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Pharmacokinetics and safety of intravitreally delivered etanercept

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Abstract *Background:* The anti-inflammatory drug etanercept may be an effective therapeutic agent in diabetic retinopathy. In order to further evaluate its potential, the pharmacokinetics and safety of this drug after intravitreal delivery were investigated. *Methods:* After intravitreal administration of etanercept in rabbits, clinical examination, electroretinography (ERG), visually evoked potentials (VEP) and histology were evaluated. The pharmacokinetics and distribution of etanercept were analyzed using fluorescence-coupled protein at 0, 2, 4, and 8 weeks after injection in vitreous, retina, and choroid. *Results:* No adverse effects and signs of toxicity were found. Etanercept showed peak concentrations after 4 weeks in the retina and choroid. *Conclusions:* Intravitreally delivered etanercept is safe and results in high concentrations in the retina and choroid over a long period of time.

Introduction

Increased leukocyte adhesion to the vascular endothelium is one of the earliest events of inflammation and neovascularization. Besides the well-known inflammatory diseases such as uveitis in various forms, diseases such as diabetic retinopathy also show signs of inflammation. In diabetic retinopathy increased leukocyte adhesion to the vascular endothelium has been demonstrated in multiple settings [3, 5, 13, 16]. The altered binding results in early blood–retina barrier breakdown, capillary nonperfusion, and endothelial cell injury and death. The adhesion is mediated in part by intercellular adhesion molecule-1 (ICAM-1) [12], which is expressed on endothelial cells and binds to α2-integrins (including CD18) expressed on leukocytes [3, 11].

Various mediators contribute to the up-regulation of endothelial cell and leukocyte adhesion molecules. Tumor necrosis factor α (TNFα) is a proinflammatory cytokine that has been implicated in this process. TNFα expression is upregulated in the extracellular matrix, endothelium, and vessel walls of fibrovascular tissue of eyes with proliferative diabetic retinopathy, and TNFα protein levels are elevated in the vitreous from eyes with this condition [7, 9, 10, 17]. The administration of neutralizing antibodies against ICAM-1 or CD18 in a rat model of diabetic retinopathy markedly reduces leukocyte adhesion and, as a consequence, blood–retina barrier breakdown and endothelial injury [5]. Administration of a soluble

TNF-receptor–Fc fusion protein (etanercept) has a similar effect, causally linking TNFα to leukocyte adhesion. Additionally, TNFα might also have a direct effect on retinal cell apoptosis and thus reduce endothelial cell death [8].

Etanercept is a soluble fusion protein consisting of the extracellular ligand-binding portion of human TNF receptor (p75) linked to an Fc portion of human IgG_1. It contains 934 amino acids and has an apparent molecular weight of 150 kDa. Its main therapeutic indication is the treatment of rheumatoid and psoriatic arthritis. Ongoing clinical studies are testing the effect of TNFα inhibitors in systemic treatment for various diseases such as uveitis, Behçet's disease, and Wegener's granulomatosis.

Due to the immunosuppressive action and reported systemic side effects of etanercept, local delivery appears favorable. Concerns have been expressed regarding the risk of lymphomas and congestive heart failure [2]. For the treatment of diabetic macular edema, substances such as triamcinolone or VEGF antagonists are being investigated in clinical trials. However, there is evidence suggesting that etanercept may be also a potent and even more specific therapeutic agent for patients with diabetic retinopathy. In the current study, we therefore investigated the pharmacokinetics and safety of local administration of the TNFα fusion protein etanercept.

Material and methods

Animals

Male pigmented rabbits (Charles River, France) weighing approximately 2,000 g were used in all experiments. All protocols abided by the ARVO (Association for Research in Vision and Ophthalmology) statement on the "Use of Animals in Ophthalmology and Vision Research" and were approved by the Animal Care and Use Committee of the Regierungspräsidium Köln. The animals were fed standard laboratory food and allowed free access to water in an air-conditioned room with a 12-h light–dark cycle. The animals were anesthetized with ketamine (40 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (4 mg/kg; Rompun, Bayer, Leverkusen, Germany) prior to all experimental manipulations.

