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Effect of a Dihydrobenzofuran Derivative on Lipid Hydroperoxide-Induced Rabbit Corneal Neovascularization

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Abstract. The aim of this study was to investigate the effect of A-3922, a dihydrobenzofuran derivative, on linoleic acid hydroperoxide (LHP)-induced corneal neovascularization (NV) in a rabbit model. Male New Zealand rabbits received intraperitoneal (i.p.) injections of 10 or 30 mg/kg per day A-3922 or its vehicle as control for 3 days. One day after i.p. injections, LHP was injected with a 30-gauge needle into the corneal stroma of the superior quadrant 4.5-mm below the limbus. Photographs of the vessels were taken for digital analysis with a surgical microscope. Vascular endothelial growth factor (VEGF) was measured using an immunoassay kit, and matrix metalloproteinase (MMP)-9 was measured by gelatin zymography in corneal samples. At 7 days post-LHP injection, the total vessel length was 26.7 ± 3.8 mm in the control animals (n = 8), 16.1 ± 0.8 mm in the A-3922 (10 mg/kg)-treated group (n = 5), and 11.4 ± 2.1 mm in the 30 mg/kg group (n = 8, $P<0.01$ *vs* control), respectively. After LHP injection, the content of VEGF and MMP-9 activity were increased in the superior cornea, but these were not influenced by A-3922 treatments. These results indicate that LHP-induced corneal NV is inhibited by treatment with A-3922 and therefore may represent a potential pharmacological intervention for ocular neovascularization disorders.

Keywords: neovascularization, lipid hydroperoxide, dihydrobenzofuran derivative, vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)

Introduction

Dihydrobenzofuran derivatives were developed for ischemic reperfusion injuries because of their antioxidant activity (1, 2), and they also inhibited lipid peroxidation (3 – 5). Lipid peroxidation induced by blue light emitting diode (LED) light exposure and 1 mM levofloxacin was inhibited in porcine retinal homogenate by the dihydrobenzofuran derivative A-3922 (6). This inhibition effect was greater than that of vitamin E under these experimental conditions.

Oxidative stress is the cause of several retinal diseases in the eye (7), including diabetic retinopathy (8), age-related macular degeneration (9), and retinopathy of prematurity (10). An increase of lipid peroxide due to oxidative stress in the eye may be closely related to various ocular disorders (11 – 15) and to abnormalities in retinal microcirculation (16). Lipid peroxides have been proposed as mediators or second messengers, whereby they induce gene expression and up-regulate various cytokines (17). Schreck et al. (18) reported that H_2O_2 and lipid hydroperoxides activate the NF-κB transcription factor in a human T cell line. Linoleic acid hydroperoxide (LHP) induced bovine aortic endothelial cell growth and basic-fibroblast growth factor release (19), and vascular endothelial growth factor (VEGF) production in cultured rabbit corneal stromal cells (20).

We previously reported that LHP induces neovascularizaion (NV) in a New Zealand albino rabbit corneal model (21, 22) through upregulation of particular cytokines, especially tumor necrosis factor-alpha (TNF-α) and VEGF. The NV response was enhanced by

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hyperglycemic conditions (23), and it was suppressed by vitamin E treatment (24).

In this study, we investigated the effect of A-3922 on LHP-induced NV in a corneal model and demonstrated a temporal change in VEGF and matrix metalloproteinase (MMP)-9 activity, which may be related to disruption of the extracellular matrix in corneal stroma.

Material and Methods

Animals

A total of 46 male New Zealand albino rabbits weighing 2.5 – 4.0 kg were used in experiments. Rabbits were allowed to acclimatize to their environment 2 weeks prior to experimentation. Rabbits were anesthetized with an intramuscular injection of 35 mg/kg of ketamine hydrochloride (Sankyo Life Tech, Co., Ltd., Tokyo) and 5 mg/kg xylazine (Sigma, St. Louis, MO, USA) at the time of LHP injection and whilst taking corneal photographs. The cornea was locally anesthetized with a topical application of 0.4% oxybuprocaine hydrochloride (Benoxil; Santen Pharmaceutical Co., Ltd., Osaka) at the time of LHP injection. All animals were treated in accordance with the Institutional Animal care and use committee of Showa University.

