

**CHEMICAL MODIFICATION OF INTERLEUKIN-10 WITH MANNOSE 6-PHOSPHATE
GROUPS YIELDS A LIVER-SELECTIVE CYTOKINE**

Heni Rachmawati, Catharina Reker-Smit, Marjolijn N. Lub-de Hooge, Annemiek van Loenen-Weemaes,
Klaas Poelstra, Leonie Beljaars

Prime affiliation:

Department of Pharmacokinetics and Drug Delivery, University Centre for Pharmacy, University of
Groningen, Groningen, The Netherlands.

Affiliations:

Department of Pharmacokinetics and Drug Delivery, University of Groningen, Groningen, The
Netherlands (HR, CRS, AvLW, KP, LB)

Dept. of Nuclear Medicine, University Medical Center Groningen, The Netherlands (MNLH).

Running Title page:

a. Running title: Design of a liver-selective form of interleukin-10

b. Corresponding author: L. Beljaars, Dept. of Pharmacokinetics and Drug Delivery, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. Tel. 0031-50-3636414; Fax. 0031-50-3633247; E-mail: E.Beljaars@rug.nl

C. Number of text pages:

Number of tables: 0

Number of figures: 8

Number of references: 39

Number of words in Abstract: 250

Number of words in Introduction: 661

Number of words in Discussion: 1419

D. List of abbreviations:

IL10, interleukin-10; HSC, hepatic stellate cells; M6P, mannose 6-phosphate; M6P/IGFII receptor, mannose 6-phosphate/insulin-like growth factor II receptor; M6P-IL10 interleukin-10 modified with mannose 6-phosphate groups; LPS, lipopolysaccharides; MMP-13, matrix metalloproteinase-13; TIMP-1, Tissue Inhibitor of Metalloproteinase-1; TGF β , transforming growth factor beta; SucHSA, human serum albumin modified with succinic acid groups; M6PHSA, human serum albumin modified with mannose 6-phosphate groups.

Abstract

Cytokines are considered as a promising immunotherapy for chronic diseases, because of their potency and fundamental roles in pathological processes. However, their therapeutic use is limited because of their poor pharmacokinetics and pleiotropic effects in various organs. These problems may be overcome by cell-specific delivery of the cytokine. This approach involves chemical modification of the protein with homing devices that recognize receptors on target cells. The cytokine interleukin-10 (IL10) may be valuable as a therapeutic cytokine for patients with liver cirrhosis. However, its rapid renal elimination and general immunosuppressive activities limit therapeutic use. We therefore aim to target this cytokine in the liver, in particular to fibrogenic hepatic stellate cells (HSC). We show that IL10 is successfully modified with mannose 6-phosphate (M6P) groups, which is a homing device for the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor expressed on activated HSC. Chemical modification did not diminish IL10 efficacy with regard to *in vitro* anti-inflammatory (LPS-stimulated TNF- α release) and antifibrotic (collagen deposition and degradation) activities. Biodistribution studies with radiolabeled M6P-IL10 and IL10 in rats with liver fibrosis showed that modification with M6P-groups induced a shift in the distribution from the kidneys (IL10) to the liver (M6P-IL10). Hepatocellular binding of M6P-IL10 occurred via M6P/IGFII receptors and scavenger receptors, indicating that not only HSC but also Kupffer and endothelial cells are target cells. IL10 did not bind to these receptors. We conclude that we prepared an active and liver-specific form of the cytokine IL10 that can be evaluated for its efficacy to treat liver diseases.

Introduction

Today's trends in drug development include the application of therapeutic proteins, such as cytokines, for the treatment of various diseases. Liver cirrhosis is a chronic liver disease for which no drugs are available to date. A liver transplant is the only effective treatment but this therapy is associated with high costs, limited availability of donor livers, and many clinical problems. New insights in the pathogenesis of fibrosis have led to many new experimental antifibrotic drugs, but so far they all failed before or during the clinical trials (Pinzani, et al., 2005). The use of cytokines for the therapy of fibrosis may be beneficial because these compounds are very potent (pM-nM range) and they play essential roles in the pathogenesis. With regard to liver cirrhosis, IL10 is relevant because potent anti-inflammatory and antifibrotic activities of this cytokine are described. The antifibrotic effects of IL-10 are thought to be mediated by modulation of extracellular matrix deposition via a downregulation of collagen production or upregulation of the expression of collagenase, in particular matrix metalloproteinase-13 (MMP-13) (Yamamoto, et al., 2001;Reitamo, et al., 1994;Wang, et al., 1998;Thompson, et al., 1998;Mathurin, et al., 2002). IL10 was tested in clinical trials in patients with hepatitis C virus-induced liver fibrosis (Nelson, et al., 2000;Nelson, et al., 2003), but major drawbacks related to the systemic immunosuppressive effects became apparent. This was noted by a flare-up of viral levels in these patients. Also, the short half-life in serum with rapid elimination by the kidneys (Rachmawati, et al., 2004a), meaning that the exposure time of this drug to liver cells is short, hampers its therapeutic use. Dose-escalation to overcome this short plasma half life or strategies to improve the circulation time (polyethylene glycol coupling) inevitably will lead to increased side effects most probably increased immunosuppressive effects on immune cells, i.e. B- and T-cells. To improve the therapeutic effects of IL10, changes in the pharmacokinetic profile, in particular the distribution in the body, are necessary (Brown, 2005). A specific delivery to the diseased target cells, thereby enhancing the concentration at the target site, will improve their efficacy *in vivo* because of an enhanced exposure to the diseased cells, a diminished excretion rate, and a reduced accumulation in the rest of the body. For IL10 the most relevant target cell with regard to fibrosis is the hepatic stellate cell. This is the major pro-fibrotic cell within the liver that produces collagens, and this cell type expresses

IL10 receptors (Mathurin, et al., 2002;Rachmawati, et al., 2004a). In vitro studies demonstrated that IL10 attenuated pro-fibrotic activities of HSC (Wang, et al., 1998;Mathurin, et al., 2002). Therefore, the pharmacokinetics of IL10 should be changed to obtain increased concentrations in this cell type in order to increase the antifibrotic activities of this cytokine.

Chemical modification of the cytokine, necessary to introduce cell-specificity, includes the risk of changing crucial structural properties and thus the biological activities of the cytokine. Recently, the feasibility of cell-specific targeting of cytokines was shown in studies that demonstrated targeting of TNF α and IFN γ to endothelial cells of angiogenic blood vessels in tumours (Curnis, et al., 2000;Curnis, et al., 2005). To that end, the cytokine TNF was fused with a targeting moiety recognizing the $\alpha v \beta 3$ receptor to improve the therapeutic index of the cytokine. Also IFN β has been targeted to inflammatory sites using a complicated strategy that involves the local degradation of a shell around the cytokine by matrixproteinases (Adams, et al., 2003). So far, no reports are available on the chemical modification of IL10 or on the use of cell-specific targeted cytokines to the fibrotic liver. The targeting of IL10 has not been tried before, while it may be highly relevant in view of the important effects of IL10 on fibrogenic cells. Only viral delivery of the IL10 gene was tested and beneficial therapeutic results were obtained in various diseased models (Hung, et al., 2005;Arai, et al., 2000;Choi, et al., 2003). In the present study, we aim to modify the IL10 protein with homing devices that recognize receptors present on the diseased (target) cells in the liver. In the past, we modified albumin with mannose 6-phosphate (M6P) groups and obtained a protein with affinity for the mannose 6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptors on the membrane of HSC (Beljaars, et al., 1999). This receptor is highly upregulated on the designated target cells: myofibroblasts and activated HSC during liver fibrosis (De Bleser, et al., 1995;Weiner, et al., 1998;Greupink, et al., 2006) In the present study, we explored the possibility to chemically modify IL10 with M6P-groups, while we aim to retain the biological activities of IL10 and to improve the pharmacokinetic features of this cytokine.

