Journal of Controlled Release 162 (2012) 84–91

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

PEGylation of interleukin-10 improves the pharmacokinetic profile and enhances the antifibrotic effectivity in CCl_4-induced fibrogenesis in mice

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ARTICLE INFO

Article history:
Received 14 February 2012
Accepted 24 May 2012
Available online 31 May 2012

Keywords:
Interleukin-10
PEGylation
Pharmacokinetic profile
Liver fibrosis
Macrophage

ABSTRACT

Liver fibrosis represents a scar formation process as a response to chronic injury and a major cause of death worldwide. To date, no drug is available for this condition. Interleukin-10 (IL-10) has potent anti-inflammatory and antifibrotic properties but its short half-life in the circulation hampers its clinical use. Our aim was therefore to modify IL-10 with polyethylene glycol (PEG) to prolong its circulation time and enhance its effectivity. IL-10 was modified with 5 or 20 kDa PEG. The biological activity was preserved after PEGylation as assessed by inhibition of TNF-α production by macrophages. *In vivo*, during CCl_4-induced fibrogenesis in mice, both 5PEG–IL-10 and 20PEG–IL-10 showed a longer circulation time compared to IL-10, which was associated with a significant increased liver accumulation. Immunohistochemical analysis of fibrotic livers of mice receiving treatment with IL-10 or its PEGylated forms, revealed a decrease in markers reflecting HSC and KC activation induced by 5PEG–IL10. Transcription levels of IL-6 were decreased upon treatment with IL-10 and both PEGylated forms, whereas IL-1β levels were only down-regulated by 5PEGIL-10 and 20PEGIL-10. We conclude that PEGylation of IL-10 is a good strategy to attenuate liver fibrosis and that 5PEGIL-10 is the most effective conjugate.

1. Introduction

Liver fibrosis is a major cause of morbidity and death worldwide and represents a sustained wound-healing process in response to chronic injury, induced by chronic viral hepatitis, drugs or alcohol abuse, non-alcoholic steatohepatitis, autoimmune, parasitic, cholestatic or metabolic diseases [1,2]. The disease is characterized by an excessive deposition of collagens, mostly types I and III [1,3], which are mostly produced by activated hepatic stellate cells (HSC), the key pro-fibrogenic cells [1–3]. The end-stage is referred to as cirrhosis. A pharmacotherapy for this disease is unavailable [4,5]. As epidemiologic studies show that the prevalence of the disease will increase significantly in the coming years, a therapy for fibrosis is warranted [6].

During disease progression, the concerted actions of many cytokines control the activity of hepatic cells leading to a balance between pro- and anti-fibrotic activities. One of the few anti-fibrotic cytokines that is produced during fibrogenesis is interleukin-10 (IL-10).

IL-10 is expressed by different immune cell-types [7] and within the liver it can be produced by nearly all resident hepatic cells [8]. It is a potent immunomodulatory cytokine that downregulates several proinflammatory cytokines in macrophages, including IL-1β and IL-6, and decreases the expression of MHC class II molecules [8,9]. *In vitro*, it downregulates collagen 1 and increases expression of matrix metalloproteases in HSC and thus promotes degradation of collagens I and III [10,11]. It is also postulated that IL-10 can prevent activation of quiescent HSC and cause apoptosis of activated HSCs [12]. In 2003, a clinical trial in patients with hepatitis B-induced fibrosis, revealed a downregulation of fibrotic parameters, but the study was abandoned due to a flare-up of viral activity [13].

The rapid renal clearance of IL-10 that results in a low plasma half-life is one of the limitations to use this cytokine for the treatment of liver fibrosis [14]. One way to avoid this effect is the chemical attachment of polyethylene glycol to the cytokine (*i.e.* PEGylation) [15]. PEGylation can enhance the therapeutical potency of peptides and proteins by prolonging their half-life in the bloodstream [16–18].