Corneal neovascularization

Rabbits were randomly divided into 3 groups: control group, 10 mg/kg A-3922 treatment group, and 30 mg/kg A-3922 treatment group. Rabbits received daily intraperitoneal injections (i.p. treatment) of 10 or 30 mg/kg A-3922 or vehicle as a control, for a total of 3 days. A-3922 was dissolved in 1% NIKKOL HCO-60 (Nikko Chemicals, Tokyo)/dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka): 8/2 (v/v). LHP was synthesized from linoleic acid (Sigma) with soybean lipoxygenase (Sigma) and oxygen (25). On the second day of i.p. injections, LHP was injected into corneal stroma 30 min after i.p. A-3922 (Nippon Soda. Co., Ltd., Odawara) or vehicle (control) treatment. The injection site on the cornea was positioned at 4.5 mm from the superior limbal arcade using a castroviejo caliper. Under a Zeiss surgical microscope (Carl Zeiss, Oberkohen, Germany), a single injection of 10 μL LHP (40 mM) was delivered to one half the depth of the cornea using a 100-μL syringe and 30-gauge needle (21). The contralateral eye received 10 μL control buffer (20 mM boric acid) which was the vehicle for the LHP.

At 1, 4, 7, 10, and 14 days after the LHP injection, vessel characteristics and distribution pattern were determined by examining anesthetized animals and photographed by using a camera mounted on a Zeiss operating microscope. Total vessel lengths of new vessels were calculated using NIH image analysis from the corneal photographs.

Sample preparation

At 6, 12, 24, and 96 h following the LHP injection, the rabbits were sacrificed with 3 ml of sodium pentobarbital (50 mg/ml). Eyes were enucleated. Aqueous humor was collected. Corneal samples were surgically removed and dissected into a 'superior' sample (include the injection site) and 'inferior' sample. Each corneal specimen was weighed and then crushed with a Cryo-press (Microtech Nichion, Co., Ltd., Chiba). The anterior segment was removed, and retina collected. These samples were stored at −80°C until further analysis. The crushed superior corneal samples were added to 150 μL of distilled water. The sample was submerged in crushed ice, sonicated with 5-s bursts at 40 W (SONIFIER cell disruptor 200; Branson, Danbury, CT, USA) and centrifuged at 1,500 × g for 10 min. The supernatant was used for VEGF and MMP determinations. To determine the pharmacokinetics of A-3922 in rabbits, 0.5 ml blood was collected from ear veins at 1, 2, 4, 6, 8, and 24 h following A-3922 i.p. injection, and each sample was centrifuged at 1,500 × g for 10 min to obtain the serum.

VEGF determination

The concentration of VEGF in the supernatant was measured using a Quantikine immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA). Protein concentrations were determined by the method of Lowry et al. (26), with bovine serum albumin as the standard.

MMP determination

MMP-2 and MMP-9 activities in superior corneal samples were measured by gelatin zymography. Samples containing 5 μg protein were mixed with sample buffer and then electrophoresed. The gels were incubated for 1 day and then stained. The zymography bands were digitized using the ATTO imaging system (ATTO Co., Tokyo), and the data were analyzed using NIH imaging software (27, 28).

Analysis of A-3922

The concentration of A-3922 was measured by HPLC analysis. Each sample (the crushed cornea inferior sample, aqueous humor, retina, or 0.2 ml serum) was added to 0.6 ml acetonitrile (Wako) and centrifuged at 20,000 × g for 15 min. Filtered supernatant samples (40 μL) were used for HPLC analysis. The HPLC analysis was performed on an LC-10A system with an SPD-10A UV-VIS detector and LC solution software (Shimadzu, Kyoto). Chromatographic conditions were

as follows: column, Shiseido CAPCELLPAK C_{18} (4.6 mm i.d. × 250 mm, UG120, 3 micrometer; Shiseido, Tokyo); mobile phase, 10 mM phosphate buffer (pH 6.8):acetonitrile = 3:7; flow rate, 1.0 ml/min; detection, 250 nm, column temperature, 40°C.

