

Suppression of Choroidal Neovascularization by Intravitreal Injection of Liposomal SU5416

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Objective: To clarify whether use of angiogenic vessel-homing peptide, Ala-Pro-Arg-Pro-Gly (APRPG)-modified liposomes encapsulating 3-([2,4-dimethylpyrrol-5-yl] methylidene)-indolin-2-one (SU5416), an angiogenesis inhibitor, can inhibit the development of experimental choroidal neovascularization (CNV) in rats.

Methods: Liposomes were prepared using the thin-film hydration method. To set up the rat experimental CNV model, intense fundus laser photocoagulation at 6 spots per eye was performed on pigmented rats. After photocoagulation, the rats were divided into 4 groups (6 rats in each group): an APRPG-liposomal SU5416 treatment group and control groups treated with a balanced salt solution, APRPG liposomes, or soluble SU5416. Each rat received a single intravitreal injection immediately after the injury. One week or 2 weeks after laser injury, the extent of CNV was evaluated by perfu-

sion with high-molecular-weight fluorescein isothiocyanate-dextran.

Results: Two weeks after injection, the CNV area was significantly ($P < .05$) smaller in the APRPG-liposomal SU5416-treated group compared with the CNV area in the balanced salt solution- and APRPG liposome-treated groups.

Conclusion: Liposomes modified by APRPG and encapsulating SU5416 constitute a potential drug formulation for CNV treatment that would require only a single intravitreal injection.

Clinical Relevance: This liposomal delivery may enable the sustained release of small molecules and be a new treatment modality for CNV.

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CHOROIDAL NEOVASCULARIZATION (CNV) is a key pathologic change, causing severe vision loss as part of the pathogenesis of several diseases. The most prevalent disease of this type is exudative age-related macular degeneration (AMD), resulting in irreversible vision loss, which primarily affects people in developed countries.^{1,2} In relation to pathomorphologic disorders, various angiogenic growth factors, such as vascular endothelial growth factor (VEGF), have been reported³⁻⁵ to be expressed in CNV tissue and in retinal pigment epithelium (RPE) cells. Vascular endothelial growth factor is believed to be one of the major regulators in a variety of physiologic and pathologic angiogenesis processes, including CNV.⁶⁻⁹ In addition, the molecular expression of VEGF and its receptors (VEGFRs) is upregulated in rat experimental CNV models.^{3,10}

Two types of endothelium-specific tyrosine kinase receptors, Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2), are known to

mediate the function of VEGF, as receptor molecules, in physiologic and pathologic neovascularization.¹¹⁻¹³ In addition, VEGFR2 is thought to mediate VEGF-induced signals such as vascular hyperpermeability and most angiogenic properties, including mitogenicity/chemotactic activity.¹⁴

To date, 2 antiangiogenic therapies have been approved for the treatment of exudative AMD: pegaptanib sodium, an oligonucleotide (Macugen; Eyetech Pharmaceuticals/Pfizer, New York, New York), and ranibizumab, an anti-VEGF antibody Fab fragment (Lucentis; Genentech/Novartis, Basel, Switzerland). Another VEGF blocker in ongoing clinical studies is VEGF trap, a fusion protein of domain 2 from VEGFR1 and domain 3 from VEGFR2. This recombinant fusion protein has a high affinity for VEGF and interacts with all VEGF isoforms as well as placental growth factors. All clinical trials to date have demonstrated satisfactory results with intravitreal delivery of these agents in patients with AMD. However,

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there are serious clinical problems with these approaches. The desired therapeutic outcome often requires frequent injections. Repeated ocular injections are generally accompanied by risk of endophthalmitis, retinal detachment, and vitreous hemorrhage. Therefore, drug formulations, and especially drug delivery systems (DDSs), need to be improved to reduce the frequency of intravitreal injection. Vascular endothelial growth factor signal transduction inhibitors have been also used as antiangiogenic factors in chemotherapy. For example, sunitinib (Sutent, SU11248, Pfizer), a small molecule inhibitor of receptor tyrosine kinases of VEGFR and platelet-derived growth factor receptor, has been approved for the treatment of cancer.¹⁵ The antiangiogenic compound SU5416 (3-[(2,4-dimethylpyrrol-5-yl) methylidene]-indolin-2-one) is a potent and selective inhibitor of VEGFR2.¹⁶ It has been shown¹⁷ to suppress VEGF-mediated angiogenesis in vitro and in vivo through the inhibition of autophosphorylation of VEGFR2 by blocking the adenosine monophosphate-binding site within the kinase domain. In contrast to the successful results of phase 1 and 2 trials for the treatment of advanced cancers, SU5416 did not show any significant clinical benefit, and some patients experienced severe toxic reactions to the solvent used for dissolving SU5416, polyethoxylated castor oil.¹⁸