Methods

Animals

Specific pathogen-free male Wistar rats (obtained from Harlan, Zeist, The Netherlands) received standard laboratory diet and housing conditions. 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.

Modification of IL10 with M6P groups

Recombinant human IL10 (rhIL10; obtained from TeBu-Bio, The Netherlands) was modified with mannose 6-phosphate (M6P) groups similar to the synthesis of M6P-albumin (Beljaars, et al., 1999). Briefly, phosphorylated p-nitrophenyl- α -D-mannopyranoside (Sigma, St. Louis, MO) was synthesized and activated with thiophosgene. Activated M6P was subsequently coupled to IL10 (figure 1A). Each time, 10 μ g-batches of rhIL10 were reacted with activated M6P in a molar ratio of M6P:IL10 = 400:1. The reaction was carried out in sodium carbonate buffer pH 9.5 at room temperature for 1 hour and proceeded at 4°C for 24 hours. Free M6P as well as all buffer components were removed by extensive filtration with Nanosep[®] centrifugal devices (10 kDa, Omega, Pall corp., Michigan), according to the manufacturer's instructions. The purified products were stored at -20°C until use.

Characterization of the protein content of the preparations was performed with NanoDrop[®] (ND-1000 UV-Vis spectrophotometer, NanoDrop Technology, USA) with a mini Bradford method according to the manufacturers instructions. This method allows quantification of very low amounts of protein. The coupling of M6P-groups to IL10 was characterized by western blot analysis with a rabbit polyclonal IL10 antibody (1:200, Santa Cruz Biotechnology, USA) as a primary antibody and horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG (1:2000, DAKO, Glostrup, Denmark) as a secondary antibody. The secondary antibody was visualized with DAB (3,3'-diaminobenzidine, Sigma).

Anti-inflammatory effects of IL10 and M6P-IL10 in vitro

RAW 264.7 cells (American Tissue Culture Center TiB-71), a mouse embryonic macrophage cell line, were cultured in a humidified atmosphere at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Belgium) supplemented with 10% fetal calf serum (FCS, Biowhittaker), 60 µg/mL of gentamicin (Invitrogen, Paisley, Scotland), 2 mM of L-glutamine (Invitrogen) and 0.48 M of L-arginine (Sigma). The cells until passage number 20 were used for experiments.

RAW 264.7 cells (1.5×10^5 cells/well, detached by cell-scraping) were cultured overnight in 96-well plates in complete medium. Then, the cells were preconditioned for 1 hour at 37°C in 200 µl serum-free DMEM. Subsequently, the cells were pre-incubated for 30 min with various concentrations of IL10 or M6P-IL10 (0-250 ng/ml) in 0.1 ml serum-free medium containing 0.5% normal mouse serum. At t = 0h, 25 ng/ml of lipopolysaccharide (LPS of E. coli, List Biological Laboratories, Campbell, CA) was added to the wells. Control cells were incubated without LPS. At t = 6h, the culture media were collected for determination of TNF-α levels.

TNF-α was determined with a TNF-α ELISA sandwich method as described (Bentala, et al., 2002). Briefly, ELISA plates were coated overnight with the monoclonal rat anti-mouse TNF-α (BD Pharmingen, San Diego, USA) in Na₂HPO₄ buffer 0.1 M, pH 6.0. Then, the samples and the standard mouse TNF-α (BD Pharmingen) were added to the plate. After addition of the capture antibody biotinylated rabbit anti-mouse-TNF-α (BD Pharmingen), streptavidin-horse radish peroxidase, and the substrate o-phenylenediamine dichloride (Sigma) were subsequently added and the absorbance was measured at 490 nm.

Antifibrotic effects of IL10 and M6P-IL10

HSC were freshly isolated from livers of normal rats (>500 g). Briefly, the liver was digested with pronase (Merck, Germany), collagenase P (Boehringer, Germany) and DNase (Boehringer) by in situ

perfusion. After several centrifuge steps, the cell suspension was subjected to a Nycodenz (Nyegaard, Norway) gradient to collect the HSC. The purity after isolation was confirmed by phase contrast microscopy and by staining of the cells with markers for all hepatic cell types. The isolated cells were cultured in DMEM containing 10% FCS, 100 U/ml penicillin (Sigma), and 100 µg/ml streptomycin (Sigma). After 6 days in culture, the cells displayed an activated phenotype as assessed by light microscopy and staining for alpha-smooth muscle actin (mouse monoclonal antibody clone 1A4 from Sigma) (Rockey, et al., 1992;Geerts, 2001). Staining of the cell cultures for IL10 receptor and M6P/IGFII receptor was performed with a rabbit polyclonal antibody against IL10 receptor (Santa Cruz) and a goat polyclonal M6P/IGFII receptor (Santa Cruz) according to standard indirect immunoperoxidase methods.

PCR analysis. To assess the effects of IL10 and M6P-IL10 on matrix deposition, HSC at 6 days after isolation were seeded in 6-wells plates (2.5×10^5 cells/well) and adhered overnight. The cells were preconditioned in FCS-free medium for 1 h, before incubation with either 0.5 ml of IL10 or M6P-IL10 (12.5 and 25 ng/ml) for 1 h. Control cells were pre-incubated with FCS-free medium. Subsequently, the cells were stimulated with TGFβ-1 (5 ng/mL, Roche Diagnostics corp., USA). After 24 h, cell fractions were harvested and used for PCR analysis. The mRNA fraction was isolated with the Qiagen RNeasy Mini Kit (Qiagen Sciencer, USA) according to the manufacturer's instructions. The mRNA concentration was determined with the NanoDrop[®]. A reverse transcriptase 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 the following primers: collagen 1a1 (5'-AGCCTGAGCCAGCAGATTGA-3' and 5'-CCAGGTTGCAGCCTTGGTTA-3'), MMP-13 (5'-AGGCCTTCAGAAAAGCCTTC-3' and 5'-GAGCTGCTTGTCCAGGTTTC-3'), TIMP-1 (5'-ACAGCTTTCTGCAACTCG-3' and 5'-CTATAGGTCTTTACGAAGGCC-3') and GAPDH (5'-CCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTCACCACCTTCTTG-3'). PCR for MMP-13 was performed in a total volume of 25 µl containing 1.5 µl of cDNA, 50 mM MgCl₂, 2.5 µl of 10x Taq DNA polymerase buffer, 10 mM dNTPs, 0.5 U Taq-DNA polymerase (Eurogenetec, Belgium) and 50 pmol/µl

of each primer. The MMP13 reaction was performed with 30 cycles and an annealing temperature of 56°C for 30s, while GAPDH PCR was performed with 26 cycles and annealing temperature of 58°C for 30s. The band intensity of the PCR products was quantified with ImageJ (NIH Image software, USA), and the gene expressions were normalized to the signal of the house keeping gene GAPDH. For collagen 1a1 and TIMP-1, cDNA was amplified by quantitative real-time PCR using the SYBR green PCR Master Mix (Applied Biosystems) and the reaction was performed in a ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Data were analyzed using the comparative threshold cycle (C_T) method as described in User Bulletin No. 2 of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Data are expressed as fold induction of the gene of interest compared with the control condition as calculated by the formula $2^{-\Delta\Delta C_T}$.

Immunohistochemical analysis. The effects of M6P-IL10 on type I collagen production was studied in cultured HSC, 6 days after isolation. After trypsinisation, HSC were cultured (5×10^3 cells/well) in 8-well cover slip chamber slides (Lab-Teks, Nunc, Rochester, USA) for 24 h. Subsequently, cells were pre-conditioned in FCS-free medium for 2 h. After this, cells were incubated with either 0.1 ml of IL10 or M6P-IL10 (12.5 and 25 ng/mL) for 24 h. One hour after addition of IL10, cells were stimulated with TGF β -1. Control cells were incubated with FCS-free medium in parallel wells. Type I collagen deposition in the wells was detected immunohistochemically with a goat polyclonal antibody against type I collagen (Southern Biotechnology Associates Inc., USA), horseradish peroxidase-conjugated rabbit polyclonal anti-goat immunoglobulin (DAKO) as a secondary antibody, and visualized with AEC (3-amino, 9 ethyl-carbazole, Sigma).