Statistical analysis

Data were compared between groups by multiple comparisons (William's) and these were expressed as a mean ± S.E.M. The differences were considered significant when $P<0.05$.

Results

Corneal neovascularization

After the LHP injection, vascular sprouts were observed in the superior quadrant extending from the limbus towards the LHP injection site. The vessel growth increased in length over 2 weeks. NV was not observed after control buffer injection. The vessels in the control group (n = 8) were longer and had extensive branching compared to those in the 10 (n = 5) or 30 mg/kg (n = 8) A-3922-treated group, up to 7 days post LHP injection (Fig. 1). The total length of vessels in the control group was 7.8 ± 1.7 mm at 4 days. This increased to 26.7 ± 3.8 mm by 7 days, after which time some vessels matured or regressed; and at 14 days, the total length of vessels was 19.9 ± 4.9 mm. At 7 days after LHP injection, the total length of vessels in the 10 mg/kg A-3922 treatment group and 30 mg/kg A-3922 treatment group was 16.1 ± 0.8 and 11.4 ± 2.1 mm, respectively ($P<0.05$ *vs* the control group) (Fig. 2). The longest vessel in the control group was 1.1 ± 0.1 mm by 4 days, which increased to 2.5 ± 0.1 mm by 7 days. After day 7, some vessels matured or regressed; and at day 14, the length was 3.3 ± 0.7 mm. On day 7 post LHP

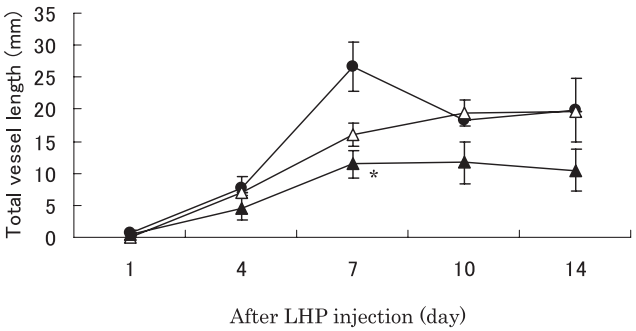

Fig. 2. Corneal neovascularization curve for total vessel length measurement. The total vessel length was suppressed by A-3922 treatment. Data represent the mean ± S.E.M. for the control group (closed circle, n = 8), the 10 mg/kg A-3922-treated group (open triangle, n = 5), and the 30 mg/kg A-3922-treated group (closed triangle, n = 8). *$P<0.05$, *vs* control group at each time point.

