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

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Chapter - 6

Chemically modified IL-10 with a liver-specific ligand: a new strategy for the treatment of liver fibrosis with a therapeutic cytokine

submitted

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Abstract

IL-10 is a pleiotropic cytokine with anti-inflammatory and antifibrotic actions. Recently, IL-10 therapy in patients with hepatitis C virus (HCV)-associated liver fibrosis seemed promising, but a flare-up of viral levels in serum was noted due to the immunosuppressive effect of this cytokine. This undesired effect is a logical consequence of the pleiotropic properties of IL-10. To circumvent this, we aimed to deliver this cytokine to its site of action i.e. hepatic stellate cells (HSCs) in the fibrotic liver. We modified IL-10 with mannose 6-phosphate (M6P), a specific ligand for the M6P/IGF-II receptor expressed on HSCs. Using Western blotting methods, successful conjugation of M6P to IL-10 (M6PIL-10) was confirmed. Addition of M6PIL-10 to LPS-activated RAW 264.7 cell cultures resulted in a dose-dependent inhibition of TNF-α release, almost equivalent to that of native IL-10. In addition, both the conjugate and IL-10 enhanced the mRNA ratio of MMP-13/TIMP-1 and reduced type I collagen deposition *in vitro*. Biodistribution studies of radiolabeled-M6PIL-10 and -IL-10 in normal rats and in rats with liver fibrosis revealed a rapid hepatic disposition of M6PIL-10, while IL-10 accumulated mostly in the kidney. Identification of the hepatic receptors that bound to M6PIL-10 *in vivo* using several competitive receptor blockers indicated binding to M6P-recognizing receptors, to scavenger- and most likely also to IL-10 receptors. We conclude that IL-10 modified with M6P retains the biological activity of IL-10 *in vitro*. *In vivo*, M6PIL-10 is efficiently targeted to the liver in contrast to IL-10. The strategy to target IL-10 to the intrahepatic receptors ensures a rapid delivery of IL-10 to the liver, while avoiding non-target sites and thereby may eliminate the undesired effects as observed during long-term IL-10 therapy in patients with liver fibrosis.

Introduction

Interleukin-10 is a potent cytokine with multiple actions in acute and chronic inflammatory processes. The receptors of this cytokine are constitutively expressed in nearly all tissues leading to many biological effects. Most of these effects are anti-inflammatory and lead to an immunosuppressive state. Antifibrogenic activities also gained attention in recent years[1-4] but the pleiotropic activities hamper the therapeutic applications of IL-10 as an antifibrotic agent. This may in fact be true for many, if not all, cytokines, that is in case that they are administered via the general circulation. All cytokines have multiple effects *in vivo* and have a short plasma half-life due to uptake in many organs. Previous pharmacokinetic studies revealed that IL-10 is rapidly cleared by the kidney, and handled to a lesser extent to the liver.[5] Based on these drawbacks, it can be deduced that a therapy with IL-10 in patients with liver disease may not yield an optimal clinical outcome. Thus, in order to increase the therapeutic efficiency of IL-10, prolongation of the plasma half-life, prevention of the renal clearance and/or an increase of uptake at the target site must be achieved. The latter strategy also separates the antifibrotic and immune-suppressive effects. Both these effects may oppose each other.

Many studies in recent years indicate that IL-10 might function as a potent antifibrotic cytokine through extracellular matrix modulation both via downregulation of type I collagen and upregulation of matrix metalloproteinase-13 (MMP-13) expression.[1,2,6-9] However, clinical studies with IL-10 attempting to reduce liver fibrosis in patients, failed due to the effects of IL-10 on immune cells, leading to an immune-compromised situation.[3] This resulted in a flare-up of underlying viral infections in patients.

The rapid clearance of IL-10 by the kidney leads to a short plasma half-life.[5] This may be avoided either by modifying the molecular weight of the cytokine (e.g. pegylation technique) or by modifying the charge, since the passage of proteins across the glomerular filtration barrier is regulated by their size and charge.[10,11] Basement membrane pores in the kidney are coated by fixed anionic charges, like glycosaminoglycans (GAGs), capable of restricting the passage of negatively charged proteins and increasing the negative charge of small proteins will therefore attenuate the renal clearance.[11] However, an even more powerful

way to improve the effectiveness of small proteins is by enhancing the accumulation at the target site based on the specific delivery to target receptors which are present at these target sites.[12,13]

Mannose 6-phosphate (M6P) is a negatively charged molecule which is also a specific ligand for the mannose 6-phosphate/insuline-like growth factor II (IGF-II) receptor.[14] This receptor is highly upregulated on fibroblast-like cells such as hepatic stellate cells (HSCs) during liver fibrosis.[15-18] Coupling of M6P to albumin caused a cell-selective uptake of this modified albumin in HSCs during fibrosis presumably mediated by the M6P/IGF-II receptor.[14] Incorporation of M6P to IL-10 (M6PIL-10) may therefore change IL-10's characteristics, including the charge, and may thus enhance the delivery of IL-10 to fibroblast-like cells via a receptor-mediated disposition. This may improve the therapeutic efficacy of this potent cytokine for diseases like liver fibrosis and it may even suppress the undesired effects during therapy, such as the immune suppressive effects.

In the present study we describe a modified IL-10 which does retain the biological activity of IL-10 *in vitro* and has a profound improved organ and cellular distribution pattern *in vivo.* This may open new ways to use this cytokine in novel therapeutic approaches for the treatment of liver fibrosis.

Materials and methods

Cells and cell line

Mouse embryonic macrophage cell line (RAW 264.7)

RAW 264.7 cells (ATCC, number TIB-71) were cultured in a humidified atmosphere at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Belgium) containing 10% fetal bovine serum (FBS, Biowhittaker, Belgium), 60 μg/mL of gentamicin (Gibco, Invitrogen), 2 mM of L-glutamin (Gibco, Invitrogen) and 0.48 M of L-arginine (Sigma, USA). The cells until passage number 20 were used for experiments.