Animal model of liver fibrosis

Rats (weighing 250–300 g) were subjected to bile duct ligation (BDL) (Kountouras, et al., 1984) under anesthesia with 40% O₂:60% N₂O combined with 0.5% Isoflurane (Abbot Laboratories Ltd, UK). Three weeks after the ligation (BDL-3), the rats were used for further experiments. At this time-point, the fibrosis in the liver is in a late stage of the disease. See also figure 6 A, in which frozen sections are

stained with a polyclonal antibody against collagen type III (Southern Biotechnology) as described in the previous paragraph.

In vivo distribution of [¹²⁵I]-IL10 or [¹²⁵I]-M6P-IL10 in rats

Radioactive labeling. IL10 and M6P-IL10 were labeled with Iodine-125 (¹²⁵I) according to standard methods (Greenwood, et al., 1963; Mather and Ward, 1987). For gamma camera studies, these proteins were labeled with Iodine-123 (¹²³I, GE Health) (Greenwood, et al., 1963). Prior to each experiment, free iodine in the preparations was removed by gel filtration with a PD-10 column (Amersham Pharmacia Biotech) to obtain dosing preparations with less than 5% free iodine.

Quantitative biodistribution studies. After anesthetizing BDL-3 rats (n=4/group) with O₂/N₂O/isoflurane, [¹²⁵I]-IL10 and [¹²⁵I]-M6P-IL10 (± 500,000 cpm/rat, diluted in saline) was intravenously injected in the penile vein. After 10 minutes, blood and major organs were harvested to determine the amount of radioactivity. 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, as described (Beljaars, et al., 2000).

To assess the receptor-mediated uptake of the modified cytokine, 5 min prior to the intravenous injection of the radio-labeled [¹²⁵I]-IL10 or [¹²⁵I]-M6P-IL10, 5 mg/kg of either M6P-HSA (n=4), succinylated human serum albumin (SucHSA, n=6), a mixture of SucHSA and M6PHSA (n=4), or HSA (n=4) was administered intravenously. M6PHSA and SucHSA were prepared and characterized according to standard procedures (Beljaars, et al., 1999; Swart, et al., 1996).

Gamma camera studies. The body distribution of IL10 and M6P-IL10 was visualized in BDL-3 rats using gamma camera analysis (N=3/group). Anesthetized rats were placed on a low-energy all-purpose collimator of a gamma camera and received intravenously a tracer amount of ¹²³I-labeled proteins. The

radioactivity was dynamically recorded from 0 to 30 min with a frame rate of one total body scan per minute.

Statistical analysis

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

RESULTS

Synthesis of M6P-IL10

The attachment of M6P-groups to IL10 (figure 1A) was demonstrated with western blot techniques. Similar to IL10, two bands were detected in the blot of M6P-IL10 corresponding to respectively the monomeric and dimeric form of the cytokine (figure 1B). A shift in the molecular weight (Mw) of the IL10-positive bands relative to native IL10, confirmed the attachment of M6P-groups. With an average Mw of 20 kDa and 40 kDa, the increase in Mw of the M6P-IL10 bands was respectively, ± 1500 and 3000 dalton. Therefore, we estimated that about 4 M6P-groups were coupled per cytokine monomer. No bands at the Mw of unmodified IL10 were detectable in the M6P-IL10 preparations.

Anti-inflammatory activities of IL10 and M6P-IL10 *in vitro*

To assess whether this M6P-modified IL10 was still biologically active, we first studied its anti-inflammatory activities using LPS-stimulated macrophages (figure 2). In these RAW 264.7 cells, LPS strongly enhanced the TNF- α production; from 79 ± 49 pg/ml for control wells (without LPS) to 10845 ± 746 pg/ml in the presence of LPS ($p < 0.01$). Co-incubation of LPS with various concentrations of IL10 significantly attenuated the TNF production of the RAW cells starting at 15 ng/ml concentrations. The TNF levels were reduced to 4292 ± 452 pg/ml at a concentration of 250 ng/ml IL10 ($p < 0.01$). Also, the chemically modified form of IL10 (M6P-IL10) reduced the TNF- α response of the macrophages in a dose-dependent manner, starting also at 15 ng/ml concentrations. At a concentration of 250 ng/ml, the TNF production in M6P-IL10 incubations was significantly reduced to similar levels as with unmodified IL10 (4784 ± 160 pg/ml; $p < 0.01$).

Antifibrotic activities of IL10 and M6P-IL10 *in vitro*

The antifibrotic activities of IL10 and M6P-IL10 were studied in fibroblast cultures. Primary rat HSC were used because these myofibroblasts are our target cells in the liver and after culturing them for 7 days

in a plastic flask they display an activated phenotype as reflected by alpha-smooth muscle actin staining (data not shown). At this time point, we confirmed by immunostaining of the HSC cultures that these cells displayed the IL10 receptor as well as the target M6P/IGFII receptor (figure 3).

We studied the effects of IL10 and modified IL10 on MMP-13, Tissue Inhibitor of Metalloproteinase (TIMP)-1 and type I collagen expression. We found that both IL10 and M6P-IL10 increased MMP13 mRNA expression levels of HSC stimulated with TGF β , whereas TIMP-1 mRNA levels were decreased by both compounds but not significantly. In vivo, the ratio between matrix degrading activity and inhibition of this activity determines the net outcome of the process. When the ratio of MMP-13 and TIMP-1 was determined (Ricke, et al., 2002; Vaillant, et al., 2001), we assessed that IL10 and M6PIL-10 both significantly enhanced this ratio (figure 4). As compared to control TGF β -treated cells, this ratio was increased 2.4 times and 1.8 times at 25 ng/ml of respectively IL10 and M6P-IL10. This means that the collagenolytic activity of these cells was enhanced by both types of IL10. Collagen 1a1 mRNA expression of the HSC was not significantly altered by M6P-IL10 or IL10 of (data not shown).

In addition, we tested whether IL10 and M6P-IL10 influenced total collagen deposition, by staining of HSC cultures for collagen type I, which is the major interstitial collagen produced by HSC in fibrogenesis. Type I collagen was present in HSC cultures at 7 days after isolation. The collagen staining was diminished by incubation of the cells with IL10 (figure 5). M6P-IL10 reduced type I collagen staining in these cultures at similar concentrations.

In vivo distribution of IL10 and M6P-IL10

We assessed the initial biodistribution 10 min after intravenous injection of [¹²⁵I]-IL10 and [¹²⁵I]-M6P-IL10 in rats with advanced liver fibrosis (figure 6). While for IL10 25 \pm 3% of the iv injected radioactive dose ended up in the liver, the hepatic amounts were significantly increased after coupling of M6P groups to IL10. Up to 54 \pm 6% of the total injected dose was detectable in the livers of these BDL rats. In addition, the uptake in the kidneys and the amount of cytokine in the blood was significantly lower for M6P-IL10

as compared to native IL10 at that same time point. These results indicate that we created a form of IL10 with higher specificity for the liver.

