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Original

Effects of Subconjunctival Injection of Anti-Vascular Endothelial Growth Factor Antibody on Oxygen-Induced Ischemic Retinopathy in a Neonatal Rat Model

Junkichi KABAYAMA[1)], Yuta SAITO[1)], Takako NAKANISHI-UEDA[2)], Toshihiko UEDA[1)], Michiko MATSUBARA[1)], Yoshihiro WADA[1)], Hideaki OKAWADA[1)], Tadashi HISAMITSU[2)] and Ryohei KOIDE[1)]

Abstract : The present study investigated the effects of subconjunctival injections of an anti-rat vascular endothelial growth factor (anti-VEGF) antibody on oxygen-induced retinopathy (OIR) in a neonatal rat model. OIR was induced by daily cycles of 80% oxygen (20.5 h), room air (0.5 h), and a progressive return to 80% oxygen (3 h) for 12 days [until postnatal day (P) 12]. On P12, rats received subconjunctival injections in their right eye of 0.1 or 1.0 μg anti-VEGF antibody (or 1.0 μg goat IgG as a control). No injections were made into the left eye. On P18, rats were killed and their retinas were removed and flat-mounted before being stained with adenosine diphosphatase. Retinal neovascularization (NV) was scored and the extent of avascular areas, as a percentage of total retinal area (%AVA), was determined using image analysis. Although there was a tendency for lower mean NV scores in eyes injected with 0.1 and 1.0 μg anti-VEGF compared with control (4.3 ± 1.1, 2.3 ± 1.0, and 6.7 ± 1.3, respectively ; n = 10–13), the difference failed to reach statistical significance. Similarly, although there was a tendency for mean %AVA to be lower in the injected eyes for both the 0.1 and 1.0 μg anti-VEGF groups compared with control (15 ± 3%, 13 ± 3%, and 25 ± 4%, respectively ; n = 10–13), the differences were not significant. Similar tendencies were observed in the contralateral eyes. Although further studies using larger numbers of rats are needed to obtain statistically significant results, the results of the present study suggest that the subconjunctival injection of anti-VEGF antibody may prove to be a useful route of administration in conjunction with intravitreal injections, which are the generally used method at present. However, careful attention should be paid to the possibility of systemic side effects.

Key words : retinopathy of prematurity, retinal neovascularization, vascular endothelial growth factor, subconjunctival injection, oxygen-induced retinopathy

[1)] *Department of Ophthalmology, Showa University School of Medicine*, 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142–8666, Japan.
[2)] *Department of Physiology, Showa University School of Medicine.*

Introduction

In recent years, developments in perinatal care have contributed to decreased mortality among low birth-weight infants. Consequently, the treatment rate for retinopathy of prematurity (ROP) has increased. Despite current treatments, ROP remains a common, potentially blinding disease in babies who are born prematurely[1]. Retinal photocoagulation, cryoretinopexy, scleral buckling, and vitreous surgery are established treatments for ROP[2]; however, surgery for severe ROP, such as retinal detachment, has a less favorable prognosis[3]. Therefore, the prevention of ROP is extremely important and several studies have investigated ways in which to reduce ROP, such as the use of vitamin E prophylaxis[4] and adjusting oxygen administration to newborns[5]. Recent clinical studies of intravitreal injections of the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab for the treatment of ROP have demonstrated good safety and efficacy in the short term for cases of severe ROP[6–8]. In addition to these small-scale clinical studies[6–8] using intravitreal bevacizumab for the treatment of ROP, bevacizumab is widely used to treat angiogenic disorders, including age-related macular degeneration, proliferative diabetic retinopathy, neovascular glaucoma, and macular edema related to retinal vein occlusion[9, 10]. However, intravitreal injections may result in several local complications, such as endophthalmitis, increased intraocular pressure, retinal detachment, and intraocular hemorrhage. Furthermore, intravitreal injections in infants carry a greater risk of lens centesis because infants have a relatively large lens volume for the eyeball. In contrast, subconjunctival injections are associated with fewer local complications, and the technique is simple, safe, and amenable to rapid bedside administration. Subconjunctival injections of bevacizumab have been reported in the treatment of corneal neovascularization, pterygium, and bleb maintenance after filtering surgery for glaucoma[11–13]; however, the use of subconjunctival bevacizumab for the treatment of ROP has not yet been reported. Thus, in the present study we investigated the effects of the subconjunctival administration of anti-VEGF antibody on retinal neovascularization (NV) and avascular areas (AVA) in a rat oxygen-induced retinopathy (OIR) model of ROP.