It has been demonstrated¹⁹ that the liposomal formulation of SU5416 does not require polyethoxylated castor oil for administration. Furthermore, a liposomal DDS using the angiogenic vessel-homing peptide Ala-Pro-Arg-Pro-Gly (APRPG) for active targeting of the drug carrier to the angiogenic site was useful in the treatment of tumor-bearing mice.²⁰⁻²² Thus, this chemical compound is expected to be a new drug for treatment of AMD if an appropriate and safe DDS is developed.

Liposomes are small lipid vesicles and one of the most advanced drug nanocarriers in DDS studies.²³ The use of liposomal vehicles to prolong clearance and limit peak concentration after intravitreal injection might avert the risks of frequent intravitreal injections.²⁴ The aim of the present study was to clarify whether SU5416 can be applied in CNV treatment using a liposomal DDS.

METHODS

ANIMALS

Sexually mature male Brown Norway rats (weight, 180-200 g; Japan SLC, Inc, Shizuoka, Japan) were maintained under 12-hour light and 12-hour dark conditions and fed a standard rat chow and water ad libitum. All experiments adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research²⁵ and Junendo University's guidelines for care and use of laboratory animals.

PREPARATION OF LIPOSOMAL SU5416

Liposomes were prepared as previously described.²¹ In brief, DPPC (dipalmitoylphosphatidylcholine), POPC (palmitoyloleoylphosphatidylcholine), cholesterol, and SU5416 solutions in chloroform were mixed (10:10:5:1 molar ratio) and dried under reduced pressure to make a thin lipid film. The APRPG peptide-

conjugated DSPE-PEG (distearoylphosphatidylethanolamine-polyethylene glycol) solution was added to the initial lipid solutions in the proportion of 10 mole percent (mol%) to phosphatidylcholine to modify the liposomes. For histochemical analysis, DiI_{C18} (Molecular Probes, Inc, Eugene, Oregon) was added to the initial lipid solutions in the proportion of 10 mol% to phosphatidylcholine. The thin lipid films were hydrated with 20mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid)-buffered saline (pH 7.4), and the liposome solutions underwent 3 freeze-thaw cycles using liquid nitrogen. The size of the liposomes was then adjusted by extruding through a 100-nm pore polycarbonate filter. The particle size and zeta potential of liposomal SU5416 were measured (Zetasizer; Malvern Instruments, Malvern, Worcestershire, England), and these values were approximately 130 nm and -2 mV, respectively.

INDUCTION OF CNV

Laser-induced CNV is widely used as an animal model for exudative AMD.²⁶ Using this model, we observed new vessels from the choroid that invaded the subretinal space after photocoagulation. Laser photocoagulation was performed in 6 spots per eye around the optic disc (wavelength, 532 nm; power, 90 mW; spot diameter, 100 μ m) using a slit delivery system (NOVUS Spectra; Lumenis, Tokyo, Japan), as described.^{27,28} After photocoagulation, the rats were divided into 4 experimental groups (6 rats in each group) and were given APRPG-liposomal SU5416, balanced salt solution (BSS), APRPG liposomes, or soluble SU5416 for administration of the drug. The SU5416 concentration used was 0.625 mg/mL. Immediately after photocoagulation, each rat received a single 10- μ L intravitreal injection using a 30-gauge needle.