PEGylation may affect the biological activity *in vitro* but *in vivo* experiments have shown that the increased circulation time can compensate this [19]. IL-10 was recently PEGylated and showed better effects than unmodified IL-10 in controlling neuropathic pain [18]. However, although IL-10 is a relevant cytokine and PEGylation has already proven its value for many cytokines, studies examining the biological effects of PEGylated IL-10 *in vivo* are scarce. Only local administration has been examined [20], and the effects have never been explored in experimental models of disease. Also a study on the optimal size of the PEG molecules, which greatly determines the circulation time and receptor binding, is completely lacking.

In the present study, we therefore modified IL-10 with two different sizes of PEG (5 kDa, 20 kDa) and examined the circulation times

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doi:10.1016/j.jconrel.2012.05.041

and biological effects in a CCl_4-induced acute model of liver fibrosis in mice. Our results show improved effects of PEGylated IL-10 compared to IL-10 on the fibrotic process and macrophages within the fibrotic liver. In particular its modification with the 5 kDa PEG molecule yields a long circulating and effective antifibrotic compound, which provides a strong basis for further studies on modified IL-10 as a therapeutic compound. Our studies also indicate that the effects of PEG–IL10 are mediated by a subset of macrophages within the liver.

2. Material and methods

2.1. Il-10 PEGylation and characterization

2.1.1. IL-10 PEGylation

Five micrograms of recombinant human IL-10 (rhIL-10, PeproTech EC Ltda., UK) was brought to a concentration of 50 μg/mL in 1× PBS buffer (0.01 M), pH 7.2–7.4. PEGylation, *via* the amine groups, was done overnight at 4 °C by adding 6.4 μL of 12.5 mg/mL 5 kDa mPEG-NHS or 50 mg/mL 20 kDa mPEG-NHS (NANOCS, USA; ratio 1:50), as described before [21]. The reaction was followed by 24 h dialysis against 1× PBS, using suitable dialysis membranes (Harvard Apparatus, Holliston, MA) to remove any unreacted PEG. Presence of unreacted IL-10 was checked in Western blots after 10% SDS-polyacrylamide gel electrophoresis and a subsequent silver staining according to Morrissey [22]. PEGylation was confirmed by barium–iodine PEGstaining of SDS-PAGE gel. Briefly, 200 ng of IL-10 and the PEGylated forms of IL-10 were loaded to the 10% SDS-PAGE. After the running, the gels were rinsed with water and fixated with perchloric acid (0.1 M) for 15 min and then washed with water. Subsequently, the gel was incubated in barium chloride (5%) for 10 min and developed using tritisol iodine solution (Sigma, Cambridge, UK). The gels were photographed using GBox (Syngene, Cambridge, UK). Products were stored at −20 °C. The protein concentrations used in the reactions were too low to be detected in protein assays and therefore the final protein concentrations were calculated based on the initial amount of IL-10 added to the reaction mixture.

2.2. Cells and cell line

2.2.1. RAW 264.7 - Mouse embryonic cell line

RAW 264.7 cells (ATCC, TIB-71) were cultured 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 of gentamicin (Gibco, Invitrogen), 2 mM of L-glutamine (Gibco, Invitrogen) and 0.48 M of L-arginine (Sigma, USA). Cells were used for experiments until a maximum of 20 passages.

2.3. Assessment of bioactivity in vitro

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 min with 50 ng/mL of IL-10, 5PEGIL-10 or 20PEGIL-10 in 0.1 mL FBS-free medium containing 0.5% normal mouse serum. At t = 0, 25 ng/mL of LPS (*E. coli*, List Biological Laboratories, INC., USA) was added to the wells. Control cells were not incubated with LPS. At t = 6 h, TNF-α levels in the culture media were determined using a sandwich TNF-α ELISA kit (BD PharMingen, USA). Assays were performed with two different batches of 5 and 20PEG–IL-10 and in triplicate for each batch.