Fluorescence labeling of etanercept

A quantity of 0.5 mg of etanercept in water was ultrafiltrated with a 20-kDa membrane by using phosphate-buffered saline (PBS) pH 7.2, then 0.1 M borate buffer pH 9.5 to a final volume of 0.5 ml. The protein concentration was 0.48 mg/ml. Then 0.15 mg fluorescein isothiocyanate (FITC) on Celite was added to the solution under stirring in the dark for 1 h. After centrifugation, the solution was applied to a Sephadex PD10 column and eluted with PBS. The yellow high-molecular fractions eluting between 3.5 and 5 ml were combined and used for the in vivo experiments.

Detection of etanercept fragments in biological samples

Eyes were enucleated and vitreous, retina, and choroid were dissected under a microscope. A quantity of 50 µl of PBS was added to the biological samples, and they were vortexed and centrifuged at 10,000 *g*. Next, 20 µl of each sample was analyzed on a Pharmacia 75HR 5/20 high-performance gel filtration column. This column operated at a flow rate of 0.4 ml/min using PBS pH 7.2. The effluent was passed through a Merck ultraviolet detector and a fluorescence spectrophotometer (490/520 nm) set up in series. The signals were recorded on a D2500 integrator (Merck/Hitachi).

Administration of etanercept

Soluble p75 TNF-α receptor–Fc fusion protein (etanercept; Enbrel, Wyeth Pharmaceuticals, Münster, Germany) was reconstituted with sterile water according to the manufacturer's instructions. Animals received either etanercept or solvent alone. The drug was administered intravitreally in a volume of 0.1 ml in sterile BSS. Injections were performed at 1.5 mm from the limbus, between ora serrata and ciliary body. Doses were calculated according to the previous experience with systemic treatment in a rat model and based on the recommendation of the manufacturer for treatment of rheumatoid arthritis in humans (Amgen and Wyeth Pharmaceuticals).

Electrophysiology

Electroretinograms (ERG) and visually evoked potentials (VEP) were elicited in rabbits under general anesthesia using single bright xenon flash light stimulation. The ERG was recorded in both eyes with corneal electrodes (jet electrodes, Universo Plastique, Switzerland). The reference electrode (Ag–AgCl, skin) was placed on the forehead. The VEP was recorded with an occipital skin electrode and a reference electrode on the forehead (both Ag–AgCl). ERG and VEP were amplified using Grass P122 amplifiers. Average waveforms were recorded with a digital storage oscilloscope. The recordings of the untreated eye served as control.

Histology

Eyes were enucleated and fixed in 4% formaldehyde for 24 h before paraffin embedding and sectioning. Serial sections were stained with hematoxylin and eosin and evaluated under a light microscope. For comparison, defined areas of each retina were captured using a CD-330 charge-coupled device (CCD) camera (Dage-MIT, Improvision, Heidelberg, Germany) attached to a Zeiss microscope (Zeiss, Oberkochem, Germany). The images were captured on an Apple G4 Computer (Apple, Cupertino, CA) and analyzed using Openlab software (Improvision). The number of cell layers was determined in a masked fashion.

Results

Clinical evaluation

After intravitreal injection of 100 µg etanercept, no clinical signs of toxicity were observed in all animals throughout the follow-up time for up to 2 months. Cornea and lens remained clear. Using indirect ophthalmoscopy, the retina appeared clinically normal. There was no vitreous haze or opacities within the vitreous cavity.

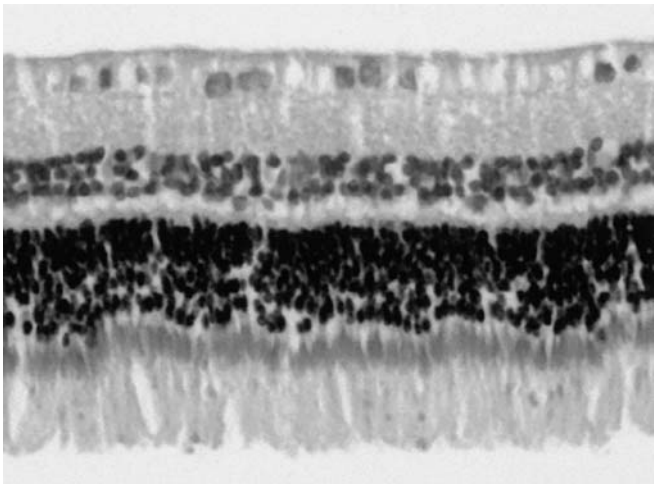

Fig. 1 Section of a rabbit retina 8 weeks after injection of 100 µg etanercept into the vitreous. No signs of toxicity are found. The retina shows normal tissue structures