Primary isolated rat hepatic stellate cells (HSCs)

HSCs were isolated from livers of Wistar rats (> 400 g; obtained from Harlan, Zeist, The Netherlands). Briefly, normal livers were digested with pronase (Merck, Germany), collagenase P (Boehringer, Germany) and DNase (Boehringer)

by in situ perfusion to isolate HSCs. Isolated cells were subsequently subjected to Nycodenz (Nyegaard, Norway) gradient ultracentrifugation to collect a pure fraction of HSCs. The purity of isolated HSCs was examined by phase contrast microscopy and the viability by trypan blue exclusion. Purity of HSC suspensions always exceeded 95%. These cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin/100 µg/mL streptomycin (Sigma, USA). After 7 days, the cells showed an activated phenotype as detected microscopically[19] and were used for experiments.

Animals and the experimental model of liver fibrosis

Specific pathogen-free male Wistar rats were used in this study. The rats received a standard diet and were housed under standard laboratory conditions. To induce liver fibrosis, rats weighing 250 – 300 g were subjected to bile duct ligation (BDL) as described by Kountouras *et al*[20], under anaesthesia with 40% O_2:60% N_2O combined with 0.5% Isoflurane (Abbot Laboratories Ltd, UK). Three weeks after the ligation (BDL-3) when extensive fibrosis is established, rats were used for further experiments. Parallel to this group, a control group did not receive this chirurgical procedure.

The studies as presented were approved by the Local Committee for Care and Use of Laboratory Animals and were performed according to strict governmental and international guidelines on animal experimentation.

Synthesis of M6P-modified IL-10 (M6PIL-10)

Activated M6P was synthesized (fig.1) according to Roche AC, *et al.*[16] The products of each step were checked with thin layer chromatography (TLC). The activated M6P was subsequently coupled to 10 µg of recombinant human IL-10 (activity of about 5.10^5units.mg^{-1}, Peprotech EC Ltd., UK). The molar ratio (M6P:IL-10) of the reaction mixture was 400:1. The reaction was carried out in sodium carbonate buffer pH 9.5 at room temperature for 1 h and then at 4°C for 24 h. Free M6P as well as all buffer components were removed by filtration with Nanosep® centrifugal devices (10 kDa, Omega, Pall corp., Michigan). The protein content of the product was determined with NanoDrop® (ND-1000 UV-Vis spectrophotometer, NanoDrop Technology,USA) with a mini Bradford method according to the manufacturer's instructions. The conjugate was further

characterized by Western blot analysis with a rabbit polyclonal IL-10 antibody (1:200, Santa Cruz Biotechnology, USA) as a primary antibody and horseradish

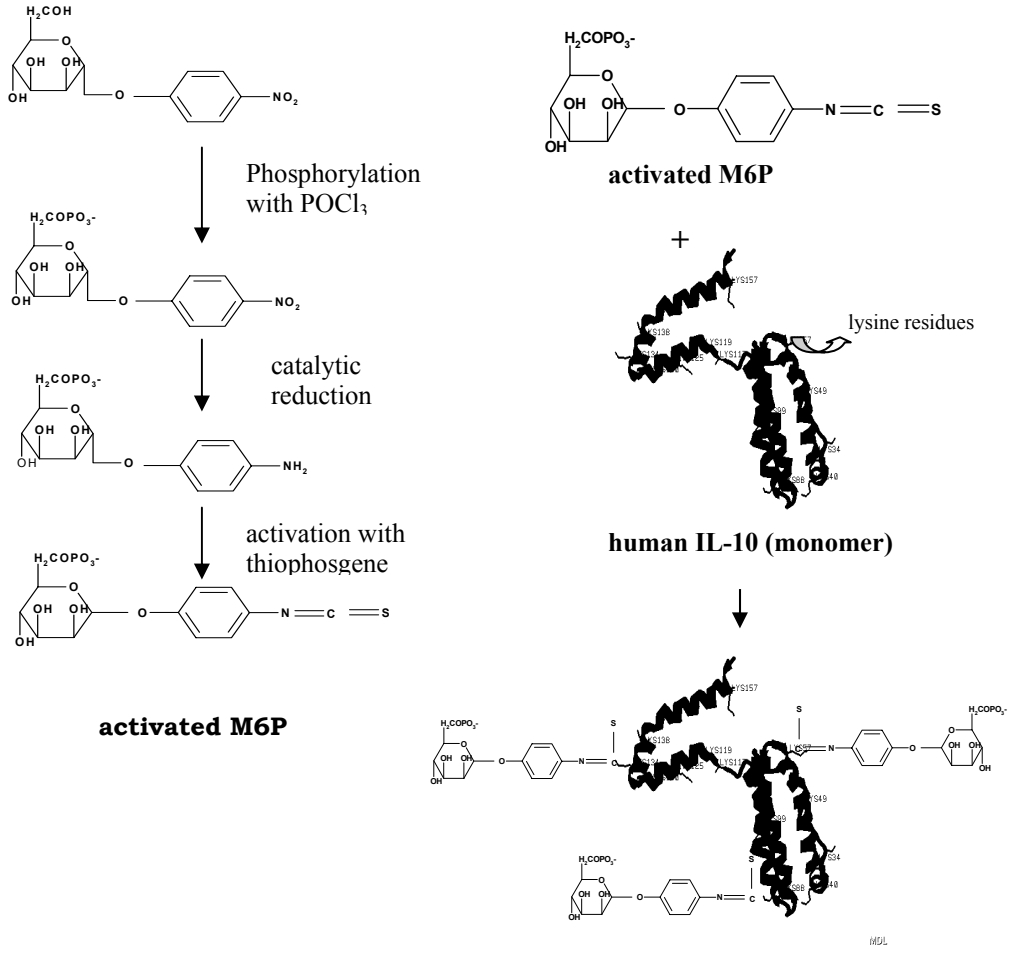

Fig.1. Scheme of reaction steps of M6P synthesis and its activation, and coupling to IL-10. Activated M6P was coupled to recombinant human IL-10 in a molar ratio of 400:1.

peroxidase-conjugated goat polyclonal anti-rabbit IgG (1:2000, DAKO) as a secondary antibody. The secondary antibody was visualized with DAB (3.3'-diaminobenzidine, Sigma).