Immunohistochemical examination of the hepatocellular localization of modified IL10, similar to previous studies with modified albumin (Beljaars, et al., 1999), was not possible, because this technique is not sensitive enough to detect the microgram dose of rhIL10 that was injected. Therefore we performed radioactive studies aiming to block the receptor-mediated uptake to assess which receptors are responsible for the uptake. In addition to the IL-10 receptor and the M6P/IGF-II receptor, we also tested whether scavenger receptors were able to bind M6P-IL10. It is known that the scavenger receptor type A is involved in the binding of polyanions (Terpstra, et al., 2000), and our construct is negatively charged, as reflected by zetapotential measurements. Figure 7 shows that co-administration of an excess of M6P-HSA, a ligand for the M6P/IGFII-receptor, resulted in a 54% reduction in the hepatic accumulation of [¹²⁵I]M6P-IL10. In addition, an excess of SucHSA, which is a ligand for the scavenger type A receptor, also significantly reduced the distribution to the liver by about 46%. Blocking the receptor-mediated uptake of [¹²⁵I]M6P-IL10 by administration of a mixture of Suc-HSA and M6P-HSA did not further reduce the hepatic radioactivity as compared to M6P-HSA alone (data not shown). The decrease in liver uptake of M6P-IL10 by co-administration of M6PHSA or SucHSA led to higher plasma concentrations of the cytokine at the same time point (data not shown). Administration of the control protein HSA did not display any effect on the hepatic accumulation of [¹²⁵I]M6P-IL10. The distribution of [¹²⁵I]IL10 was not influenced by M6PHSA, SucHSA or HSA, which indicated that the M6P/IGFII receptor and the scavenger receptor were not involved in the hepatic uptake of unmodified IL10 (figure 7).

Finally, gamma camera studies were used to visualize the body distribution. Already two min after iv injection, high levels of [¹²³I]M6P-IL10 were observed within the liver region, with very low levels in the kidneys. Hepatic levels remained high for at least 30 min. In contrast, native [¹²³I]-IL10 rapidly accumulated in the kidneys with low uptake in livers (fig. 8), which is in agreement with previous studies (Rachmawati, et al., 2004a; Andersen, et al., 1999). Coupling of M6P-groups to IL10 induced a shift in biodistribution of IL10 from the kidneys to the fibrotic liver.

DISCUSSION

IL10 seems an attractive therapeutic protein for the treatment of liver diseases, but the unfavorable pharmacokinetic profile, its possible uptake by many different cell types, the multiple immunomodulatory activities, and its role in normal physiological processes limit the clinical use. Therefore, we aimed at the preparation of a liver-selective form of interleukin-10 by chemical modification of the protein with M6P groups. M6P is a specific ligand for M6P/IGF-II receptor, and the expression of this receptor is highly upregulated on the cell membrane of HSC during liver injury (De Bleser, et al., 1995; Greupink, et al., 2006). Previously, M6P-modified human serum albumin (M6P-HSA) has been prepared and successfully applied as a drug carrier to HSC *in vitro* and *in vivo* (Beljaars, et al., 1999; Beljaars, et al., 2001). In the present study, we used this approach for the stellate cell-targeting of the antifibrotic cytokine IL10. A recombinant human IL10 was used in the present studies, because human IL10 is active on both rodent and human cells (Moore, et al., 2001), and because clinical have been done (Nelson, et al., 2000) and will be done in patients, the human form is the most suitable in pre-clinical studies.

In the past, some cytokines have been chemically modified in order to obtain products with an improved pharmacokinetic profile (Schrama, et al., 2006; Rachmawati, et al., 2004b) or improved therapeutic profile (Curnis, et al., 2000; Curnis, et al., 2005; Adams, et al., 2003). The most well-known therapeutic cytokine with regard to liver diseases is Pegasys[®], which is interferon α 2a modified with polyethylene glycol (PEG) groups. Substitution of cytokines with PEG moieties prevents rapid renal elimination, which results in compounds with prolonged plasma levels (Harris and Chess, 2003; Veronese and Pasut, 2005). This strategy to improve pharmacokinetics has also been applied to some other cytokines, such as IL2, IL6, GM-CSF, and TNF, but not for IL10 yet. However, this strategy only prevents rapid clearance. An even more powerful way is to improve the therapeutic profile of cytokines by enhancing the accumulation at the target site based on the delivery to specific target receptors. With this strategy, also applied in the current study, side-effects in other parts of the body will be reduced, because elevated amounts of cytokine in the blood will be present only for a short time after injection. In contrast, after PEGylation of proteins, elevated plasma concentrations can be measured for several hours. That delivery of IL10 may be

promising, can be derived from a few studies exploring the therapeutic potential of IL10 after viral delivery (Hung, et al., 2005;Arai, et al., 2000;Choi, et al., 2003). In these gene targeting studies, viral-produced IL10 significantly attenuated the fibrotic process.

The present study shows successful coupling of M6P to IL10, which was confirmed by chemical characterization with western blot analysis. The sugar moiety was attached to the ϵ NH₂-groups present in the lysine residues in the protein backbone of IL10. Human IL10 contains 13 lysine residues in total. Not all these residues are readily accessible for chemical modification. With our characterizations, we estimated that about 4 molecules of M6P were attached to IL10. Of note, the lysines at position 34 and 57 in the IL10 protein are predicted to interact with the IL10 receptor and are therefore involved in IL10 signaling (Reineke, et al., 1998). Coupling of M6P-groups to these lysine amino acids might therefore block the biological activities of this cytokine. With our *in vitro* systems to examine anti-inflammatory and antifibrotic activities of IL10, we assessed that M6P-IL10 was still biologically active. First, we examined the effect of M6P-IL10 on RAW cells that constitutively express the IL10 receptor but have no M6P/IGF-II receptors. These receptor expressions were confirmed immunohistochemically (data not shown). RAW cells are highly responsive to LPS, leading to their activation followed by the release of proinflammatory mediators such as TNF- α . Inhibition of TNF- α by M6P-IL10 was observed at similar concentrations as used for IL10. This indicates that M6P-IL10 is still able to bind to IL-10 receptors.

Secondly, we tested the antifibrotic effect of this conjugate on the target cell, that is, the activated HSC. This cell contains both IL10 and M6P/IGFII receptors as tested immunohistochemically. With respect to this, relevant parameters are TIMP-1, MMP-13 and type I collagen, because these key parameters of the fibrotic process are regulated by IL10 (Mathurin, et al., 2002;Schaefer, et al., 2003). In advanced liver fibrosis, a complex process resulting from an imbalance between matrix deposition and degradation leads to enhanced collagen deposition. The fibrillar collagen type I is one of the most abundant fibrous matrix constituents. During the fibrogenesis, the activity of MMP-13, the principal protease capable of cleaving fibrillar collagens, decreases. At the same time, TIMP-1, the inhibitor that blocks MMP-13 activity, is over-expressed. Under conditions in which TIMP-1 levels are increased and MMP13 are decreased, there

is an increased deposition of fibrillar collagens in the liver. The imbalance between MMP-13 and TIMP-1 can be expressed as MMP-13/TIMP-1 ratio (Ricke, et al., 2002; Vaillant, et al., 2001). In this study, we observed antifibrotic effects of both IL10 and M6P-IL10 via the regulation of MMP-13 and TIMP-1 mRNA expressions. In addition, we found an effect of IL10 and M6P-IL10 on the type I collagen deposition by HSC in culture. These results indicate that M6P-IL10 is pharmacologically active and exerts antifibrogenic effects on activated HSC.

Specific delivery to target receptors will yield compounds with an enhanced accumulation at the target site due to receptor-mediated removal of the compound from the circulation. Coupling of M6P-groups to IL10 resulted in a modified protein that was rapidly eliminated from the blood, as illustrated by the fact that less than 10% was present in plasma at 10 minutes after intravenous injection, and most of the compound ($54\pm 6\%$ of the radioactive dose) was traced back in the fibrotic liver, and more importantly, in the target cells, that is, in HSC. M6P-modified albumin displayed a similar distribution pattern and plasma elimination rate (Beljaars, et al., 1999). As previously reported (Rachmawati, et al., 2004a), the cytokine IL10 itself was rapidly cleared by the kidneys through glomerular filtration, with only minor uptake in the liver. As shown in figure 7, we found that the hepatic accumulation of M6P-IL10 is mediated by several hepatic receptors. In addition to the M6P/IGF-II receptor and the IL-10 receptor present on HSC, also the scavenger receptor appears to be involved in the removal of this cytokine from the blood, because succinylated albumin, a well known ligand for this scavenger receptor, attenuated the liver uptake of M6P-IL10. This is not surprising since after coupling of M6P to NH₂-groups of lysine, a protonated group (NH₃⁺) is replaced by a negatively charged group (PO₄³⁻). Scavenger receptors are found on Kupffer and sinusoidal endothelial cells, and also on activated HSC (Schneiderhan, et al., 2001; Adrian, et al., 2006). The combination of SucHSA and M6PHSA did not induce a complete blockage of liver uptake, even after administration of very high doses. This residual hepatic uptake likely reflects IL10 receptor-mediated uptake. Since high amounts of IL10 cannot be administered, complete IL10 receptor blockage can not be achieved. However, studies with RAW cells show that our construct does bind to the IL10 receptor and this receptor is expressed in fibrotic livers (Rachmawati, et al., 2004a).