VISUALIZING AND QUANTIFYING CNV

One week or 2 weeks after injury, blood flow was visualized by vascular perfusion with high-molecular-weight (2×10^6 -MW [2-MMW]) fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich Corp, St Louis, Missouri) using the method described by D'Amato et al.²⁹ Briefly, animals were anesthetized with intraperitoneal injection of sodium pentobarbital, 30 mg (Nembutal; Dainippon Sumitomo Pharma Co, Ltd, Osaka, Japan) per kilogram of body weight. The left ventricle was then perfused with approximately 50 mL of lactated Ringer solution, followed with 20 mL of lactated Ringer solution containing 10% (wt/vol) gelatin and 5-mg/mL FITC-dextran. After perfusion, treated eyes were enucleated and fixed with 4% paraformaldehyde solution for 20 minutes. Retinal pigment epithelium-choroid-sclera flat mounts were obtained by hemisecting the eye and peeling the neural retina away from the underlying RPE. Radial cuts allowed the eyecup to be laid flat onto a microscope slide with the RPE side facing up. Flat mounts were fixed by mounting medium (Vectashield; Vector Laboratories, Inc, Burlingame, California), and the specimens were examined with a microscope (Biorevo BZ-9000; Keyence, Osaka, Japan) equipped with an FITC-detecting filter unit. The CNV areas were photographed with a charge-coupled device camera and captured with an image analysis system (BZ-II; Keyence). The FITC-dextran-perfused vessels representing neovascularization were quantified by an experienced clinician (M.H.) delineating the perimeter of the fibrovascular membranes.

ACCUMULATION OF FLUORESCHEIN LIPOSOMAL SU5416 IN CNV

To investigate the accumulation of APRPG-liposomal SU5416 in CNV, double-labeling experiments including DiI_{C18} (red fluo-

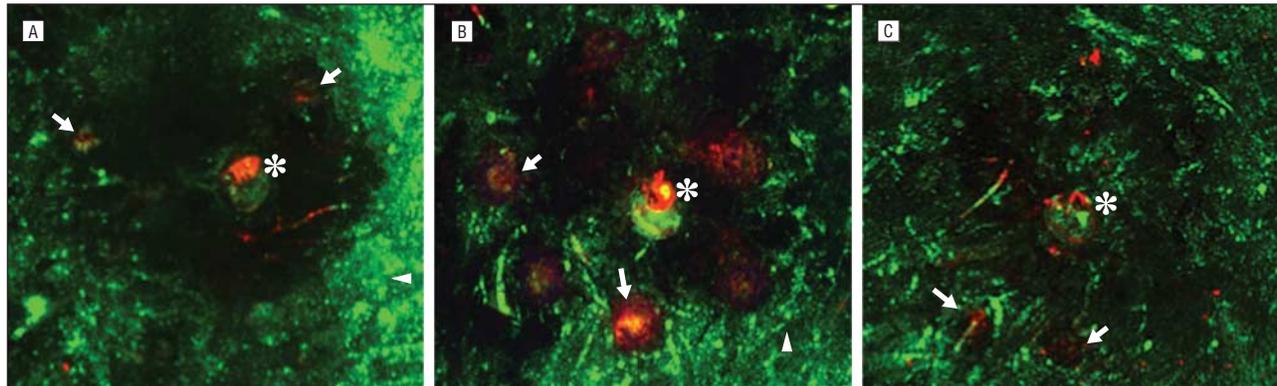


Figure 1. Liposomal SU5416 (3-[(2,4-dimethylpyrrol-5-yl) methylidene]-indolin-2-one) (red) and choroidal vascularization (green) double labeling. Localization of (Ala-Pro-Arg-Pro-Gly [APRPG]-free) liposomal SU5416 (A) and APRPG-liposomal SU5416 (B) 4 days after intravitreal injection, as well as APRPG-liposomal SU5416 at 2 weeks after injection (C). Asterisks indicate optic nerve; arrows, choroidal neovascularization region; arrowheads, normal choroidal vessels.

rescence) were performed. Just after laser injury, (APRPG-free) liposomal SU5416 and APRPG-liposomal SU5416 were intravitreally injected in rats. Four days or 2 weeks after injection, the accumulation of liposomal SU5416 in choroidal flat-mount was examined using an epifluorescent microscope.

STATISTICAL ANALYSIS

Experimental results of the quantitative analysis are expressed as the mean (SE). Data were analyzed by analysis of variance with the Tukey test. A probability of $P < .05$ was considered statistically significant.

RESULTS

To evaluate the effect of vascular-targeted liposomal SU5416 on CNV, the morphologic effect of the compound was examined by angiography using 2-MMW FITC-dextran and by epifluorescent microscopy. The rat experimental model was chosen for this study to determine the pharmacologic effect of the liposomal chemical compound on CNV, since this animal model of laser-induced CNV has been widely used^{30,31} in the evaluation of experimental AMD treatments. The rat model of laser-induced CNV has a high reproducibility.