In vitro studies

Anti-inflammatory effects of IL-10 and M6PIL-10

RAW 264.7 cells (1.5×10^5 cells/well) were cultured overnight in 96 wells plates (Costar®, Corning Inc., USA). First, the cells were preconditioned for 1 h at 37°C in 0.2 mL of FBS-free medium. After this, cells were preincubated for 30 minutes with various concentrations of IL-10 or M6PIL-10 (500;250;100;50;25;12.5;6.25;3.125;1.56;0.78 ng/mL) in 0.1 mL FBS-free medium containing 0.5% normal mouse serum. At t = 0 h, 25 ng/mL of LPS (*E.coli* K12, D31m4(Re), List Biological Laboratories, Inc., USA) was added to the wells. Control cells were incubated with the same concentration of LPS. At t = 6 h, TNF-α levels in the culture media were determined with a sandwich TNF-α ELISA kit (BD PharMingen, USA). Assays were performed with two batches of M6PIL-10 and in triplicate for each batch.

Antifibrotic effects of IL-10 and M6PIL-10

To investigate the antifibrotic effects of IL-10 or M6PIL-10, HSCs at 6 days after isolation were cultured in 6 wells plates ($2.5\text{x}10^5$ cells/well) for 24 h in the conditions as described above. Prior to the experiments, the cells were preconditioned in FBS-free medium for 2 h. Subsequently, cells were preincubated with either 0.5 mL of IL-10 or M6PIL-10 (12.5 and 25 ng/mL) for 1 h. Control cells were preincubated with FBS-free medium. At t = 0 h, the cells were co-incubated with TGFβ-1 (5 ng/mL, Roche Diagnoctics corp.,USA) for 24 h. Then, mRNA was isolated from HSCs with a mini column according to the manufacturer's instruction (RNeasy® mini kit, Qiagen Sciencer, USA). mRNA concentrations were determined with NanoDrop®. Integrity of mRNA samples was checked by electrophoresis on a 2% agarose gel and the absence of cDNA in the samples was verified by performing a PCR on the mRNA of the samples using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) while omitting the reverse-transcriptase step.

The effect of IL-10 and M6PIL-10 on the mRNA levels of MMP-13 and TIMP-1

mRNA levels of MMP-13 and TIMP-1 were determined with reverse transcriptase-PCR techniques. An RT reaction (Sensiscript RT kit, Qiagen Benelux

B.V., Netherlands) was performed with 25 ng of total RNA in 20 µL of reaction volume to obtain 20 µL of cDNA. cDNA was examined with following primers: MMP-13 primers (5-AGGCCTTCAGAAAAGCCTTC-3 and 5-GAGCTGCTTGTCCAGGTTTC-3), TIMP-1 primers (5-ACAGCTTTCTGCAACTCG-3 and 5-CTATAGGTCTTTACGAAGGCC-3) and GAPDH primers (5-CCATCACCATCTTCCAGGAG-3 and 5-CCTGCTTCACCACCTTCTTG-3). PCR for MMP-13 was performed in a volume of 25 µL containing 1.5 µL of cDNA, 50 mM $MgCl_2$, 2.5 µL of 10x Taq DNA polymerase buffer , 10 mM dNTPs, 0.5 unit of Taq DNA polymerase (Eurogenetec, Belgium) and 50 pmol/µL of each primer. PCR was performed with 30 cycles and an annealing temperature of 56^0C for 30 seconds, while GAPDH PCR was performed 26 cycles and an annealing temperature of 58^0C for 30 seconds. The band intensity of the PCR products was quantified with a computer program ImageJ (NIH Image software, USA). TIMP-1 gene was quantified by real-time PCR techniques with a high-throughput real-time PCR system (ABI 7900HT sequence Detection System, Applied Biosystems, CA, USA). The PCR mixture (20µL) contained 1.25 µL cDNA, primers (1 µM concentration of each primer), and 2x SyberGreen master mix (Applied Biosystem). An initial denaturing step at 95°C for 10 minutes was followed by 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C (a measuring step) for 40 seconds. Each measurement was performed in three replicates. Data were analysed with the SDS software 2.1 (Applied Biosystems). The gene expressions were normalized to the signal of the house keeping gene GAPDH.

The effect of IL-10 and M6PIL-10 on the protein level of type I collagen

To study the effects of M6PIL-10 on type I collagen synthesis, HSCs were cultured (5 x 10^3 cells/well) in 8-well coverslip chamber slides (Lab-Teks, Nunc, Rochester, USA) for 24 h. Prior to the experiments, cells were preconditioned in FBS-free medium for 2 h. After this, cells were incubated with either 0.1 mL of IL-10 or M6PIL-10 (12.5 ng/mL) for 24 h. Control cells were incubated with FBS-free medium in parallel wells. Type I collagen synthesis was examined with immunostaining methods using a goat polyclonal antibody against type I collagen.

Immunostaining for type I collagen

Prior to the immunostaining protocol, cells were fixed in acetone:methanol (1:1) for 15 minutes at RT. Immunostaining of type I collagen was performed with a standard method using a goat polyclonal anti-collagen I antibody (1:40) as a primary antibody (Southern Biotechnology Associates Inc., USA) and a horseradish peroxidase-conjugated rabbit polyclonal anti-goat immunoglobulin (RAGPO 1:20, DAKO) as a secondary antibody. The immunoreaction was visualized with 3-amino-9-ethylcarbazole (AEC, Sigma, USA).

In vivo studies

Radiolabeling of IL-10 and M6PIL-10

The ^{125}I-labeling of IL-10 and M6PIL-10 was performed with a standard chloramine-T method according to Greenwood FC.[21] Prior to each experiment, free ^{125}I was removed by gel filtration using a PD-10 column (Amersham Pharmacia Biotech), by eluting with phosphate buffer (0.2 M, pH 7.4) to obtain a dosing preparation with less than 5% free ^{125}I.

