Treatment of experimental autoimmune uveoretinitis with intravitreal injection of infliximab encapsulated in liposomes

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ABSTRACT

Aims To evaluate the safety and efficacy of intravitreal injection of liposomes encapsulating infliximab in experimental autoimmune uveoretinitis (EAU) rats.

Methods Liposomes containing infliximab were prepared and characterised for mean particle size, entrapment efficiency, polydispersity index (PDI) and zeta potential. In vitro release profile and the stability of infliximab-lip were evaluated. EAU rats were intravitreally injected with saline, infliximab, infliximab-lip or unloaded liposomes. Clinical signs and ocular histological sections were graded. Infliximab concentrations were determined with competitive ELISA. Safety of the intravitreal injections was evaluated by electroretinography (ERG) and histopathological examination. Retinal biodistribution and clearance of rhodamine-conjugated liposomes containing infliximab were evaluated with a laser scanning confocal microscope.

Results The mean particle size of infliximab liposomes was 351.3±58 nm and entrapment efficiency was 90.65%±2.68%. PDI and zeta potential of infliximab liposomes were 0.386 and −20.8±9.78 mV, respectively. Stability test data showed that the infliximab-lip was stable for 60 days at room temperature. In EAU rats, intravitreal injection of infliximab and infliximab-lip greatly reduced intraocular inflammation determined by clinical scores and histopathological analyses (n=4). The mean concentrations of infliximab decreased quickly in infliximab injection group and were lower than those in infliximab-lip injection group (n=4 eyes, $p<0.05$ after 3 days post injection). No retinal toxic effects were detected after intravitreal injection of infliximab-lip in ERG (n=4 rats, $p>0.05$) and histopathological sections compared with normal rats. Confocal microscopy showed that fluorescent liposomes were observed in almost every layer of the retina and remained detectable for >30 days after injection.

Conclusions Intravitreal injection of liposomal infliximab can prolong the persistence of the drug in vitreous body and demonstrated a satisfactory safety and significant therapeutic potentials in EAU. The use of biodegradable particles for therapeutic antibody delivery may provide a promising approach for the treatment of ocular diseases.

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INTRODUCTION

Tumour necrosis factor alpha (TNF-α) is a multifunctional cytokine primarily released by macrophages, T lymphocytes and natural killer cells and it plays an important role in inflammation and immunity.[1] Anti-TNF-α therapy was proven to be effective in the treatment of rheumatoid arthritis,[2] Crohn's disease,[3] Behcet's disease[4] and ankylosing spondylitis.[5]

Infliximab is a human-mouse chimeric IgG1 antibody that inhibits the active soluble TNF-α and was shown to be effective in the treatment of patients with refractory uveitis.[6–9] However, adverse effects have been described when systemically administrated, including development of autoimmunity, demyelinating disease, propensity for infections, infusion reactions, neutropenia-thrombocytopenia and increased risk for malignancy.[10] Therefore, local application such as intravitreal injection with minimal systemic absorption is preferred.[11] Intraocular injection may also increase the amount of drug available to the retina and choroid and is routinely used for intraocular delivery of therapeutic molecules such as corticosteroids, antibiotics and anti-vascular endothelial growth factor agents.[12–14] Intravitreal injection of infliximab proved to be effective in suppressing the ongoing process of uveitis and improving the best-corrected visual acuity in patients with sight-threatening uveitis.[15] However, repeated injections would be necessary to achieve prolonged therapeutic level of infliximab, which may lead to adverse effects and complications.[16] In addition, high dose of intravitreal infliximab at the time of infusion may induce severe uveitis.[17] To improve the efficacy and safety of intravitreal delivery, a controlled drug-release system is required that are biodegradable and can maintain the therapeutic concentration of drugs within the vitreous body.

Recently, liposomes are of great interest for efficient drug delivery to intraocular tissues due to excellent biocompatibility and sustained drug release.[18] In this study, liposomes were prepared for intravitreal delivery of infliximab in an animal model of EAU.

MATERIALS AND METHODS

Preparation and characterisation of infliximab-lip

Liposomes containing infliximab (Remicade; Roche, Mannheim, Germany) were prepared by reverse-phase evaporation method.[19] Briefly, a mixture of 12 mg 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 8.1 mg cholesterol and 1 mg DSPE-PEG-NH_2 was dissolved in dichloromethane (4 mL). Infliximab was dissolved in phosphate buffered saline (PBS) to achieve a concentration of 3 mg/mL. Also, 4 mL of PBS

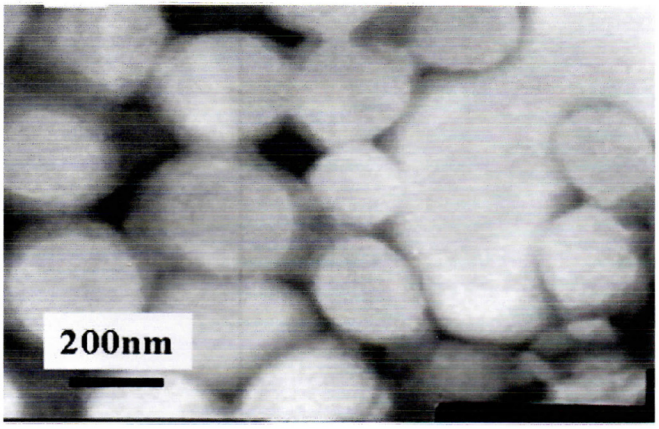

Figure 1 Transmission electron microscope image of the infliximab liposomes showed the circular shape with narrow size distribution.

