL-10 at the renal site. We studied acute and more chronic effects of IL-10 in a rat model of glomerulonephritis (**chapter 4** respectively **chapter 5)**. We used anti-Thy 1 antibody to induce glomerular disease. Anti-Thy 1 nephritis is caused by the binding of Thy 1 IgG to the mesangial cell surface, resulting in complement-dependent glomerular damage.[19-23] Within hours, a destruction of mesangial cells occurred and this was accompanied by acute inflammatory reactions. As described in **chapter 4**, we demonstrate that despite the short half-life, a single iv dose of IL-10 suppresses

inflammatory processes during acute glomerulonephritis induced by anti-Thy 1 after 24 h. A possible anti-inflammatory mechanism underlying this acute effect of IL-10 may occur via inhibition of glomerular ICAM-1 expression, resulting in reduced macrophage recruitment as reflected by reduced CD14 staining (**chapter 4**). The reduced MMP-13 and proteinuria after IL-10 treatment indicates that this action of IL-10 is beneficial to preserve glomerular integrity. However, despite decrease in inflammatory parameters at the protein levels and the glomeruloprotective effects in IL-10-treated rats, an effect of IL-10 on the expression of many genes was not found in the time frame studied in this experiment.

In a subsequent study we examined the effects of IL-10 in advanced glomerulonephritis in rats (**chapter 5**). A high amount of IL-10 accumulated within one minute in the kidney and remained present in this tissue up to 60 minutes (gammacamera results), yet this accumulation probably reflects uptake in tubular cells. So, in view of the short plasma half-life, we assessed the effect of a daily administration of IL-10 on a chronic process like glomerulosclerosis. We administered IL-10 from day 4 to day 6 and sacrificed the animals at day 7. In this time frame, initiation of disease has taken place, and acute inflammation is already strongly diminished but the process of glomerulosclerosis is still rapidly ongoing. As presented in **chapter 5**, we found potent effects of IL-10 treatment in this model. IL-10 interfered with almost all of the parameters that eventually affect matrix deposition. However, the inhibition of the fibrotic process evidently did not lead to attenuation of the glomerular damage; proteinuria was not reduced by IL-10 treatment. IL-10 treatment did also not affect the mRNA levels for various examined genes in the time frame defined in this experiment.

In view of the results presented in **chapter 4** and **chapter 5**, we can conclude that IL-10 exerts acute and more chronic effects *in vivo* within the kidney. The extensive renal clearance of IL-10, however limits a clinical application of this cytokine for the treatment of chronic liver diseases like liver fibrosis. In order to enhance the effectivity of this cytokine for the treatment of liver fibrosis, this cytokine has to be delivered to this organ to overcome the normal low uptake. We designed a modified form of IL-10 with a specific ligand for the M6P/IGF-II receptor. This receptor is highly expressed on activated HSC. The coupling reaction of mannose 6-phosphate (M6P) to IL-10 was performed with

micrograms of protein. Characterization of the product is therefore a major challenge since this limited amount of protein does not allow the use of classical methods. Determination of mannose 6-phosphate groups in the conjugate was unsuccessful with classical sugar assays and with classical phosphate assays. Determination of a successful coupling with a mass spectrophotometry also failed due to the limited amount of the coupling products. Western blotting method was therefore used to characterize the conjugate because of its sensitivity and its specificity. Using this method, two bands of M6PIL-10 representing a monomeric and a dimeric conjugate were detected. Also native IL-10 was characterized by a monomeric and a dimeric form, of which only the latter is bioactive.[24] In addition to immunodetection, we also performed bioassays to assess the activity of the conjugate *in vitro*. In this *in vitro* system, we used RAW cells which constitutively express IL-10 receptors but have no M6P/IGF-II receptors and primary isolated HSC which expresses both IL-10 and M6P/IGF-II receptors. The inhibitory effect of M6PIL-10 on the release of TNF-α by LPS-stimulated RAW cells was almost equivalent to that of IL-10, indicating that pharmacological activity of IL-10 on an important inflammatory parameter is preserved in the modified-IL-10. In addition, the inhibitory effect of the conjugate on the collagen deposition and on the induction of MMP-13/TIMP-1 mRNA ratio in HSC (two crucial parameters of fibrogenesis) was also almost equivalent to that of IL-10. The effectivity of the conjugate on HSC reveals a binding capacity of the conjugate to the IL-10 receptor.