Biodistribution and intra-hepatic uptake of [^{125}I]IL-10 or [^{125}I]M6PIL-10 in rats

The biodistribution study of [^{125}I]IL-10 or [^{125}I]M6PIL-10 was carried out in normal and BDL-3 rats (N = 4). The hepatic uptake of [^{125}I]IL-10 or [^{125}I]M6PIL-10 in the liver was assessed only in BDL-3 rats since M6P/IGF-II receptor expression is enhanced in fibrotic livers. In the latter study, 5 minutes prior to the iv injection of radiolabeled-proteins, 5 mg/kg of either succinylated human serum albumin (sucHSA, N = 6), $M6P_{24}$HSA (N = 4) or a mixture of sucHSA and $M6P_{24}$HSA (N = 4) was administered intravenously. Control animals received either vehicle (PBS) or HSA (5 mg/kg, N = 6) before injection of radiolabeled-proteins. The biodistribution and intra-hepatic distribution of [^{125}I]IL-10 and [^{125}I]M6PIL-10 was determined 10 minutes after administration of a tracer amount of radiolabeled proteins. The total radioactivity in each tissue was measured with a gamma counter (Riastar Gamma Counting System, Packard Instrument Company, Meriden, CT) and then corrected for blood-derived radioactivity in that tissue. This correction factor was calculated from biodistribution studies with

HSA, a protein that remains in the circulation during the time frame of this experiment.

Statistical analysis

Data were presented as mean ± SD. All data were subjected to an unpaired, two-tailed distribution student t-test. Differences were considered significant at $p<0.05$.

Results

Characterization of M6PIL-10

Western blotting was performed to assess the coupling of M6P to IL-10 (fig.2). Two bands of M6PIL-10 products were detected. The molecular weight (MW) of the bands, ± 20 kDa and ± 40 kDa respectively corresponded to monomeric and dimeric forms of the conjugate. IL-10 was also characterized by two bands representing a monomeric and a homodimeric forms.

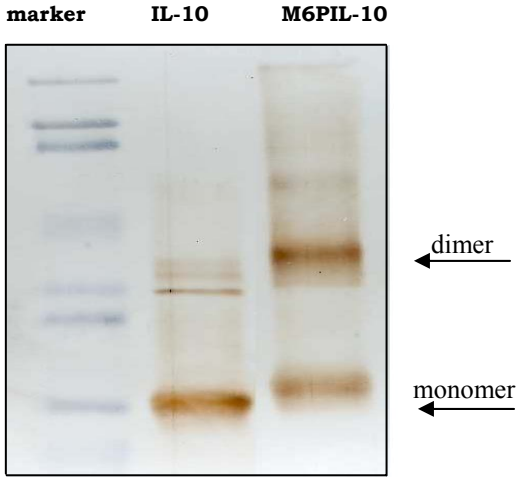

Fig.2. Western blotting of M6PIL-10. Note the increase in molecular weight of both monomeric (± 20 kDa) and homodimeric (± 40 kDa) forms of M6PIL-10 as compared to the monomeric and homodimeric forms of IL-10 (18.5 kDa and 37 kDa, respectively).

TNF-α release by LPS-stimulated RAW 264.7 cells is inhibited by IL-10 and M6PIL-10 in a dose-dependent manner

To study the anti-inflammatory effects of IL-10 and M6PIL-10, we used LPS-stimulated RAW 264.7 cells. In this study we observed a strong TNF-α

response induced by LPS and this response was inhibited by IL-10. Also, M6PIL-10 attenuated LPS-induced RAW cell activation. Both IL-10 and M6PIL-10 reduced TNF-α response in a dose-dependent manner and a maximal inhibition of 60% was shown for both proteins at the highest dose used in this experiment (fig.3).

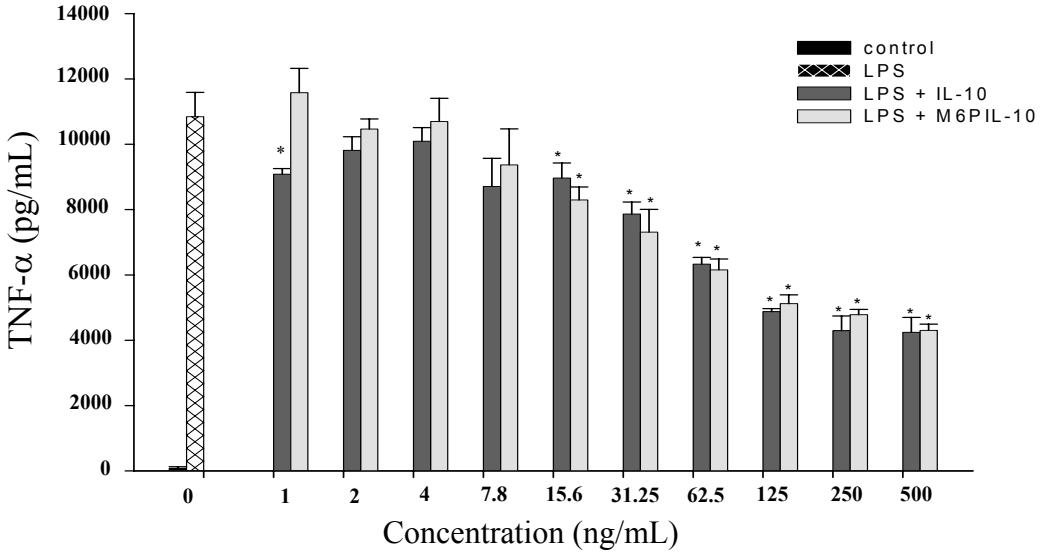

Fig.3. Effect of IL-10 or M6PIL-10 on RAW 264.7 cells. Cells were preincubated with various concentrations of IL-10 or M6PIL-10 30 min prior to the LPS challenge (25 ng/mL). Four hours after LPS addition, TNF-α levels in media were measured with a capture ELISA method. M6PIL-10 showed an equivalent inhibitory effect in TNF-α release as IL-10 (60% inhibition at a concentration of 500 ng/mL). Values are expressed as mean ± SD. *p<0.05 compared to LPS-stimulated control.