2.4. Animals and experimental model of liver fibrosis

Pathogen-free male C57BL/6 mice weighing 20 to 22 g, purchased from Harlan (Zeist, The Netherlands) were used in this study. Animals were housed under laboratory conditions and received *ad libitum* normal diet. The experimental protocols for animal studies were approved by the Ethical Animal Committee of the University of Groningen. To induce acute liver fibrosis, mice received a single intraperitoneal injection of CCl_4 (1 mL/kg in olive oil). Animals were either used for distribution studies or effect studies.

2.4.1. Distribution studies

IL-10, 5PEGIL-10 and 20PEGIL-10 were labeled with ^{125}I according to standard methods [23]. Before each experiment, free iodine was removed with a PD-10 column (GE Healthcare). 72 h after the CCl_4 injection (1 mL/kg in olive oil), mice (n = 3 per group per time-point) were anesthetized with O_2/N_2O/isoflurane and received an i.v. injection (penile vein) of [^{125}I]IL-10, [^{125}I]5PEGIL-10 and [^{125}I]20PEGIL-10 (± 1,000,000 cpm per animal, in saline). After 15 min, 3 h and 24 h, blood and livers were harvested to check the amount of radioactivity. A gamma counter (Riastar Gamma Counting System; PerkinElmer Life and Analytical Sciences, Boston, MA) was used to measure the total amount of radioactivity in blood samples and livers. Liver samples were corrected for blood-derived radioactivity, as previously described [24].

2.4.2. Effect studies

To assess the effects of IL-10 and PEGylated forms of IL-10, on fibrotic parameters *in vivo*, we injected, *via* penile vein, CCl_4 treated-mice with vehicle (PBS), IL-10, 5PEGIL-10 or 20PEGIL-10, all in a dose of 10 μg/kg/day (n = 6 per group), 24 h and 48 h after CCl_4 administration. Subsequently, animals were sacrificed 24 h after the last injection (72 h after CCl_4 administration). Organs were taken out and prepared for further analysis.

2.4.3. Immunohistochemistry

To access the amount of activated HSCs, livers were fixated in 4% formalin and embedded in paraffin. After deparaffinization, 4 μm sections were stained for α-SMA according to standard methods using a monoclonal antibody (Sigma-Aldrich),and a biotinylated secondary anti-mouse IgG antibody. Staining was visualized using the Vectastain Elite ABC Mouse IgG kit (Vector Laboratories, Burlingame, CA). Expression of type III collagen, macrophages and antigen-presenting cells was examined in acetone fixated cryostat sections (4 μm) according to standard indirect immunoperoxidase methods [25] with goat anti-collagen III (SouternBiotech, USA), rat anti-F4/80 (Serotec) or rat anti-MHC class II (Santa Cruz Biotechnology, USA) IgGs, respectively. Staining was quantified by image analyzing techniques, using multiple alignment analysis (MIA). The results were given as percentages of positive-stained areas divided by the total area of the section.

2.4.4. Real-time PCR

Isolation of total RNA from mice livers was performed using the RNeasy kit (QIAGEN, Hilden, Germany), and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, NC). The reverse transcriptase reaction was done using random primers (Promega). The transcription levels of mice IL-1β (forward 5′GCCAAGACAGGTCGCTCAGGG3′ and reverse 5′CCCCCACACGTTGACAGCTAGG3′) and IL-6 (forward 5′TGATGCTGGTGACAACCACGGC3″ and reverse 5′TAAGCCTCCGACTTGT3′) were detected by quantitative real-time PCR methods with SensiMix™ SYBR kit (Bioline) on an ABI 7900HT apparatus (Applied Biosystems, Foster City, CA).

Quantification of data was performed *via* comparative ΔΔ Ct calculation (β-actin as housekeeping gene), and the gene expression levels in livers from CCl_4 treated animals were set at baseline.

2.4.5. Western blotting

Proteins were isolated from frozen tissue (30–40 mg) and samples (100 μg) were separated in a 10% sodium dodecyl sulfate-polyacrylamide (SDS) gel. Subsequently, proteins were transferred to a PVDF membrane, according to standard procedures, for 2 h. β-actin (Sigma, A5316, 1:5000) was used as a control for equal loading of