Methods

Animal model

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research: http://www.arvo.org/. The Institutional Committee of Animal Care and Use at Showa University approved the study protocol.

Female Sprague-Dawley rats at 14 days gestation were purchased from CLEA Japan (Tokyo, Japan). Rats were divided into two groups: those exposed to room air and those exposed to oxygen. In both groups, 13 or 14 neonatal rats were kept with a dam in each cage after birth. The dams were rotated between the room air- and oxygen-exposed groups every 2 days during the experimental period. Retinal NV was induced in neonatal

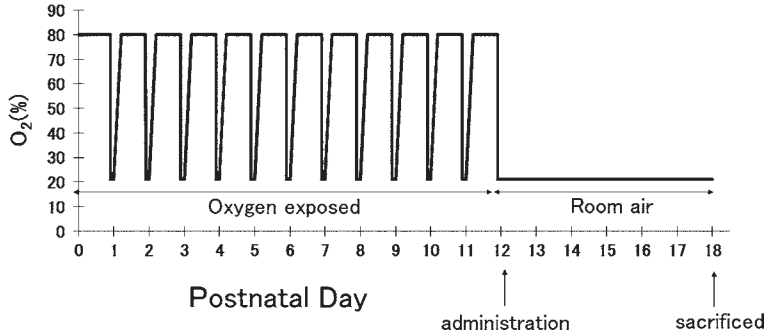

Fig. 1. Treatment schedule for the oxygen-induced retinopathy (OIR) model. Rats were exposed to daily cycles of 80% oxygen (20.5 h), room air (0.5 h), and a progressive return to 80% oxygen (3 h) until postnatal day (P) 12, and then placed in room air until P18. On P12, rats were administered a subconjunctival injection of anti-vascular endothelial growth factor (VEGF) antibody.

rats using a standard protocol, as described previously[14, 15]. The oxygen-exposed rats were exposed from birth until postnatal day (P) 12 to daily cycles of 80% oxygen (20.5 h), room air (30 min), and a progressive return to 80% oxygen (3 h) in an Oxycycler (Biosperix, Lacona, NY, USA). On P12, the oxygen-exposed rats were placed in room air until the end of experiments at P18 (Fig. 1).

Treatment schedule

On P12, rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (15 mg / kg) and xylazine (25 mg / kg). The right eye was injected subconjunctivally with 0.1 or 1.0 μg anti-rat VEGF antibody (R&D Systems, Minneapolis, MN, USA) or 1.0 μg normal goat IgG (R&D Systems) as a control. The left eye was not injected (contralateral eye).

Retinal processing

On P18, some rats were killed by intraperitoneal injection of 100 μL / 10 g body weight sodium pentobarbital (50 mg / mL). Both eyes were enucleated and then fixed for 24–48 h in 4% paraformaldehyde (Polyscience, Warrington, PA, USA) in 0.1 M cacodylate buffer, pH 7.2 (Sigma, St. Louis, MO, USA). Anterior segments were removed and retinas with intact ora serratas were carefully dissected, with care taken to remove the hyaloid vessels and any remaining vitreous. The retinas were stained with adenosine diphosphatase (ADPase) and flat-mounted[14]. Digital images of ADPase-stained retinas were obtained using a camera and scanner (Nikon, Tokyo, Japan), with retinal NV scored according to the method of Hasebe *et al*[15], e.g. score 4 : long ridge, 3 : short ridge, 2 : five or more glomerular buds, 1 : less than five glomerular buds, and 0 : none observed. The AVA was also determined in ADPase-stained retinas as the mean percentage of total retinal area using image J software (National Institutes of Health, Bethesda, MD, USA).