The effects of IL-10 and M6PIL-10 on the gene expression of MMP-13 and TIMP-1

The antifibrotic effect of IL-10 and M6PIL-10 was subsequently studied in cultures of primary HSCs. We used 7 days cultured cells because these cells represent activated stellate cells as reflected by alpha smooth muscle actin staining (data not shown). These particular cells are the major producer of collagen during liver fibrosis and therefore the major target cells for antifibrotic therapies. We studied the expression of two key parameters which are important in the fibrogenesis: matrix degradation and synthesis. MMP-13, one of the most important ECM-degrading enzymes, has been detected in HSCs.[22,23] TIMP-1 as

one of the major MMPs inhibitors, is highly upregulated during fibrogenesis and is capable to block MMP-13 activity.[23] The expression of type I collagen represents the matrix deposition. In this study, we used TGFβ-1, a fibrogenic cytokine, to induce the fibrogenesis to mimic the situation *in vivo*.[23,24] We investigated the effect of IL-10 and M6PIL-10 on mRNA expression for MMP-13 and TIMP-1 in the presence of TGFβ-1. The results are shown in figure 4 and 5. As displayed in figure 4, we found an effect of IL-10 and M6PIL-10 on the mRNA ratio of MMP-13/TIMP-1. Quantification of PCR products with ImageJ software yielded an upregulation by 1.8-fold and 2.4-fold for IL-10 and 1.6-fold and 1.8-fold for M6PIL-10 at low and higher concentrations, respectively, as compared to TGFβ-1 alone.

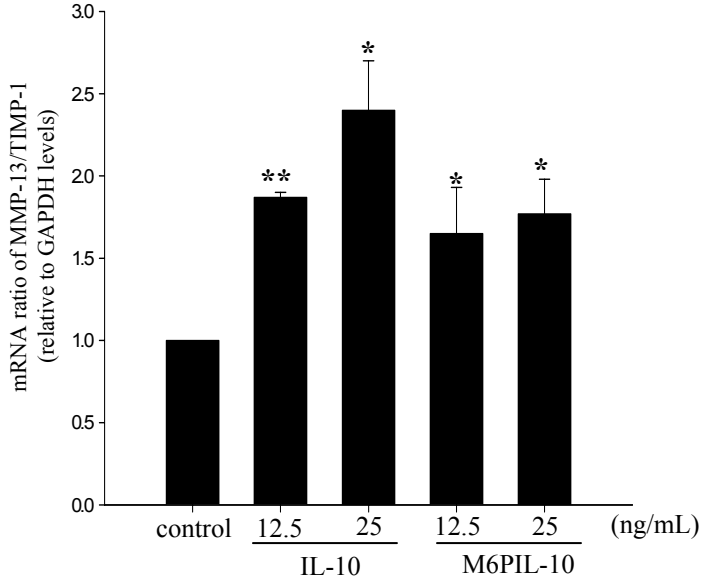

Fig.4. Effect of IL-10 or M6PIL-10 on the mRNA levels of MMP-13 and TIMP-1 in primary isolated rat HSCs. mRNA ratio of MMP-13 to TIMP-1 was calculated with the mRNA data on MMP-13 and TIMP-1 after normalizing to the mRNA levels of GAPDH. Addition of IL-10 or M6PIL-10 in the presence of TGFβ-1 significantly enhanced mRNA ratio of MMP-13/TIMP-1 as compared to TGFβ-1 only (control). *p<0.05; **p<0.01.

Immunostaining of type I collagen on HSCs

We also tested whether IL-10 and M6PIL-10 influenced collagen deposition *in vitro*. This deposition is the net result of matrix production and MMP-induced matrix degrading activity. Therefore we examined type I collagen deposition in

cultures of HSC treated with IL-10 or M6PIL-10 with immunostaining methods. We observed type I collagen deposition in HSCs cultures at day-7 and this deposition was diminished by IL-10 (figure 5). M6PIL-10 at same concentration as IL-10 also reduced type I collagen deposition clearly.

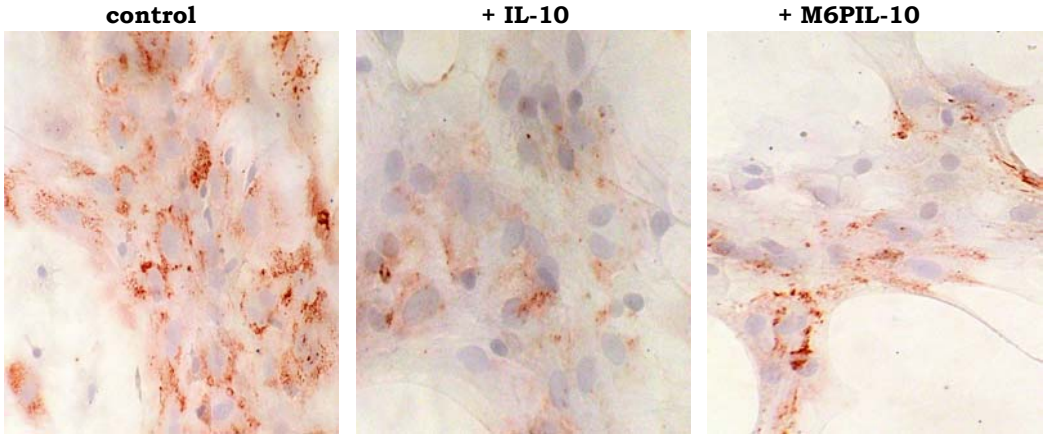

Fig.5. Type I collagen deposition in primary isolated rat HSCs as detected with immunostaining methods. Type I collagen is present on HSCs after 7 days in culture. Deposition of collagen is attenuated by IL-10 or M6PIL-10 at the same concentration (12.5 ng/mL). Original magnification: x200.

Biodistribution and intra-hepatic accumulation of [^{125}I]IL-10 and [^{125}I]M6PIL-10

We compared the biodistribution of [^{125}I]IL-10 and [^{125}I]M6PIL-10 in normal and BDL-3 rats.