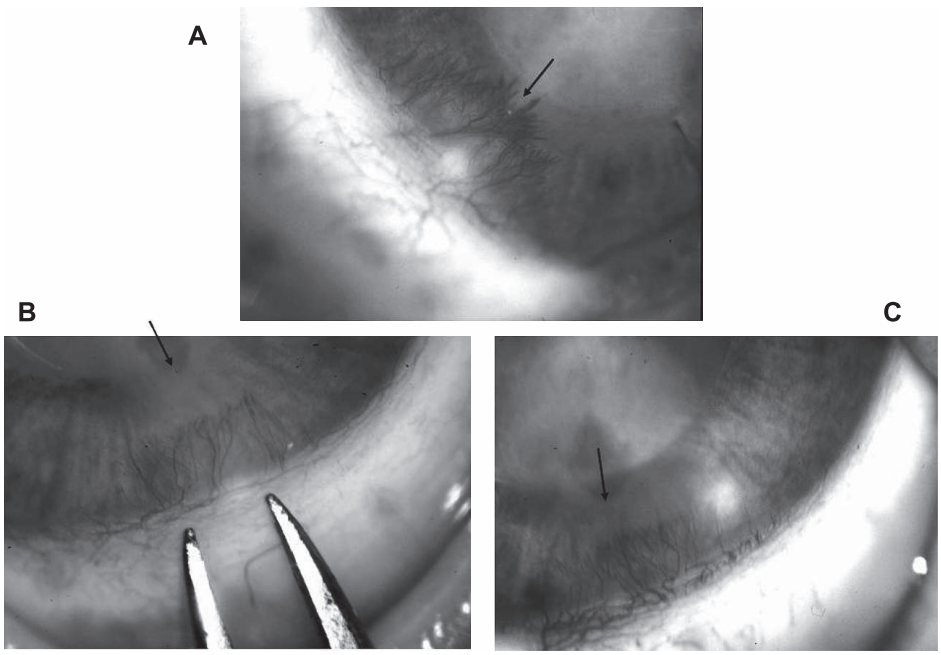

Fig. 1. Photographs of corneal NV at 7 days after LHP injection. A: control (without A-3922) rabbit. B: 10 mg/kg A-3922-treated rabbit. C: 30 mg/kg A3922-treated rabbit. The vessels in the control rabbit were longer and have extensive branching as compared to those in the rabbits treated with 10 or 30 mg/kg A-3922, i.p. The width of the castroviejo caliper was 2 mm (B). The arrowhead shows the injection point of LHP in each case.

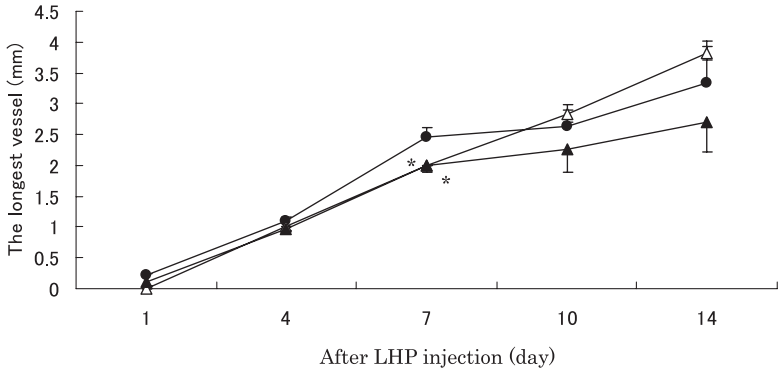

Fig. 3. Corneal neovascularization curve for the longest vessel length measurement. The longest vessel length was suppressed by A-3922 treatment. Data represent the mean ± S.E.M. for the control group (closed circle, n = 8), the 10 mg/kg A-3922-treated group (open triangle, n = 5), and the 30 mg/kg A-3922-treated group (closed triangle, n = 8). **P*<0.05, *vs* control group at each time point.

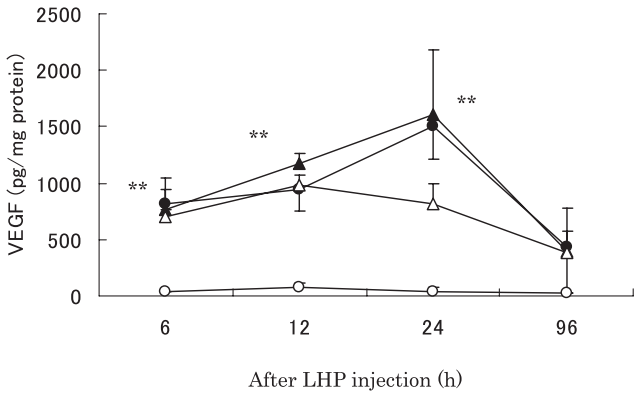

Fig. 4. Effect of A-3922 treatment on VEGF contents in the superior site cornea after LHP injection. Data represent the mean ± S.E.M., n = 11 for each point. The levels of VEGF increased significantly (***P*<0.001, *vs* control buffer injection) increased at 6, 12, and 24 h after LHP injection in each group (closed circle: control group, open triangle: 10 mg/kg A-3922, closed triangle: 30 mg/kg A-3922) compared to the control buffer injection group. The open circle shows the change of VEGF levels after control buffer injection (n = 3).

injection, the lengths of the longest vessels were significantly decreased in the 10 mg/kg and 30 mg/kg A-3922 treatment group to 2.0 ± 0 and 2.0 ± 0.1 mm, respectively (*P*<0.05) (Fig. 3).