So we conclude that M6P-recognizing receptors, scavenger receptors and IL10 receptors are involved in the hepatic uptake of M6P-IL10. From this, we can deduce a distribution of M6P-IL10 to Kupffer cells, endothelial cells and hepatic stellate cells. This is from a therapeutic point of view beneficial with regard to its anti-inflammatory and antifibrotic activities *in vivo*.

Delivery of IL10 to different receptors creates a complex situation, because only the IL10 receptor is relevant for the biological effects of M6P-IL10 and binding to the other receptors will not induce any IL10-mediated effect. We propose that delivery of high amounts of IL10 in the vicinity of the IL10 receptor may be beneficial. IL10 receptor expression is low, even after its upregulation during fibrosis. Scavenger receptor and M6P/IGFII receptor expression is relatively very high, in particular during fibrosis. M6P-IL10 bound in high concentrations to the target cell or to neighboring cells may trigger the IL10 receptor via contact between adjacent cells or via receptor cross-talk during membrane perturbations, similar to the situation of a cytokine coupled to an antibody as the targeting device (Schrama, et al., 2006; Penichet and Morrison, 2001). A study of pharmacological effects of M6P-IL10 in rats with liver fibrosis would clarify whether M6P-IL10 is able to induce effects *in vivo* after binding to the intrahepatic receptors. The assessment of *in vivo* efficacy and improvement by targeting of IL10 will be the ultimate goal of our studies. To demonstrate this, chronic studies are required, and at the moment we are up-scaling the synthesis to examine the effects in animal models of liver fibrosis.

In summary, we successfully prepared a novel liver-selective form of IL10, M6P-IL10, which displays the biological activities of IL-10. M6P-IL10 is efficiently distributed to relevant cell types in the liver that is to HSC, endothelial and Kupffer cells. The chemical modification of the cytokine IL10 may be a novel approach in the use of cytokines for diseases.

ACKNOWLEDGMENTS

We thank J.H. Pol and H. ter Veen (Department of Nuclear Medicine, Academic Hospital Groningen, The Netherlands) for technical assistance in the radioactive labeling of IL10 and M6P-IL10 and gamma camera studies.

References

- Adams G, Vessillier S, Dreja H and Chernajovsky Y (2003) Targeting cytokines to inflammation sites. *Nat Biotechnol* **21**:1314-1320.
- Adrian JE, Poelstra K, Scherphof GL, Molema G, Meijer DK, Reker-Smit C, Morselt HW and Kamps JA (2006) Interaction of targeted liposomes with primary cultured hepatic stellate cells: Involvement of multiple receptor systems. *J Hepatol* **44**:560-567.
- Andersen SR, Lambrecht LJ, Swan SK, Cutler DL, Radwanski E, Affrime MB and Garaud JJ (1999) Disposition of recombinant human interleukin-10 in subjects with various degrees of renal function. *J Clin Pharmacol* **39**:1015-1020.
- Arai T, Abe K, Matsuoka H, Yoshida M, Mori M, Goya S, Kida H, Nishino K, Osaki T, Tachibana I, Kaneda Y and Hayashi S (2000) Introduction of the interleukin-10 gene into mice inhibited bleomycin-induced lung injury in vivo. *Am J Physiol Lung Cell Mol Physiol* **278**:L914-L922.
- Beljaars L, Molema G, Schuppan D, Geerts A, De Bleser PJ, Weert B, Meijer DKF and Poelstra K (2000) Successful targeting of albumin to rat hepatic stellate cells using albumin modified with cyclic peptides that recognize the collagen type VI receptor. *J Biol Chem* **275**:12743-12751.
- Beljaars L, Molema G, Weert B, Bonnema H, Olinga P, Groothuis GMM, Meijer DKF and Poelstra K (1999) Albumin modified with mannose 6-phosphate: A potential carrier for selective delivery of antifibrotic drugs to rat and human hepatic stellate cells. *Hepatol* **29**:1486-1493.
- Beljaars L, Olinga P, Molema G, De Bleser P, Geerts A, Groothuis GMM, Meijer DKF and Poelstra K (2001) Characteristics of the hepatic stellate cell-selective carrier mannose 6-phosphate modified albumin (M6P(28)-HSA). *Liver* **21**:320-328.
- Bentala H, Verweij WR, Huizinga-van d, V, Van Loenen-Weemaes AM, Meijer DK and Poelstra K (2002) Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. *Shock* **18**:561-566.
- Brown LR (2005) Commercial challenges of protein drug delivery. *Expert Opin Drug Deliv* **2**:29-42.
- Choi YK, Kim YJ, Park HS, Choi K, Paik SG, Lee YI and Park JG (2003) Suppression of glomerulosclerosis by adenovirus-mediated IL-10 expression in the kidney. *Gene Ther* **10**:559-568.
- Curnis F, Gasparri A, Sacchi A, Cattaneo A, Magni F and Corti A (2005) Targeted delivery of IFN γ to tumor vessels uncouples antitumor from counterregulatory mechanisms. *Cancer Res* **65**:2906-2913.
- Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A and Corti A (2000) Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). *Nat Biotechnol* **18**:1185-1190.
- De Bleser PJ, Jannes P, Buul-Offers SC, Hoogerbrugge CM, van Schravendijk CF, Niki T, Rogiers V, van den Brande JL, Wisse E and Geerts A (1995) Insulinlike growth factor-II/mannose 6-phosphate receptor is expressed on CCl₄-exposed rat fat-storing cells and facilitates activation of latent transforming growth factor-beta in cocultures with sinusoidal endothelial cells. *Hepatology* **21**:1429-1437.

- Geerts A (2001) History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis* **21**:311-335.
- Greenwood FC, Hunter WM and Glover JS (1963) The preparation of ¹³¹I labelled human growth hormone of high specific radioactivity. *Biochem J* **89**:114-123.
- Greupink R, Bakker HI, van Goor H, de Borst MH, Beljaars L and Poelstra K (2006) Mannose-6-phosphate/insulin-Like growth factor-II receptors may represent a target for the selective delivery of mycophenolic acid to fibrogenic cells. *Pharm Res* **23**:1827-1834.
- Harris JM and Chess RB (2003) Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* **2**:214-221.
- Hung KS, Lee TH, Chou WY, Wu CL, Cho CL, Lu CN, Jawan B and Wang CH (2005) Interleukin-10 gene therapy reverses thioacetamide-induced liver fibrosis in mice. *Biochem Biophys Res Commun* **336**:324-331.
- Kountouras J, Billing BH and Scheuer PJ (1984) Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. *Br J Exp Pathol* **65**:305-311.
- Mather SJ and Ward BC (1987) High efficiency iodination of monoclonal antibodies for radiotherapy. *J Nucl Med* **28**:1034-1036.
- Mathurin P, Xiong S, Kharbanda KK, Veal N, Miyahara T, Motomura K, Rippe RA, Bachem MG and Tsukamoto H (2002) IL-10 receptor and coreceptor expression in quiescent and activated hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* **282**:G981-G990.
- Moore KW, Waal Malefyt R, Coffman RL and O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**:683-765.
- Nelson DR, Lauwers GY, Lau JY and Davis GL (2000) Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. *Gastroenterology* **118**:655-660.
- Nelson DR, Tu Z, Soldevila-Pico C, Abdelmalek M, Zhu H, Xu YL, Cabrera R, Liu C and Davis GL (2003) Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology* **38**:859-868.
- Penichet ML and Morrison SL (2001) Antibody-cytokine fusion proteins for the therapy of cancer. *J Immunol Methods* **248**:91-101.
- Pinzani M, Rombouts K and Colagrande S (2005) Fibrosis in chronic liver diseases: diagnosis and management. *J Hepatol* **42 Suppl**:S22-S36.
- Rachmawati H, Beljaars L, Reker-Smit C, Loenen-Weemaes AM, Hagens WI, Meijer DK and Poelstra K (2004a) Pharmacokinetic and biodistribution profile of recombinant human interleukin-10 following intravenous administration in rats with extensive liver fibrosis. *Pharm Res* **21**:2072-2078.
- Rachmawati H, Poelstra K and Beljaars L (2004b) The use of cytokines and modified cytokines as therapeutic agents, in *Recent Research Developments in Immunology* pp 191-217, Research Sign Post, Kerala, India.