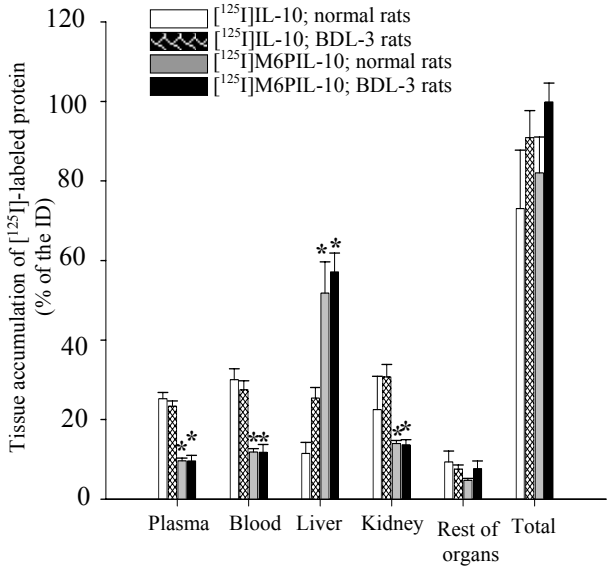

Fig.6. Biodistribution of [^{125}I]IL-10 or [^{125}I]M6PIL-10 in normal and BDL-3 rats, 10 min after iv injection of a tracer amount of radiolabeled-proteins. Values represent mean ± SD of 4 rats per group. *p<0.05 as compared to [^{125}I]IL-10.

The liver accumulation of radiolabeled-IL-10 in BDL-3 rats increased approximately 2-fold as compared to normal rats while the distribution to the kidney did not change.

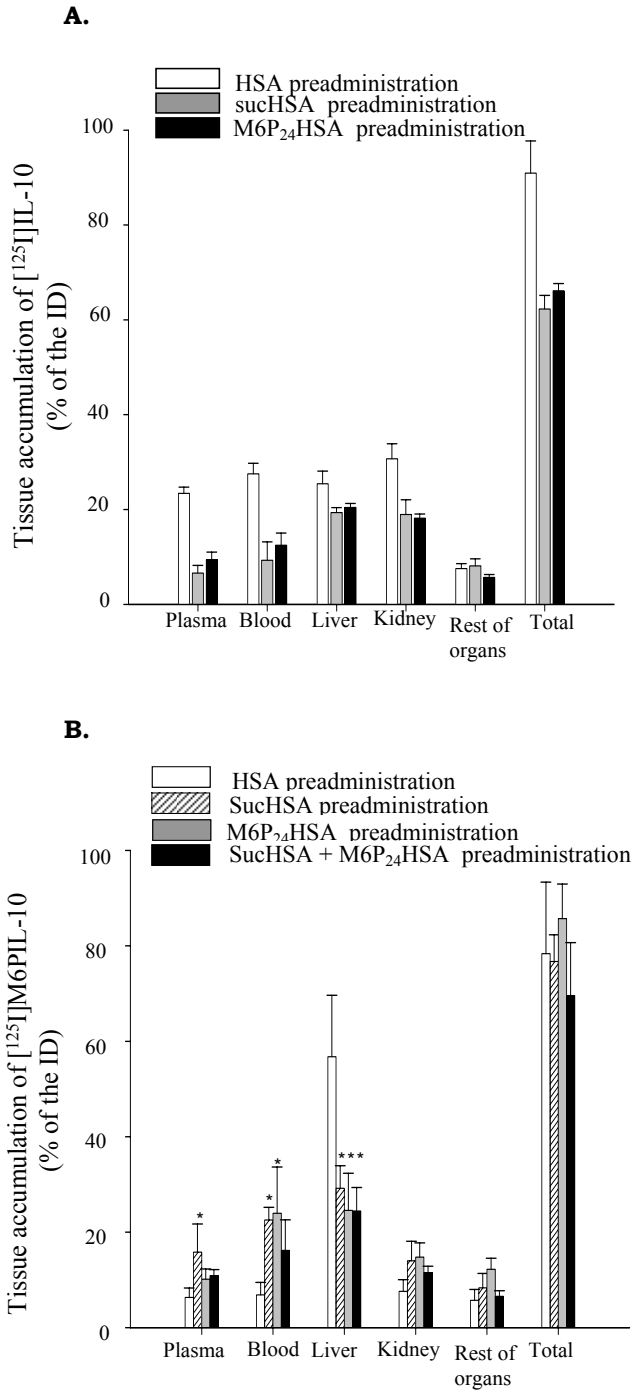

Fig.7. Intra-hepatic disposition of [^{125}I]IL-10 (**A**) or [^{125}I]M6PIL-10 (**B**) in BDL-3 rats, 10 min after iv injection of a tracer amount of radiolabeled-protein. Five min before administration of these proteins, sucHSA or M6P$_{24}$HSA were administered to show receptor specificity. HSA served as a control protein. Values represent mean ± SD of 2 rats (IL-10) or 4 rats (M6PIL-10). *p<0.05 as compared to HSA preadministration.

In contrast, the biodistribution of [^{125}I]M6PIL-10 differed significantly from native IL-10. Approximately 55% of the injected dose of radiolabeled M6PIL-10 was found in the liver, both in normal and BDL-3 rats, whereas uptake in the kidney was significantly lower for M6PIL-10 compared to native IL-10 (fig.6). This was associated with reduced plasma levels of M6PIL-10 at 10 min compared to IL-10. From this result we conclude that in normal and BDL-3 rats, the liver is responsible for the uptake of M6PIL-10 and not the kidney, in contrast to IL-10 itself. We hypothesize that the hepatic uptake of this conjugate is mediated through multiple specific receptors that are present in the liver. Several intrahepatic receptors might account for this uptake. Based on the characteristics of M6PIL-10, these are: IL-10 receptor, the scavenger receptor, and the M6P/IGF-II receptor.[5,15,17]