Fresh tissue preparation

On P12, 3 h after anti-VEGF antibody treatment (P12 + 3h), as well as on P13, P14, and P18, rats were killed as described above. Blood was collected from the left ventricle and plasma was obtained by centrifugation at 6,700 × g for 5 min at 4°C. Eyes were enucleated and the retinas were isolated under a microscope before being placed in 100 μL tissue protein-extraction reagent (T-PER ; Thermo Fisher Scientific, Rockford, IL, USA) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA ; 1 : 100) for VEGF protein and goat IgG analysis. The tissue was homogenized, centrifuged at 9,700 × g for 15 min at 4°C, and the lysate collected. Lysate protein concentrations were determined using a BCA protein assay reagent kit (Thermo Fisher Scientific) with bovine plasma albumin as the standard.

Quantification of VEGF protein and IgG

VEGF was assayed using a Quantikine rat VEGF Immunoassay kit (R&D Systems), whereas the retinal and plasma concentrations of anti-rat VEGF goat antibody were determined using a goat IgG ELISA Quantitation Kit (BETHYL, Montgomery, TX, USA). Both assays were performed in accordance with the manufacturers' instructions.

Statistical analysis

Statistical analyses were performed with the Kruskal-Wallis test or ANOVA. All data are shown as the mean ± SE. Results were considered significant at $P < 0.05$.

Results

Body weight increased similarly in rats treated with 0.1 or 1.0 μg anti-rat VEGF antibody (anti-VEGF) or 1.0 μg IgG (control). OIR retinas exhibited neovascular changes and avascular areas (Fig. 2). The NV scores in the injected and contralateral eyes were 6.7 ± 4.7 and 7.2 ± 2.5, respectively, in the control group (n = 10), 4.3 ± 3.1 and 4.2 ± 3.0, respectively, in the 0.1 μg anti-VEGF-injected group (n = 13 ; $P = 0.07$ vs. control), and 2.3 ± 1.6 and 2.8 ± 3.0, respectively, in the 1.0 μg anti-VEGF-injected group (n = 12 ; $P = 0.07$ vs. control Fig. 3). Although there was a tendency for dose-dependent decreases in NV in both the injected and contralateral eyes in the antibody-injected groups, the differences failed to reach statistical significance ($P = 0.07$). Similarly, there was a tendency for dose-dependent decreases in %AVA in both the injected and contralateral eyes in the antibody-injected groups, but the differences were not statistically significant ($P = 0.07$). Specifically, %AVA in the injected and contralateral eyes was 25 ± 4% and 25 ± 4%, respectively, in the control group ; 15 ± 3% and 15 ± 3%, respectively, in the 0.1 μg anti-VEGF-injected group ; and 13 ± 3% and 12 ± 3%, respectively, in the 1.0 μg anti-VEGF-injected group (Fig. 4). In the 1.0 μg anti-VEGF-injected group, there was a significant increase in VEGF protein from P12 to P13 in OIR retinas following subconjunctival injections (from 86.6 ± 43.0 to 544.4 ± 54.9 pg /