- Reineke U, Sabat R, Volk HD and Schneider-Mergener J (1998) Mapping of the interleukin-10/interleukin-10 receptor combining site. *Protein Sci* **7**:951-960.
- Reitamo S, Remitz A, Tamai K and Uitto J (1994) Interleukin-10 modulates type I collagen and matrix metalloproteinase gene expression in cultured human skin fibroblasts. *J Clin Invest* **94**:2489-2492.
- Ricke WA, Smith GW and Smith MF (2002) Matrix metalloproteinase expression and activity following prostaglandin F(2 alpha)-induced luteolysis. *Biol Reprod* **66**:685-691.
- Rockey DC, Boyles JK, Gabbiani G and Friedman SL (1992) Rat hepatic lipocytes express smooth muscle actin upon activation in vivo and in culture. *J Submicrosc Cytol Pathol* **24**:193-203.
- Schaefer B, Rivas-Estilla AM, Meraz-Cruz N, Reyes-Romero MA, Hernandez-Nazara ZH, Dominguez-Rosales JA, Schuppan D, Greenwel P and Rojkind M (2003) Reciprocal modulation of matrix metalloproteinase-13 and type I collagen genes in rat hepatic stellate cells. *Am J Pathol* **162**:1771-1780.
- Schneiderhan W, Schmid-Kotsas A, Zhao J, Grunert A, Nussler A, Weidenbach H, Menke A, Schmid RM, Adler G and Bachem MG (2001) Oxidized low-density lipoproteins bind to the scavenger receptor, CD36, of hepatic stellate cells and stimulate extracellular matrix synthesis. *Hepatology* **34**:729-737.
- Schrama D, Reisfeld RA and Becker JC (2006) Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov* **5**:147-159.
- Swart PJ, Beljaars E, Smit C, Pasma A, Schuitemaker H and Meijer DKF (1996) Comparative pharmacokinetic, immunologic and hematologic studies on the anti-HIV-1/2 compounds aconitylated and succinylated HSA. *J Drug Target* **4**:109-116.
- Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J and Van Berkel TJ (2000) Hepatic and extrahepatic scavenger receptors: function in relation to disease. *Arterioscler Thromb Vasc Biol* **20**:1860-1872.
- Thompson KC, Trowern A, Fowell A, Marathe M, Haycock C, Arthur MJ and Sheron N (1998) Primary rat and mouse hepatic stellate cells express the macrophage inhibitor cytokine interleukin-10 during the course of activation In vitro. *Hepatology* **28**:1518-1524.
- Vaillant B, Chiamonte MG, Cheever AW, Soloway PD and Wynn TA (2001) Regulation of hepatic fibrosis and extracellular matrix genes by the th response: new insight into the role of tissue inhibitors of matrix metalloproteinases. *J Immunol* **167**:7017-7026.
- Veronese FM and Pasut G (2005) PEGylation, successful approach to drug delivery. *Drug Discov Today* **10**:1451-1458.
- Wang SC, Ohata M, Schrum L, Rippe RA and Tsukamoto H (1998) Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate cells. *J Biol Chem* **273**:302-308.
- Weiner JA, Chen A and Davis BH (1998) E-box-binding repressor is down-regulated in hepatic stellate cells during up-regulation of mannose 6-phosphate/insulin-like growth factor-II receptor expression in early hepatic fibrogenesis. *J Biol Chem* **273**:15913-15919.

Yamamoto T, Eckes B and Krieg T (2001) Effect of interleukin-10 on the gene expression of type I collagen, fibronectin, and decorin in human skin fibroblasts: differential regulation by transforming growth factor-beta and monocyte chemoattractant protein-1. *Biochem Biophys Res Commun* **281**:200-205.

FOOTNOTES

- a. This project was supported by grants from Foundation of Technical Sciences (STW, Utrecht, The Netherlands), Islamic Development Bank (IDB) (Jeddah, Saudi Arabia) and Ubbo Emmius program (University of Groningen, The Netherlands).
- b. Reprint requests: Dr. L. Beljaars, Dept. of Pharmacokinetics and Drug Delivery, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: E.Beljaars@rug.nl
- c. -

LEGENDS FOR FIGURES

Fig. 1. A. Synthesis of M6P-IL10. Thiophosgene-activated mannose 6-phosphate (1) is coupled to the lysine amino acids (lys) of recombinant human IL10 (2) to obtain the product M6P-IL10 (3). B. Western blot analysis of M6P-IL10 and IL10 using an antibody against human IL10. Note the increased molecular weight of both monomeric (20 kDa) and dimeric (40 kDa) forms of M6P-IL10 as compared to IL10 (18.5 kDa and 37 kDa, respectively).

Fig. 2. Concentration-dependent effect of IL10 (white bars) and M6P-IL10 (black bars) on the TNF- α production by LPS-stimulated RAW 264.7 cells (hatched bar). Control non-stimulated cells produced only 79 \pm 49 pg TNF- α /ml. Note that IL10 and M6P-IL10 inhibit TNF- α response at similar concentrations. Values are expressed as mean \pm SD, * = p<0.05 (n=5).

Fig. 3. Immunohistochemical detection of the expression of IL10 receptors (A) and M6P/IGFII receptors (B), stained in red, in cultures of rat HSC at 6 days after isolation.

Fig. 4. Effect of IL-10 (black bars) and M6PIL-10 (white bars) on the ratio of MMP-13 and TIMP-1 mRNA in TGF- β stimulated primary isolated rat HSC. Addition of IL10 or M6P-IL10 significantly enhanced mRNA ratio of MMP-13/TIMP-1, indicating higher collagenolytic activity in these wells, as compared to control cells (hatched bars). Values are expressed as mean \pm SD, * = p<0.05 (n=3).

Fig. 5. Effect of IL10 and M6P-IL10 on the collagen type I staining in TGF-stimulated primary isolated rat HSC (n=3). Immunoreactivity for collagen type I is abundantly present in untreated HSC cultures (A.), whereas this staining is strongly reduced by IL10 (B.) and M6P-IL10 (C.). Original magnification: 20x10.

Fig. 6. A. Histological images of matrix deposition (cryostat sections stained for collagen type III, the major constituent of liver ECM) in normal rats and in BDL-3 rats. B. Biodistribution of [¹²⁵I]IL10 or [¹²⁵I]M6P-IL10 in rats with extensive liver fibrosis (BDL-3 weeks), at 10 min after iv injection of a tracer amount of radiolabeled-proteins. Note the significantly increased liver distribution after modification of IL10 with M6P-groups. Values represent mean ± SD, n= 4 rats per group, *=p< 0.05.