In order to identify the target receptor for M6PIL-10, we examined the intra-hepatic disposition of radiolabeled-conjugate in BDL-3 rats after co-administration of several receptor blocking proteins. Co-administration of $M6P_{24}$HSA was used to assess receptor-mediated uptake since non-specific uptake would not be blocked. HSA served as a control protein. As a scavenger receptor blocking protein, we used the negatively charged sucHSA molecule. Preadministration of $M6P_{24}$HSA reduced the hepatic accumulation of [^{125}I]M6PIL-10 for approximately 50% (fig 7B). In addition, preadministration of sucHSA also reduced the intra-hepatic by 50%. However, blocking the hepatic uptake of [^{125}I]M6PIL-10 by administration of a mixture of sucHSA and $M6P_{24}$HSA did not reduce uptake any further. The hepatic accumulation of [^{125}I]M6PIL-10 after blockade with sucHSA/$M6P_{24}$HSA was almost equivalent to that of [^{125}I]IL-10 in BDL-3 group. In contrast to [^{125}I]IL-10, preadministration of either $M6P_{24}$HSA or sucHSA did not influence the hepatic uptake of [^{125}I]IL-10 (fig 7A). HSA did not have an effect on M6PIL-10 nor on IL-10 uptake in any of the organs tested.

Discussion

We prepared a liver-selective form of interleukin-10: M6PIL-10. M6P is a specific ligand for M6P/IGF-II receptor.[14,25,26,27] In the liver, the expression of this receptor is upregulated during liver injury, particularly by fibroblast-like cells, the major extracellular matrix-producing cell.[15,17] Previously, M6P-modified HSA ($M6P_x$HSA) has been prepared and successfully delivered to HSC *in vitro* and *in*

vivo.[14] We now designed M6PIL-10 for receptor-mediated delivery of IL-10 to the liver, as a new strategy to treat liver fibrosis. IL-10 is known as a cytokine synthesis inhibitory factor with potent anti-inflammatory and antifibrotic activities.[1-4,6-8] To date, IL-10 is investigated clinically for the treatment of HCV-associated liver fibrosis in patients who do not respond to interferon-based therapy.[3,7] The preliminary results were promising but an immunosupressive action of this cytokine was also noted. This undesired effect during IL-10 therapy is a consequence of the pleiotropic activities of IL-10. The targeting of this cytokine to the liver is, therefore, one resolution to minimize this side effect while increasing its efficacy at the target site. This is also necessary to prevent rapid clearance of IL-10 by the kidney within a few minutes which makes it quite unsuitable for the treatment of chronic liver diseases.[5]

We report here the successful coupling of M6P to IL-10, which was confirmed by Western blot analysis. This activated sugar was coupled to lysine residues in IL-10. The monomeric IL-10 possesses 13 lysine residues (fig.1), and the lysines at position of 34 and 57 are predicted to be in contact with the IL-10 receptor leading to IL-10-induced activities.[28] The blockade of this binding site by particular compounds might reduce or even block the biological activities of this cytokine. In our *in vitro* systems, we tested the biological activities of M6PIL-10 and we observed that this conjugate retained much of the activities of IL-10 (figs.3,4, and 5). First, we examined the effect of M6PIL-10 on RAW cells that constitutively express IL-10 receptors but have no M6P/IGF-II receptors. These cells are highly responsive to LPS, leading to their activation followed by the release of proinflammatory mediators. Inhibition of the release of these proinflammatory agents like TNF-α by M6PIL-10 (fig.3) was almost equivalent to that of IL-10. This result indicates that M6PIL-10 is pharmacologically active and binds to IL-10 receptor.

Secondly, we tested the antifibrotic effect of this conjugate on the target cell, that is, the HSC. We isolated HSC from liver and tested the effect of cytokines on activated HSC. Relevant parameters in this study are TIMP-1, MMP-13 and type I collagen as they are key factors during fibrogenesis[2,22,23] and are produced by HSCs.[22] Liver fibrosis is a complex process resulting from an imbalance between matrix deposition and degradation. In advanced liver fibrosis, the level and/or activity of MMP-13, the principal protease capable of cleaving native

fibrillar collagens, decreases, while TIMP-1 which blocks the MMP-13 activity is overexpressed.[22,29,30] Under conditions in which TIMP-1 is overexpressed or upregulated, there is increased accumulation of collagens in particular type I collagen as the most abundant fibrous matrix constituent. The imbalance between MMP-13 and TIMP-1 is commonly expressed as MMP-13/TIMP-1 ratio.[31] In several studies, it has been reported that IL-10 exhibits its antifibrotic action via the regulation of MMP-13 and TIMP-1 expression, thereby increasing the MMP-13/TIMP-1 ratio.[2,6,8,31,32] In this study, we observed an effect of both IL-10 and M6PIL-10 on the induction of mRNA levels for MMP-13/TIMP-1 ratio. In addition, we found an effect of IL-10 as well as M6PIL-10 on the type I collagen deposition by HSCs cultures. This result indicates that M6PIL-10 is pharmacologically active and has antifibrogenic effects on activated HSC.

In addition to the *in vitro* studies, in the present study we also report on the biodistribution of IL-10 and its modified form in rats. As previously reported, IL-10 was rapidly cleared by the kidney through glomerular filtration, in normal and BDL-3 rats, and some uptake by the liver was also found.[5] The hepatic accumulation of this cytokine seems to be mediated by IL-10 receptors, since there seems to be a correlation between upregulation of the IL-10 receptor in BDL-3 rats and an increase of its hepatic accumulation in this group. As compared to [^{125}I]IL-10, the hepatic uptake of [^{125}I]M6PIL-10 in normal and BDL-3 rats was markedly increased (fig.7), and associated with a significant reduction in the renal uptake. This can be explained by the size and charge selectivity of the glomerular basement membrane. IL-10 is a small protein (37 kDa as a dimeric form) and like most cytokines it will undergo a rapid renal clearance which is highly depended on glomerular filtration. Conversely, the charge barrier will restrict the filtration of negatively charged molecules like M6PIL-10 (molecular weight is slightly higher than IL-10, fig.2). Thus, the decrease of renal uptake of ^{125}IM6P-IL-10 seems to be due to a change in charge rather than in size, as confirmed by zeta potential measurements (data not shown). In addition to the decline in renal disposition, we also observed a significant decline in plasma half-life of [^{125}I]M6PIL-10 as compared to [^{125}I]IL-10. This reflects rapid uptake of the conjugate elsewhere in the body i.e the liver. Indeed, within 10 minutes 55% of [^{125}I]M6PIL-10 was found in this organ in contrast to unmodified IL-10 which accumulated for 11% (normal) and 25% of the dose (BDL-3) in the liver. Thus, by

modifying IL-10 with M6P, we could prevent the renal clearance of IL-10 while we markedly enhanced the level of this cytokine in the liver.