The improvement of drug concentration at the desired site of action is a major goal in designing targeted drug delivery systems. We describe in **chapter 6** respectively **chapter 7**, the preferential hepatic homing of M6PIL-10 in normal rats and in rats with liver fibrosis (BDL). By coupling M6P to IL-10, the biodistribution profile of IL-10 clearly shifted from the kidney to the liver. A study on the acute effects of M6PIL-10 in nephritic rats induced by anti-Thy 1 IgG, yielded an absence effect of the conjugate (data not shown), in contrast to unmodified IL-10. This result confirms the biodistribution data, that is, M6PIL-10 concentration is too low within the kidney to exhibit a biological effect in this model of kidney disease.

In this study, we could not identify a cell-selective delivery of the conjugate using standard double immunostaining methods. This is due to the fact that high concentrations of IL-10 can not be achieved *in vivo*. We therefore performed an *in vivo* study to identify the target receptor for M6PIL-10. Radiolabeled IL-10 and M6PIL-10 was administered to rats together with several agonists for the putative target receptors for M6PIL-10. Competitive hepatic uptake between M6PIL-10 and various receptor blocking proteins indicated that three different receptors in the liver i.e. the M6P/IGF-II, the scavenger and most likely the IL-10 receptors contributed to the hepatic uptake of our conjugate (**chapter 6**). The blockade of hepatic accumulation of [^{125}I]M6PIL-10 with either $M6P_{24}HSA$ or sucHSA clearly reflects this involvement of M6P/IGF-II and scavenger receptors. In contrast, preadministration of either $M6P_{24}HSA$ or sucHSA did not influence the hepatic uptake of [^{125}I]IL-10. Although $M6P_{24}HSA$ and sucHSA inhibited M6PIL-10 uptake, there was no additive effect when both proteins were combined. Since IL-10 receptors are present in the liver (**chapter 3**) and since our experiments with RAW cells indicate that M6PIL-10 also binds to this receptor (**chapter 6**), IL-10 receptor is most likely also involved in the hepatic disposition of this conjugate. Blocking the IL-10 receptor with an excess amount of IL-10 is needed to prove this hypothesis, but these high sustained concentrations can not be achieved *in vivo* due to the rapid clearance of IL-10.

As three different receptors are responsible for the hepatic uptake of the conjugate, a complex interaction between M6PIL-10 and the target cell is anticipated. To illustrate this complexity, a proposed model is depicted in chapter 6 (fig.8). The accessory receptors depicted in this figure are the M6P/IGF-II receptor and the scavenger receptor.

The pharmacological activities of M6PIL-10 that we demonstrated in this thesis indicate that M6PIL-10 binds to IL-10 receptors. Even an improvement of the therapeutic effectiveness of the conjugate in BDL-1 rats, as compared to unmodified IL-10 was noted on some parameters (**chapter 7**). This can be explained by an enhanced concentration at the target cell (chapter 7, fig.1). Since IL-10 receptor density is low, even after its upregulation during disease[24,25], a sustained increased concentration of IL-10 around the target cell is important for the biological activity. In the *in vivo* studies, liver fibrosis in rats was induced by

ligation of the bile duct. This procedure leads to inflammatory responses in the liver and proliferation of bile ducts.[26-28] These processes are subsequently followed by proliferation of HSC and portal fibroblasts and a large production of matrix components by this cell type. Positive stainings for α-SMA, desmin/GFAP and type III collagen around the portal areas reveal this fibrogenesis process, whereas induction of iNOS, IL-10 receptor, and DAB staining in the portal area and around the necrotic area reveals the hepatic inflammatory activity. Induction of the mRNA levels for TNF-α, MMP-13, ICAM-1, procollagen type I, TGFβ-1, α-SMA, and TIMP-1 compared to normal liver also clearly reflect the fibrogenesis in the first week after ligation. Treatment with either IL-10 or M6PIL-10 was given for 3 consecutive days from day 4 to day 6 after induction of the disease. In this time frame, both IL-10 and M6PIL-10 suppressed liver inflammation and fibrosis reflected a reduction in several parameters examined. IL-10 and M6PIL-10 significantly reduced DAB positive staining in the portal area and M6PIL-10 showed slight superior effect than IL-10 on some of the parameters. In particular, the superior effect of M6PIL-10 on the reduction of collagen deposition as compared to IL-10 (chapter 7, fig.5) might indicate that a liver-selective delivery of IL-10 improves the therapeutic efficiency of this cytokine. However, additional dose-response studies are required to address this issue. No significant differences on the mRNA levels for various genes between untreated and treated groups were seen. The data for IL-10 and M6PIL-10 were similar in this respect.