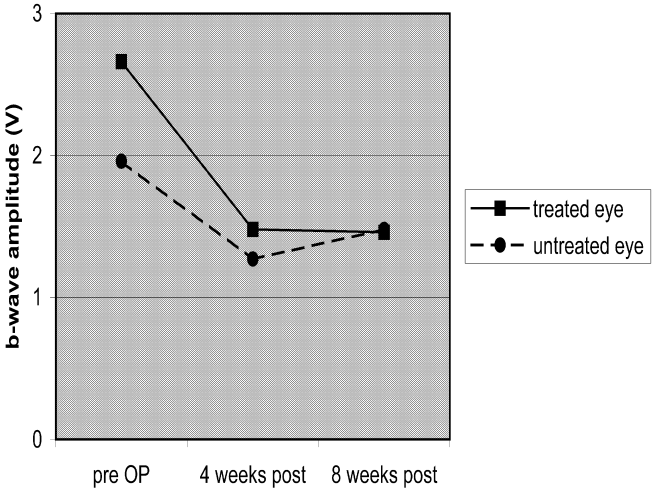

Fig. 2 The b-wave amplitudes in ERG recordings. Both treated and untreated eyes were measured 4 and 8 weeks after injection of 100 µg etanercept

Histology

The histology of both treated and control eyes after intravitreal administration of 100 µg etanercept were not distinguishable and showed normal anatomy (Fig. 1). No signs of toxicity were found. Quantification of retinal layers demonstrated no difference in either group.

Electrophysiology

A decrease in ERG b-wave amplitude was observed in both treated and control eyes 4 and 8 weeks after injection