As discussed above, the hepatic accumulation of IL-10 seems to be mediated by IL-10 receptors. As shown in figure 7, we found that hepatic accumulation of M6PIL-10 was probably mediated by three different receptors in the liver: M6P/IGF-II-, scavenger-, and most likely the IL-10 receptor. The blockade of hepatic accumulation of [^{125}I]M6PIL-10 with either $M6P_{24}$HSA or sucHSA clearly reflects the involvement of M6P/IGF-II[14] and scavenger receptors. Scavenger receptors, which can bind a variety of anionic macromolecules including M6PIL-10, are expressed on nonparenchymal cells of the liver (endothelial, Kupffer cells, and HSC).[33-35] As shown by others, highly succinylated albumins are extensively taken up by rat livers.[33] Most likely, the same scavenger receptor is responsible for the hepatic disposition of M6PIL-10 as well. Although $M6P_{24}$HSA and sucHSA inhibited M6PIL-10 uptake, there was no additive effect when both proteins were combined. We inferred therefore that there must be another receptor involved as well. Since IL-10 receptors are present[5] and since our experiments with RAW cells indicate that M6PIL-10 also binds to this receptor (fig.3), this is a likely candidate for being also involved in the hepatic disposition of this conjugate. Further studies for instance after blocking the IL-10 receptor with an excess amount of IL-10 will be needed to prove this hypothesis, but these high concentrations can not be achieved *in vivo*. In figure 8 we propose a model that reflects the involvement of these three putative receptors, and propose several possibilities about how M6PIL-10 reaches its target receptor. Delivery of IL-10 to these different receptors creates a complex situation, because only the IL-10 receptor is revealed for the biological effects. We argue that these other receptors may act accessory receptors. Contacts with neighbouring cells or membrane perturbations may lead to secondary interactions with the IL-10 receptors on HSC. Despite the increased expression of IL-10 receptors during fibrosis[5], the relative density of IL-10 receptor in diseased liver remains very low[36] compared to scavenger and M6P/IGF-II receptors density.[3,37] Thus, the delivery of relative high amounts of IL-10 to the vicinity of its receptor may therefore be quite relevant. A study of pharmacological effects of M6PIL-10 *in vivo* would clarify whether M6PIL-10 binds effectively to its receptor. Additional studies are therefore necessary to prove this hypothesis.

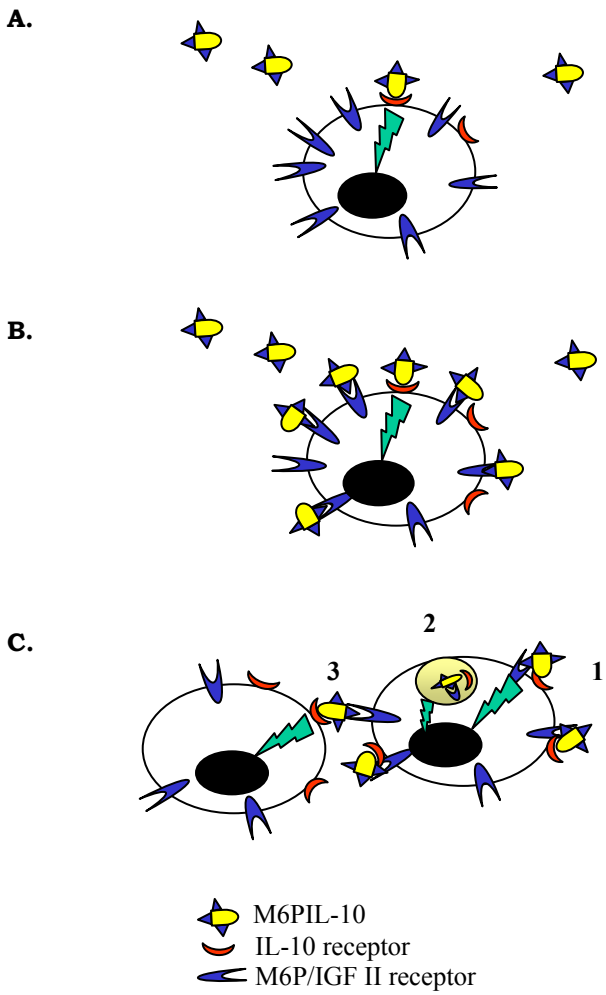

Fig.8. Schematic representation of the binding of modified IL-10 to IL-10 receptor (fig A) or to accessory receptors (fig. B). Binding of M6PIL-10 to both receptors leads to a high cytokine density on the target cell. After binding of M6PIL10 to the accessory receptors, interaction with the IL-10 receptor may take place via 3 possible routes (fig C): [1] via receptor cross-talk in the fluid plasma membrane, [2] after membrane perturbations e.g. in the early endosomes, or [3] by activation of the neighbouring cell.

In summary, we successfully prepared a novel liver-selective form of IL-10 (M6PIL-10) that retains the biological activities of IL-10 *in vitro*. M6PIL-10 is efficiently targeted to the liver to M6P/IGF-II-, scavenger- and most likely to IL-10 receptors. The strategy to target a potent therapeutic cytokine, IL-10, to these receptors ensures a rapid delivery of this cytokine to relevant cell type in the liver,

while avoiding non-target sites thereby may eliminate the known adverse events of IL-10. Since activated HSCs play a key role during fibrogenesis, effective delivery of this antifibrogenic cytokine may be a new step in the design of a therapeutic protein to treat liver fibrosis.

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