Conclusion

The studies presented in this thesis describe all activities in designing a targeting system for a therapeutic cytokine, from the documentation of the potential effects of this cytokine in various diseases, the synthesis of a cell-selective form of IL-10, the pharmacokinetic and organ distribution profile including receptor interactions *in vivo*, to the testing of the targeted cytokine in diseased animals. Cytokine-based research is revolutionizing the treatment of several diseases including liver fibrosis. However, the classical problem with the clinical use of cytokines is that the administration of these proteins must be by injection, either intravenously or subcutaneously. In addition, cytokines often have short plasma half-lives, due to rapid renal excretion and proteolytic degradation in plasma, whereas their activity on cells is usually most optimal

after long exposure times. As a consequence, they have to be administered frequently, which makes them very expensive as a drug. The clinical use of many cytokines is also limited because of their pleiotropism. Because their receptors are expressed in a number of cells and tissues, systemic application of the cytokines can easily result in undesired effects. The strategy to target the cytokine to its receptor ensures a rapid delivery of the cytokine to the site of action, while avoiding non-target sites and thereby may eliminate undesired effects mostly observed during long-term cytokine therapies. This can be achieved by coupling a receptor-selective ligand to the cytokine. Using mannose 6-phosphate (M6P), we selectively delivered a potent antifibrotic cytokine, IL-10, to the liver. The conjugate is pharmacologically active *in vitro* and in an animal model of liver fibrosis *in vivo*.

A short-term daily single dose of M6PIL-10 during liver fibrosis induced by bile duct ligation revealed potent effects of the conjugate in controlling the inflammatory and the fibrotic processes. IL-10 and its modified form influenced several parameters associated with the fibrotic process, and eventually affected the deposition of fibrous tissue in the liver. We even found some beneficial effects of M6PIL-10 in controlling these processes as compared to unmodified IL-10. This result indicates that an efficient delivery of IL-10 to the target cell in a diseased organ is possible after its modification with a HSC-selective ligand.

Future perspectives

Many types of endogenous proteins, such as cytokines, are produced and released to act locally in the body, thus exhibiting the desired activity among their multiple functions. However, the exogenous administration of such proteins into the systemic circulation may result in serious side effects due to their low targeting efficiencies to the site of action. This could be one of the stumbling blocks that hinder their clinical application. A selective delivery of the proteins to the site of action is therefore necessary to overcome such problems. As described in this thesis, IL-10 exerts potent antifibrotic effects both *in vitro* and in a model of liver fibrosis in rats. The hepatic targeting system for IL-10 using a HSC-selective ligand, M6P, shows great potential for future therapeutic applications for the treatment of liver fibrosis. Since up till now, no antifibrotic drugs are approved

due to safety problems, a potent antifibrotic effect of a liver-selective form of IL-10 during liver fibrosis offers great new opportunities.

In addition to this liver-selective form of IL-10, another interesting strategy to achieve a local sustained high level of IL-10 during hepatic fibrogenesis is now ongoing in animal experimentations.[29-31] IL-10 gene transfer has recently been proposed to have potential therapeutic applications since local gene expression can yield high local protein level for a prolonged period of time (up to months). This sustained IL-10 expression within the liver may also be a promising strategy for the treatment of chronic liver diseases like liver fibrosis. In particular liver fibrosis, where systemic effects of drugs often oppose the therapeutic effects within the liver, may benefit from such a cell-selective delivery of drugs.

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