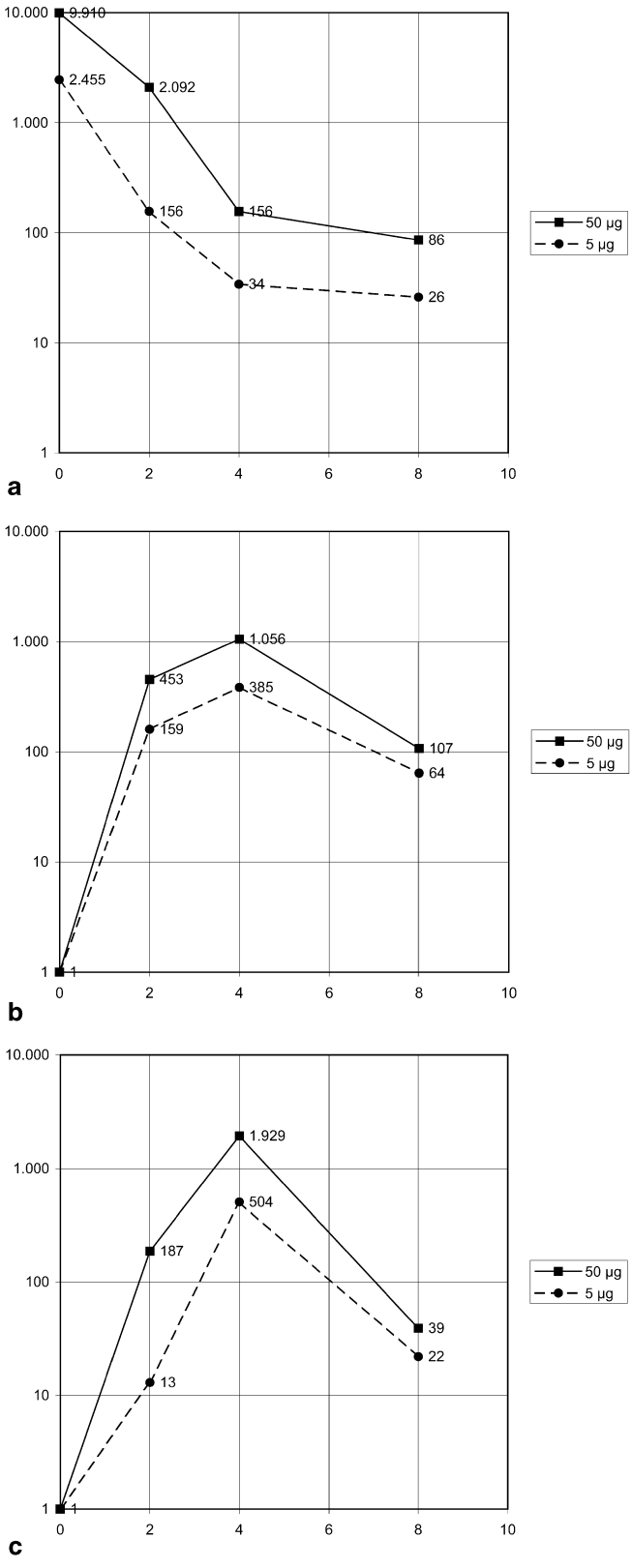

Fig. 3a–c Etanercept in various tissues at 0, 2, 4, and 8 weeks after injection (amount of protein in relative fluorescence units): **a** in vitreous; **b** in retina; **c** in choroid

of 100 μg etanercept. Comparison of treated and control eyes showed no reduction of b-wave amplitude as an effect of treatment (Fig. 2). VEP could be elicited before and after treatment.

Pharmacokinetics

Etanercept was measured in vitreous, retina, and choroid at 0, 2, 4, and 8 weeks after injection of 5 μg and 50 μg fluorescein-coupled etanercept into the vitreous. In the vitreous, a peak value was measured directly after injection which gradually declined to the 8th week after injection. In both the retina and the choroid, the highest values were found 4 weeks after injection. But even 8 weeks after injection, etanercept was still found in retina and choroid (Fig. 3).

Discussion

Experiments with etanercept in a rat model of diabetic retinopathy demonstrated that the drug reduces leukocyte adhesion, blood–retina barrier breakdown and endothelial injury [8]. Therefore, diabetic retinopathy may be a new therapeutic indication for this agent apart from its main indication in rheumatoid arthritis. As the intravitreal administration would minimize systemic side effects and deliver the drug locally, the pharmacokinetics and safety of this route of administration were investigated.

After intravitreal injection of high doses of etanercept (100 μg per eye), no signs of toxicity were found on evaluation of data from clinical examination, histology, ERG, and VEP. Etanercept did not induce any pathological changes after an observation time of up to 8 weeks. Function and structure were the same as in control eyes. The vitreous showed no opacities or bands formation. Such signs of toxicity can be found, for example, with intravitreal amphotericin B injections. With increasing doses (10–50 μg), retinal ganglion cell loss and focal necrosis of the nerve fiber layer became also apparent [4]. Daunomycin used for inhibiting proliferative vitreoretinopathy has been reported to be toxic at doses of 5 μg in the vitreous [6].

Fluorescence-labeled etanercept was injected in two doses (5 μg and 50 μg) intravitreally and the amount of the protein was determined in vitreous, retina, and choroid at 0, 2, 4, and 8 weeks after injection. By using gel filtration, only the amount of intact etanercept was determined. Therefore, a decrease in biological activity by decay of etanercept can be ruled out. In the vitreous, the amount of etanercept gradually declined up to the 8th week after injection. In both the retina and the choroid, a slow accumulation was found with a peak at 4 weeks. After 8 weeks, etanercept was still clearly detectable. The pharmacokinetics of etanercept after intravitreal delivery ensure high concentrations of the drug in the target tissues over several weeks and offer the chance of a successful therapy. The relatively high molecular size of etanercept results in slower clearance than, for example, with triamcinolone [14, 15]. After injection of 400 μg triamcinolone acetonide, the half-life as determined by HPLC was 1.6 days [15]. Dexamethasone, another glucocorticoid, has a half-life of only 2.5 h in physiologic saline [1, 18].

In summary, the results show that intravitreally delivered etanercept is safe and leads to high concentrations in the retina and choroid over several weeks.

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