As an initial experiment, we examined the accumulation of liposomal SU5416 after intravitreal injection by using DiI_{C18} (red fluorescence) to clarify whether the efficacy of liposomal SU5416 might be enhanced by the angiogenic-homing peptide (APRPG) or only by sustained release from liposomes. Four days after laser injury and intravitreal injection, significant amounts of APRPG-liposomal SU5416 recruited to the developing CNV region were compared with the control (APRPG-free liposomal SU5416) (**Figure 1** A and B). The APRPG-liposomal SU5416 remained in the CNV region 2 weeks after intravitreal injection (**Figure 1** C).

The typical CNV observed by fluorescent microscopy in the present study is shown in **Figure 2**. Two weeks after the injury, CNV was observed as relative hyperfluorescence areas overlying the laser lesions in the RPE-choroid-sclera flat mounts. Although CNV in the group treated with soluble SU5416 appeared to be somewhat inhibited (**Figure 2** C), the inhibitory effects on the induced CNV were enhanced when the animals

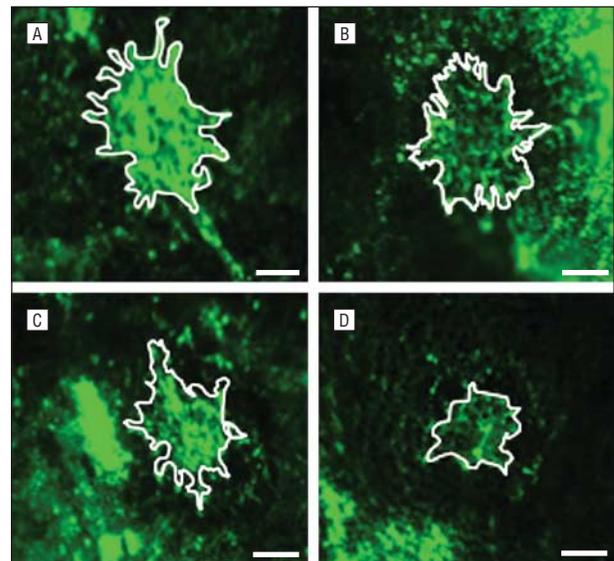


Figure 2. Morphologic changes of laser-induced choroidal neovascularization (CNV) after intravitreal injection. Typical fluorescein angiogram of retinal pigment epithelium 2 weeks after injury treated with balanced salt solution (A), Ala-Pro-Arg-Pro-Gly (APRPG) liposomes (B), soluble SU5416 (3-[(2,4-dimethylpyrrol-5-yl) methylidene]-indolin-2-one) (C), or APRPG peptide-modified liposomal SU5416 (D). Regions of CNV are outlined by a solid line. Bars represent 100 μm .

were treated with APRPG peptide-modified liposomal SU5416 (**Figure 2** D) compared with the controls (**Figure 2** A and B).

Attempts were then made to quantify the analysis by measuring the CNV areas in images of each experimental group. One week after laser injury and intravitreal injection, neovascular regions in RPE showed that the area of CNV in the soluble SU5416-treated group (19 598 [1128] μm^2) was significantly smaller than that in each of the other 2 groups (BSS, 25 092 [1584] μm^2 ; APRPG liposomes, 24 987 [1641] μm^2). No significant difference was observed between the group treated with APRPG-liposomal SU5416 (20 177 [1339] μm^2) and each of the other control groups (BSS and APRPG liposomes) (**Figure 3** A).

Data obtained from traced neovascular regions in RPE 2 weeks after injury showed that the area of CNV in the APRPG-liposomal SU5416-treated group (28 431 [2237]