Fig. 7. Influence of receptor antagonists on the hepatic accumulation of [¹²⁵I]-IL10 or [¹²⁵I]-M6P-IL10. Five min before administration of a tracer amount of these radiolabeled cytokines to BDL-3 rats, an excess amount of M6PHSA (dotted bars) or SuchSA (striped bars) was administered to identify the involvement of M6P/IGFII and scavenger receptors in the hepatic uptake of the studied cytokines. HSA served as the control protein in these incubations (black bars). Values represent mean ± SD, n=4 rats per group, *=p<0.05.

Fig. 8. Gamma camera images of the body distribution of [¹²³I]IL10 and [¹²³I]M6P-IL10 in BDL-3 rats. Pictures show an overlay of recordings from t=20 to t=30 minutes after i.v. injection. The intensity of radioactivity per area is indicated by a color varying from dark blue (low intensity) to white (high intensity). The images show a high accumulation of M6P-IL10 in the liver region (L) in contrast to IL10, which is mostly distributed to the kidneys (K). n=3/group

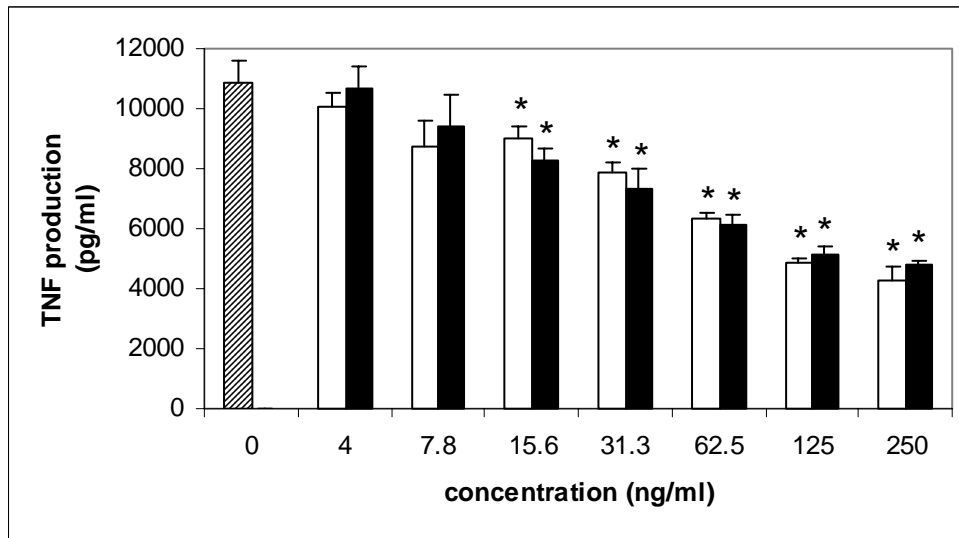


FIGURE 2

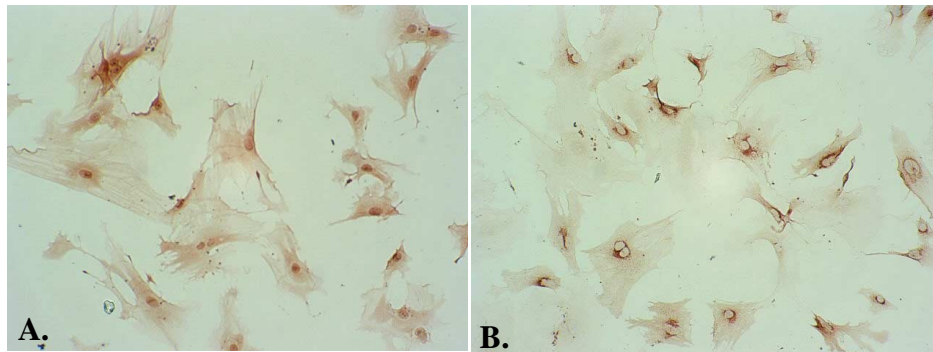


FIGURE 3

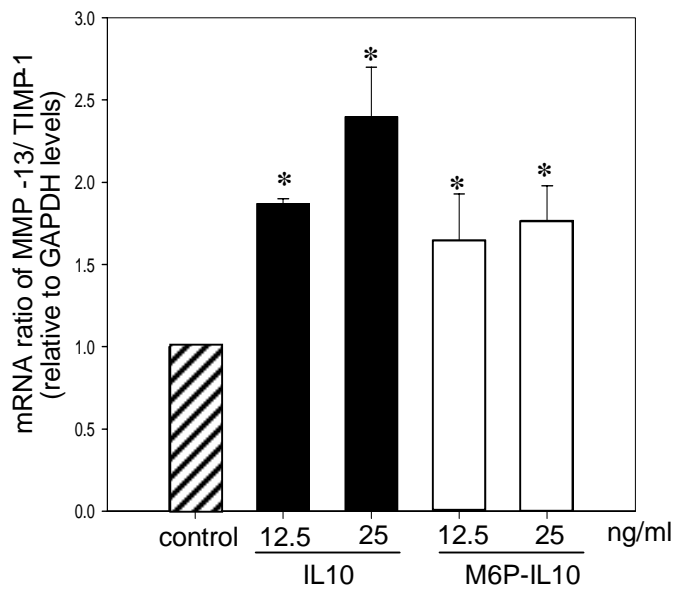


FIGURE 4

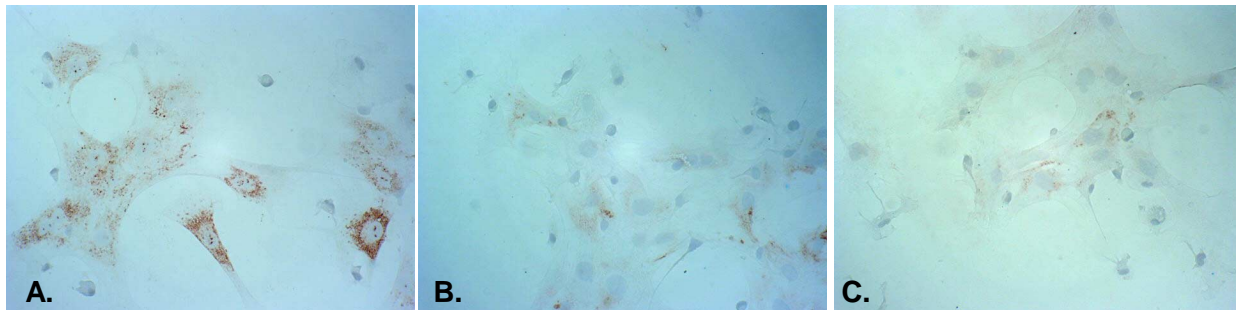


FIGURE 5

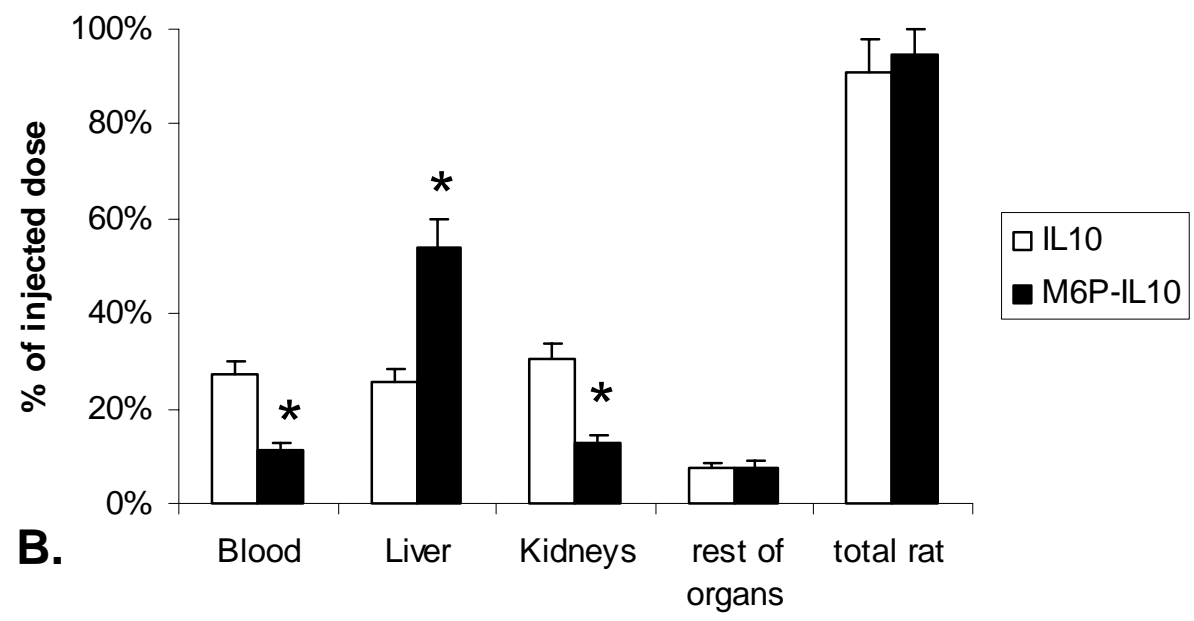
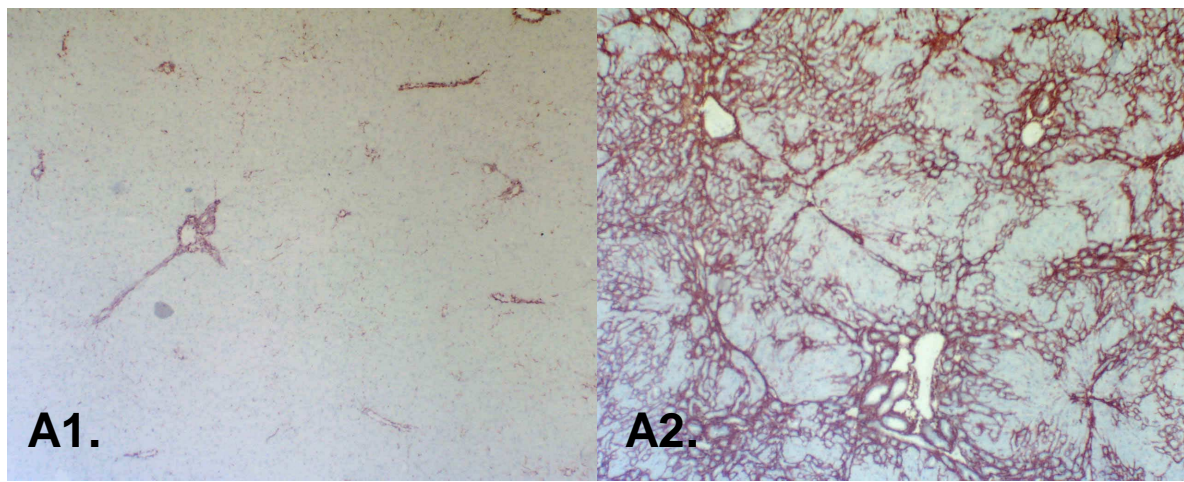


Figure 6

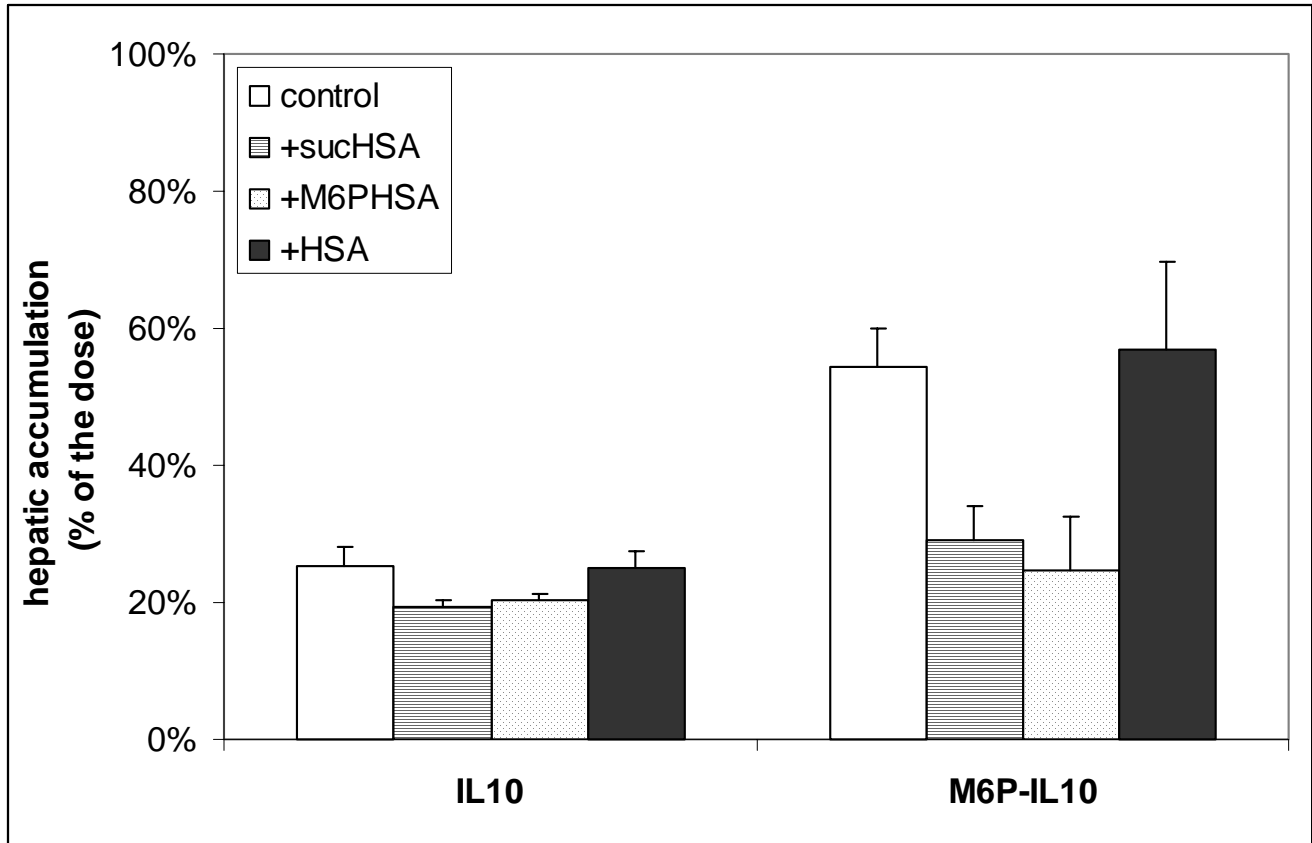


FIGURE 7

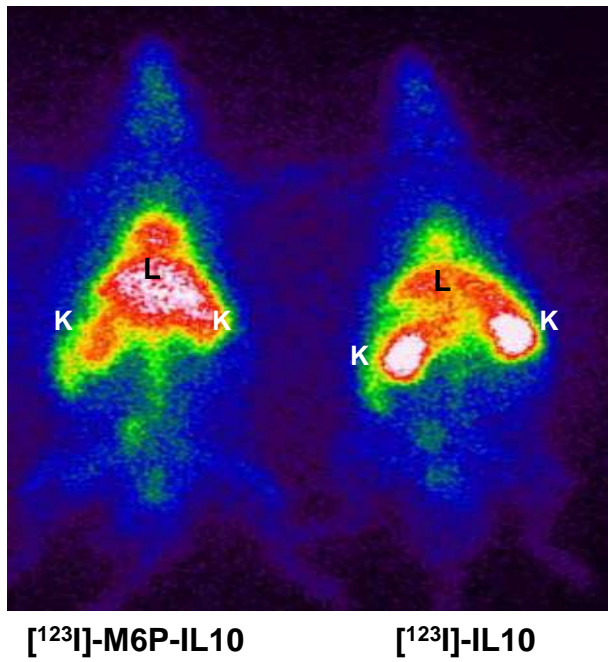


FIGURE 8