VEGF determination

The level of VEGF protein in the superior corneal samples increased significantly at 6, 12, and 24 h following LHP injection (*P*<0.001) and then declined gradually from 96 h (Fig. 4). High levels of VEGF were observed in the 30 mg/kg A-3922 treatment group at 12 (1177.3 ± 82.2 pg/mg protein) and 24 h (1608.1 ± 573.3 pg/mg protein), whilst lower levels were observed in the 10 mg/kg A-3922 treatment group at 24 h (810.4 ± 180.9 pg/mg protein) after LHP injection. These data were not significantly different when compared to the control group.

MMP determination

Figure 5A shows the activity of pro-MMP-2 and -9 in superior corneal samples by zymography. Bands corresponding to MMP-2 and -9 were quantified by densitometric scanning for their gelatinolytic activity. The detected activities of MMP-2 or -9 after 6 h post LHP injection were 100%, because MMP-9 activities following injection with control buffer were negligible. Injection with control buffer did not influence MMP-2 or -9 activity. MMP-2 activity was not affected by corneal LHP injections and A-3922 treatment (Fig. 5B). MMP-9 levels were significantly increased at 6 h after the LHP injection in superior corneal samples (*P*<0.01 *vs* the control buffer injection), and then decreased gradually till 96 h. There were no significant difference of MMP-9 levels between A-3922 treatment groups and the control group (Fig. 5C).

Distribution of A-3922

In the rabbit, A-3922 serum level increased slowly after 30 mg/kg, i.p. injection, peaked at 8 h (1.31 ± 0.30 μg/ml, n = 3), and then decreased gradually. The serum level at 24 h was 0.55 ± 0.16 μg/ml (Fig. 6). A-3922 in the cornea was not determined at 8 h after 30 mg/kg i.p. injection. A-3922 levels in the aqueous humor and the retina were 0.79 ± 0.12 μg/ml and 1.62 ± 0.48 μg/g, respectively (Table 1).

Discussion

Angiogenesis is a complex process that is essential for many physiological functions (29). Oxidative stress is one of the triggers of angiogenesis, via upregulation of NV-formation-related cytokines by transcriptional regulation involving NF-κB and AP-1; and the productions of these cytokines are increased by lipid peroxide in aerobic organisms. Moreover, lipid peroxides can participate in continuous propagation reactions with polyunsaturated fatty acids or undergo 1-electron reduction and oxygenation via Fenton and Haber-Weiss