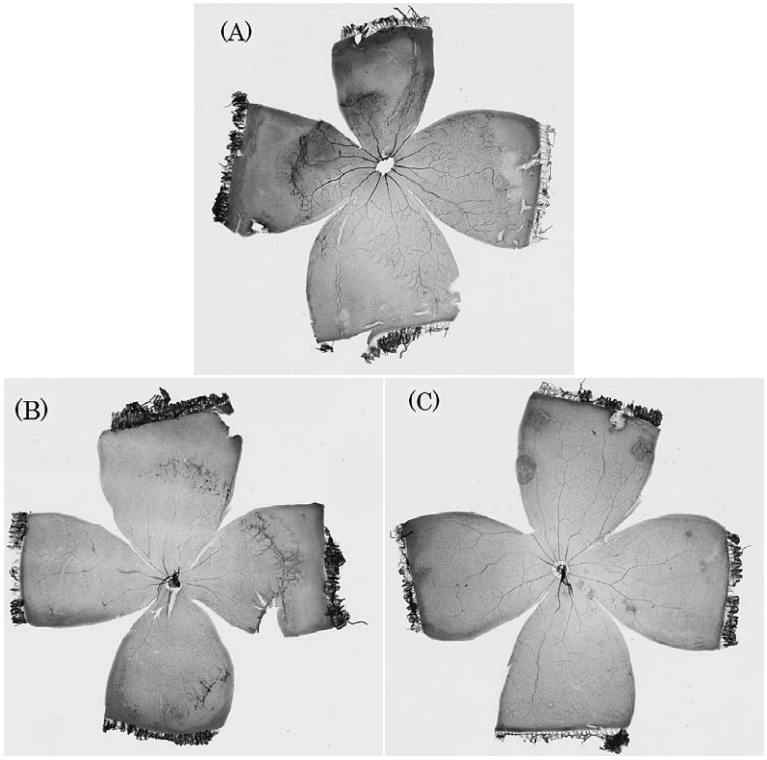

Fig. 2. Typical examples of flat-mounted adenosine diphosphatase (ADPase)-stained retinas on postnatal day (P) 18 in oxygen-induced retinopathy (OIR). (A) Control (1.0 μg IgG-injected) eye (neovascularization (NV) score 7; extent of avascular areas as a percentage of total retinal area (%AVA) 40.4%); (B) 0.1 μg anti-vascular endothelial growth factor (VEGF) antibody-injected eye (NV score 10, %AVA 15.5%); and (C) 1.0 μg anti-VEGF antibody-injected eye (NV score 0, %AVA 0%).

mg protein; n = 3; $P = 0.001$), as well as in the contralateral eye (516.2 ± 169.9 pg / mg protein; n = 3; $P = 0.001$), but levels decreased on P14 in both the injected and contralateral eyes (196.4 ± 65.1 and 219.4 ± 28.7 pg / mg protein, respectively; n = 3; Fig. 5). Thus, the anti-VEGF injections did not affect VEGF levels in either eye. In the retina, antibody concentrations in the injected and contralateral eyes increased gradually to 71.3 ± 41.1 and 92.5 ± 54.4 ng / mL, respectively, on P13 (n = 4), and to 76.7 ± 38.3 and 101.7 ± 51.0 ng / mL, respectively, on P14 (n = 3). On P18, the IgG concentrations in the injected and contralateral eyes had decreased to 0 and 48.3 ± 48.3 ng / mL, respectively (n = 3).

Discussion

Following subconjunctival injection, drugs must penetrate the sclera and choroid to affect eye function. The molecular weight of anti-human VEGF antibodies for clinical ophthalmologic use is in the range 50–150 kDa, which means they are able to penetrate a human