containing 10 µL infliximab (aqueous phase) was added into organic phase. The mixture was sonicated and the resultant opalescent dispersion was rotary evaporated to remove the organic solvent (dichloromethane). The suspension was stored at 4°C. Rhodamine-conjugated liposomes (infliximab-Rh-lip) were also prepared as mentioned except that rhodamine B (0.3%) was added in PBS. For sterility, all these steps were performed under aseptic conditions. All glassware was sterilised by autoclaving, PBS was passed through a 0.22 µm membrane filter, and the entire procedure was carried out in a laminar flow hood.[20]

The mean particle size, polydispersity index (PDI) and zeta potential of unloaded and infliximab-loaded liposomes were determined by a laser scattering method (Mastersizer 2000, Malvern, UK). The formation and dispersion of liposomes were characterised by transmission electron microscopy (JEM-100CXII, JEOL, Japan). Encapsulation efficiency is defined as follows:

Entrapment efficiency (%)=$(W_o–W_n/W_o)\times 100$

where W_o is the total amount of infliximab used in the preparation and W_n is the amount of free infliximab detected in the supernatant.

In vitro release profile of infliximab from infliximab-lip was evaluated and the solution including infliximab-lip was dispersed in PBS at 37°C and 80 r/min. Samples were withdrawn at 1 hour, 3 hour, 6 hour, 10 hours, 1 day, 2 days, 4 days and 7 days and ultracentrifuged at 20 000 rpm. The samples were then subjected to ultraviolet and visible spectrophotometer analyses.

The stability of infliximab-loaded liposomes was evaluated by determining the particle size, PDI, zeta potential, cholesterol and infliximab leakage at room temperature (25°C±2°C) for a period of 90 days.

Induction of EAU in Lewis rats

Eight-week to ten-week-old inbred female Lewis rats, each weighing 150–180 g, were used in this study. All rats were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. EAU was induced by injection of 30 µg interphotoreceptor retinoid binding protein (IRBP) R16 peptide (a uveitogenic peptide) emulsified in complete Freund's adjuvant (Sigma, St. Louis, Missouri, USA) containing 2.5 mg/mL killed *Mycobacterium tuberculosis* H37Ra into the left hind footpads of the Lewis rats, as we described previously.[20]

Intravitreal injection protocols

Ten days after induction of EAU, Lewis rats were anaesthetised by intraperitoneal injection of 0.15 mL pentobarbital. Pupils were dilated by instillation of one drop of tropicamide 5%. One drop of tetracaine 1% was administered for local anaesthesia. Rats were randomly assigned to 1 of 4 groups and were treated with a single intravitreal injection (5 µL) of PBS, infliximab (40 µg), infliximab-loaded liposomes (encapsulated infliximab 40 µg) or unloaded liposomes in both eyes using a sterile microsyringe, as previously described.[21] Four rats chosen randomly from each group were examined with a slit-lamp biomicroscopy for 25 days, whereas the other rats in these groups were humanely killed at different times after injection for further study. Four normal rats were injected intravitreally with infliximab-lip to determine the safety of the treatment.

To quantify the concentration of infliximab in the vitreous body of injected eyes and peripheral blood, EAU rats that received infliximab or infliximab-lip were humanely killed at 1 hour, 6 hours, 24 hours, 3 days, 7 days, 14 days, 21 days, 28 days, 45 days and 60 days after intravitreal injection. The eyes were immediately enucleated and the vitreous bodies were collected and infliximab concentrations were determined with competitive ELISA, as described previously,[22] with a lower detection limit of 0.01 ng. Samples of blood were also collected and the concentrations of infliximab were measured in the sera of rats intravitreal injected with infliximab-lip and free-infliximab.

Clinical and histopathological studies after intravitreal injection

Eyes were examined daily after IRBP R16 immunisation independently by two blinded observers to assess the clinical signs of uveitis using a slit-lamp biomicroscope. Clinical signs were scored as follows: 0, normal; 1, minimal signs of inflammation with occasional cells in the anterior chamber; 2, presence of mild exudate in the anterior chamber; 3, moderate exudates in the anterior chamber or moderate exudate in the posterior chamber; 4, large exudate within the anterior chamber or massive posterior chamber hypopyon; 5, gross orbital oedema and exophthalmos in addition to large exudate within the anterior chamber or massive posterior chamber hypopyon.[23]

EAU rats were humanely killed on days 4 and 7 after intravitreal injection. The eyes were immediately enucleated in each group and fixed in 10% formaldehyde. Sections (5 µm thick) were stained with H&E and were examined with a light microscope. Histological grading was as follows: 0, no inflammatory cell infiltration and no destruction of the retina; 1, minimal cell infiltration in the retina and choroid but no destruction; 2, partial and mild destruction of the outer retina; 3, moderate destruction of the outer retina; 4, extensive and severe destruction of the outer retina and partial destruction of the inner retina; 5, complete destruction of the entire retina.[24] Histological assay was also performed in the eyes of normal rats after intravitreal injection of infliximab-lip and infliximab in solution to investigate the safety of the administration.

Electroretinography

Retinal function was evaluated by scotopic electroretinography (RETI-port, Roland Consult, Germany) at 2, 7 and 30 days after intravitreal injection of infliximab-lip in normal rats. ERG was also detected in normal rats receiving intravitreal injection of infliximab in solution and those without intravitreal injection. Rats were dark adapted for 12 hours after