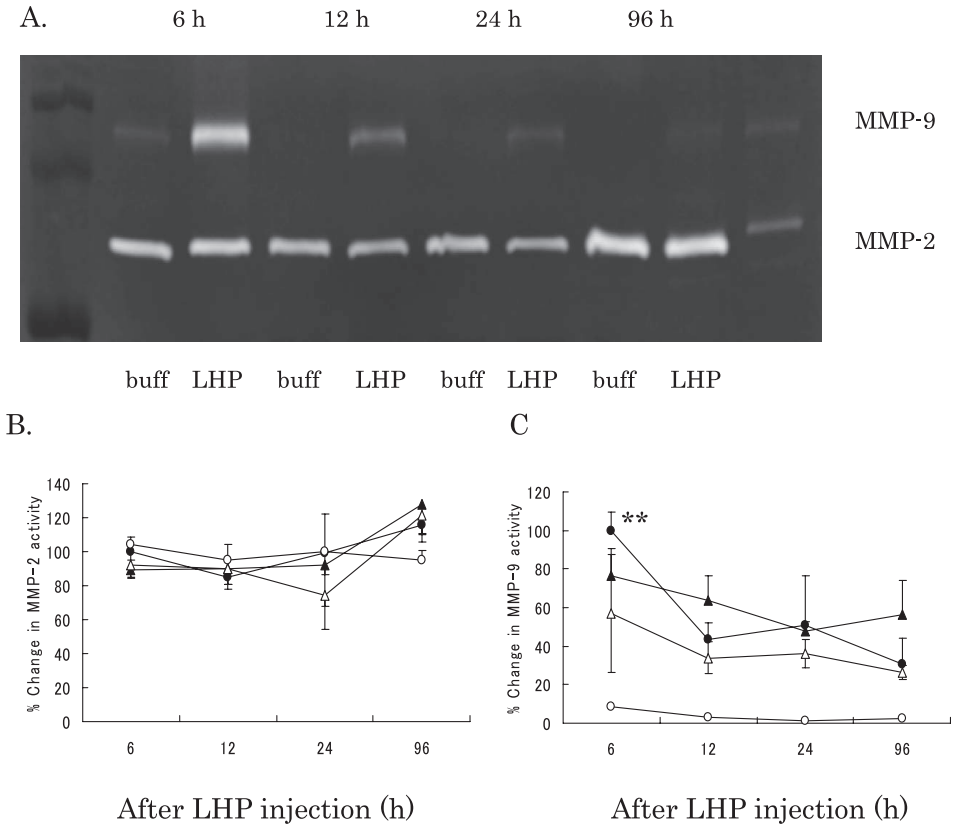

Fig. 5. Matrix metalloproteinases determination. A: Gelatin zymography of superior corneal samples. The two bands in the last lane on the right side correspond to human recombinant pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa), the standards. Illustrated is the production of pro-MMP-2 and MMP-9 at each time point after LHP or control buffer (buff) injection. B and C: Bands corresponding to MMP-2 and -9 were quantified by densitometric scanning for their gelatinolytic activity. Detected activities of MMP-2 or -9 after 6 h post LHP injection were 100%. Data represent the mean ± S.E.M., n = 4 for each point. Closed circle: control group, open triangle: 10 mg/kg A-3922, closed triangle: 30 mg/kg A-3922. The open circle shows the MMP-2 or -9 activity after control buffer injection. MMP-2 activities in the superior corneal samples after LHP injection did not change by LHP injection in each group (B). MMP-9 activities in the superior corneal samples significantly increased (**P<0.001, *vs* control buffer injection) at 6 h after LHP injection (C).

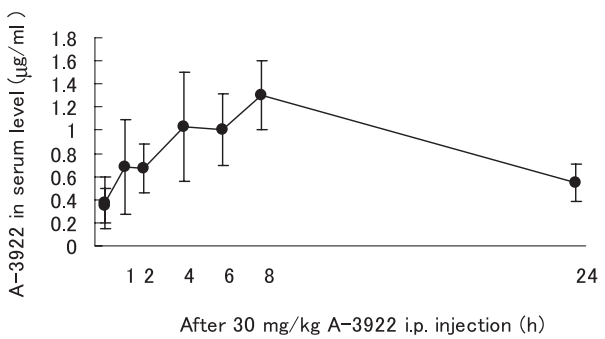

Fig. 6. Serum levels of A-3922 in rabbits. A-3922 was administered intraperitoneally to rabbits at a dose of 30 mg/kg body weight, and serum was taken at various intervals. The concentration of A-3922 was measured using an HPLC system. Data represent the mean ± S.E.M., n = 3 for each point.

Table 1. Concentration of A-3922 in the eye at 8 h after 30 mg/kg, i.p. injection

Aqueous humor	Cornea	Retina
0.79 ± 0.12 μg/ml	Not determined	1.62 ± 0.48 μg/mg

The mean ± S.E.M. (n = 3) is reported for each point.

type reactions (30) to generate additional hydroxyl radicals and start a chain reaction.

The dihydrobenzofuran derivative A-3922 demonstrated good inhibition against lipid peroxidation induced by blue-light and 1 mM levofloxacin in procine retinal homogenate. Its activity was greater than that of vitamin E under similar experimental conditions (6). A-3922 did not influence the absorption of LHP at 234 nm. That is, A-3922 does not deoxidize LHP and, therefore, may act as a chain-breaking antioxidant in the mem-

branes.