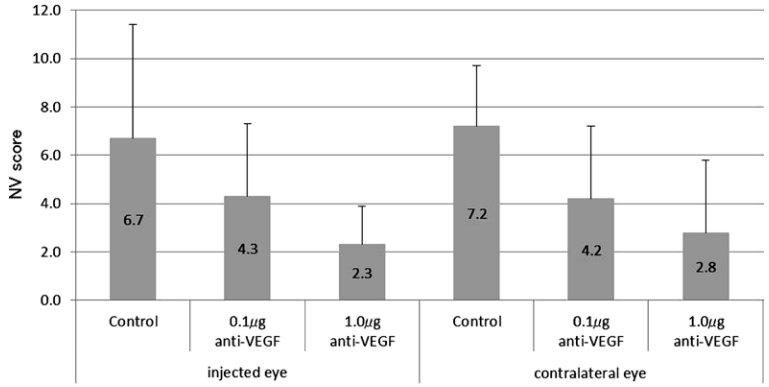

Fig. 3. Effects of subconjunctival injections of anti-vascular endothelial growth factor (VEGF) antibody on the neovascularization (NV) score in oxygen-induced retinopathy (OIR). The NV score tended to decrease in a dose-dependent manner in both the injected and contralateral eyes ($P = 0.07$). Data are the mean ± SE (n = 10 in the control group; n = 13 in the 0.1 μg anti-VEGF group; n = 12 in the 1.0 μg anti-VEGF group).

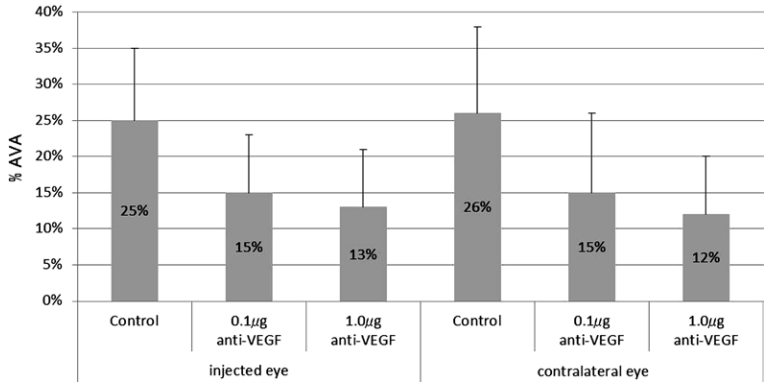

Fig. 4. Effects of subconjunctival injections of anti-vascular endothelial growth factor (VEGF) antibody on the extent of avascular areas as a percentage of total retinal area (%AVA) in oxygen-induced retinopathy (OIR). The %AVA tended to decrease in a dose-dependent manner in both the injected and contralateral eyes ($P = 0.07$). Data are the mean ± SE (n = 10 in the control group; n = 13 in the 0.1 μg anti-VEGF group; n = 12 in the 1.0 μg anti-VEGF group).

sclera[16]. Subconjunctival anti-VEGF antibody injections should be particularly efficacious in infants because the sclera is thinner in infants' eyes than in adults. In the present study, the antibody concentration in the retina of the injected eye started to increase after injection of the antibody, peaking by 24 h. However, the pharmacokinetics of the effects of the antibody on the retina following subconjunctival injection should take into consideration both drug penetration through the sclera and choroid, as well as drug delivery via the

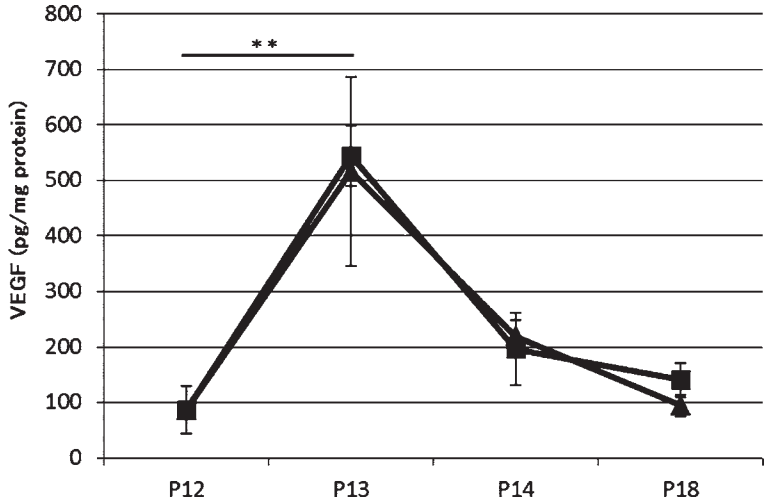

Fig. 5. Effects of subconjunctival injections of anti-vascular endothelial growth factor (VEGF) antibody on retinal VEGF content. VEGF levels increased significantly on postnatal day (P) 13 in both the antibody-injected (closed squares) and contralateral (closed triangles) eyes. Data are the mean ± SE pg / mg protein [n = 3 for each point except for the contralateral eye on P14 (n = 2) and P18 (n = 2)]. $^{**}P < 0.01$ compared with P12 values each group.

Table 1. The concentration of goat IgG in retina.

OIR	Postnatal Day Age				
	P12	P12 + 3h	P13	P14	P18
injected eye (ng/mL)	0 ± 0 (n = 3)	21.5 ± 4.5 (n = 2)	71.3 ± 41.1 (n = 4)	76.7 ± 38.3 (n = 3)	0 ± 0 (n = 3)
contralateral eye (ng/mL)	0 ± 0 (n = 3)	10.0 ± 10.0 (n = 2)	92.5 ± 54.4 (n = 4)	101.7 ± 51.0 (n = 3)	48.3 ± 48.3 (n = 3)

systemic circulation. For example, Nomoto *et al* have reported that plasma concentrations of bevacizumab increase immediately after subconjunctival administration in rabbits[17]. In the present study, antibody concentrations in the plasma had increased by 3 h after injection (data not shown), and concentrations in the contralateral retina increased similar to those in the injected eye (Table 1). These results suggest that the injected antibody moves freely from the subconjunctiva to the systemic circulation and may thus influence other organs. VEGF is an important physiological regulator of angiogenesis in health and disease[18]. Therefore, anti-VEGF antibody in the systemic circulation could potentially inhibit physiological angiogenesis in various organs in premature infants. In the present study, subconjunctival injections of anti-rat VEGF antibody tended to reduce pathological retinal NV, suggesting that the antibody did not constrict the development of physiological retinal angiogenesis. In fact, normal retinal vascular development was observed in all animals in

the room air-exposed groups (NV was not observed following subconjunctival injection of anti-rat VEGF; data not shown). Geisen *et al* have reported no significant difference in retinal AVA on P18 in a 50/10 OIR rat model compared with controls injected with anti-rat VEGF antibody into the vitreous on P12[19]. The antibody we used in the present study inhibits all isoforms of rat VEGF, and Ishida *et al* have reported that $VEGF_{120}$ is necessary for the development of normal retinal vessels[20]. The antibody doses used in the present study were sufficient to control a pathological NV, but may not be effective in inhibiting the physiological development of retinal angiogenesis. Lee *et al* suggested that intravitreal bevacizumab injection may be a safe and effective therapy at gestational ages of 34+6 weeks, and that using bevacizumab before a gestational age of 32 weeks may inhibit peripheral retinal vasculogenesis[21]. Mintz-Hittner and Kuffel also reported that the growth of normal retinal vessels after intravitreal injection of bevacizumab is often slower than the growth seen in very premature infants[22]. Therefore, the timing of bevacizumab treatment may be an important factor for peripheral retinal vasculogenesis. Further investigations are needed to determine the effects of the anti-VEGF antibody on local organs and physiological development in premature infants.

In conclusion, the results of the present study suggest that subconjunctival injections of anti-VEGF antibody decrease retinal NV and retinal AVA in a rat model of OIR. A higher concentration of the anti-VEGF antibody treatment would make a significant decrease retinal NV and retinal AVA. It is necessary to investigate a dose increasing study in the future. When administered via subconjunctival injections, anti-VEGF antibody may be transported systemically into the intraocular tissue of both the injected and contralateral eyes; however, it may also induce local complications. Further studies on the subconjunctival route of administration of anti-VEGF antibody, as an alternative to intravitreal administration, are needed to define the appropriate dose and interval to minimize any systemic side effects.

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