Armstrong et al. (31) reported severe retinal damage when lipid peroxide (>1 mM) was injected into rabbit vitreous and later discovered that relatively low concentrations of lipid hydroperoxide can induce a sub-lethal transient pathological effect. NV was elicited by oxidized C-18 linoleic fatty acid, but not by non-oxidized C-18 lipid (saturated or unsaturated) or hydroxyl C-18 linoleic fatty acid. Oxidized C-18 linoleic fatty acid also produced retinal NV (32), corneal NV (21), or choroidal NV (16) through the upregulation of several NV cytokines: transforming growth factor-beta (TGF-β), TNF-α, platelet-derived growth factor (PDGF), interleukin-1 (IL-1), basic-fibroblast growth factor (b-FGF), and VEGF (32). Of these three models, the corneal NV model is the most stable, reproducible, and convenient as an observation model. Therefore, we used the corneal NV model for our study of anti-NV agents.

In our experiment, treatment with 30 mg/kg A-3922 inhibited the LHP-induced corneal NV. A reduction was seen in both the combined total vessel length, as well as the length of the longest vessel. With regards to the longest vessel, the relative reduction in the A-3922 treatment group, compared with the control, was 11% at 4 days, 19% at 7 days, and 19% at 14 days after LHP injection. Vitamin E treatment has previously shown a similar result (24), and the relative reduction in length of the longest vessel was 17% at 7 days and 2% at 14 days, when compared to the control. These results suggest that A-3922 has a greater inhibitory effect on LHP-induced NV compared to vitamin E, in this particular model. In the vitamin E treatment model, the rabbits were fed chow (RC4; Oriental Yeast Co., Ltd., Tokyo) containing 0.02% vitamin E, and the plasma concentration of vitamin E was stable at 4.16 μg/ml, and was 1.05 μg/g in corneal tissue (24). In our experiment, at 8 h after treatment with 30 mg/kg A-3922, i.p., the concentration of A-3922 in plasma was 1.31 ± 0.30 and 0.79 ± 0.12 μg/ml in aqueous humor, but was not detected in the cornea. It is possible that A-3922 affects corneal NV via the aqueous humor, as the aqueous humor can supply the cornea with oxygen and nutrition.

In the LHP-induced corneal NV model, it appears that LHP mediates vascular growth by increasing the concentration of angiogenic cytokines (21). VEGF is probably the most important cytokine in the pathogenesis of NV because it enhances endothelial proliferation, migration, basement membrane degradation, and permeability (33). In this experiment, the levels of VEGF peaked in the superior corneal samples at 24 h after LHP injection; and these concentrations were slightly decreased in the 10 mg/kg A-3922 treatment group, but were not changed in the 30 mg/kg treatment group.

MMPs are also key enzymes for NV. Disruption of the extracellular matrix is considered to be an initial event in NV and is coupled to the production and activities of MMPs (34). In LHP-induced retinal NV models, MMP-9 was elevated in the retina, and peaked at 24 h after LHP intra vitreous injection (28, 35). In our study, MMP-9 activity significantly increased at 6 h after LHP injection in the superior site, and these activities returned to baseline after 96 h. A-3922 treatment did not show any effect on MMP-9 activities.

The concentration of VEGF did not change after vitamin E or A-3922 treatment 24 h after LHP injection. However, Miyazawa et al. (36) suggested that vitamin E partially regulates intracellular VEGF signaling and demonstrated antiangiogenic effects in bovine aortic endothelial cells. Vitamin E succinate inhibits melanoma angiogenesis in mice through the suppression of the expression of VEGF, VEGF receptor-1, and VEGF receptor-2 (37). These results suggest that the LHP-induced NV response in rabbit cornea was due not only to the VEGF increase, but also from other angiogenic cytokines. A-3922 may have effects on these cytokines that are produced by LHP injection and may act partially through oxidation stress reduction due to its strong antioxidant activity.

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