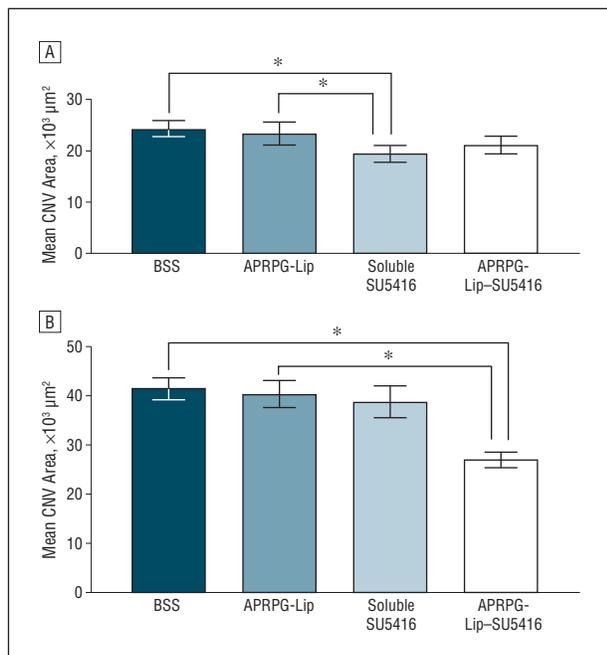


Figure 3. Quantitative analysis of the choroidal neovascularization (CNV) area in each experimental group 1 week (A) and 2 weeks (B) after intravitreal injection. The results are expressed as simple mean (SE) of CNV area (n=6). *Statistical significance between indicated groups at $P < .05$. APRPG-Lip indicates Ala-Pro-Arg-Pro-Gly-modified liposomes; BSS, balanced salt solution; and SU5416, 3-([2,4-dimethylpyrrol-5-yl] methylidene)-indolin-2-one.

μm^2) was significantly smaller than that in each of the other 2 groups (BSS and APRPG liposomes). However, no significant difference was observed between the group treated with soluble SU5416 (38 160 [3550] μm^2) and that treated with APRPG liposomes (40 788 [2845] μm^2) or BSS (421 437 [2468] μm^2) (Figure 3B).

COMMENT

The data from this study clearly demonstrate that a single intravitreal injection of the vascular-targeted liposomal SU5416 significantly reduced laser-induced CNV in the experimental rat model. This implies that the liposomal compound may be useful in the treatment of CNV in clinical practice via its inhibitory effect on VEGF. Indeed, intravitreal injections using liposomal chemicals such as antibiotics (clindamycin,^{32,33} gentamicin,³⁴ and amikacin³⁵), a polyene antifungal (amphotericin B^{36,37}), an immunosuppressant (cyclosporine³⁸), and antimetabolites (flourouracil, fluorouridine^{39,40}) have been reported to be effective.

SU5416 inhibits the phosphorylation of VEGFR2 in a dose-dependent manner in vitro.¹⁸ It is assumed that SU5416 inhibits neovascularization by blocking VEGFR2 downstream signal transduction in vivo. Previous animal model studies on SU5416 demonstrated its tumor-suppressive effect. For example, a 73% decrease in tumor volume has been demonstrated¹⁸ by SU5416 administration in a null/null mouse model after rat C6 glioma cells were transplanted into the colon serosa. The inhibition of VEGFR2 phosphorylation by in-

traperitoneal injection of SU5416 once daily for 2 weeks has been shown in the rat model, and SU5416 also showed a tumor-suppressive effect in a rat experimental CNV model.⁴¹ In addition, apoptosis has been induced in murine CNV endothelial cells after administration of SU5416 by intraperitoneal injection every other day for 14 days.²⁸

Treatment using soluble SU5416 inhibited CNV development 1 week (but not 2 weeks) after injection compared with APRPG-liposomal SU5416 (Figure 3). Because APRPG-liposomal SU5416 was observed at the CNV region 2 weeks after intravitreal injection (Figure 1C), these results suggest that APRPG-liposomal SU5416 has a long-standing effect for CNV reduction.

Based on the inhibitory effects of SU5416 on CNV in vivo, the present study evaluated the validity of a novel DDS—a single intravitreal injection of an angiogenic vessel-homing peptide, APRPG—for the active targeting of drug carriers to angiogenic sites. Healthy choroidal vessels in the APRPG-liposomal SU5416-treated group were not noted (Figure 2). However, Shimotake et al⁴² recently reported that VEGF inhibition could affect normal vessels as well as angiogenic vessels; further studies concerning the effect of liposomal SU5416 on healthy tissues are necessary to clarify the issue.

We believe that the liposomal DDS provided significant basic data for clinical use for the following reasons: (1) the efficiency of the liposomal method used in this study was satisfactory for use in an in vivo study, (2) it is believed that APRPG-liposomal SU5416 may be stable in the vitreous humor, and (3) APRPG has been reported²¹ in studies of tumor therapies to be effective in active targeting of drug carriers to angiogenic sites in the vitreous body.

Although further investigation of the precise mechanism and direct molecular basis of the effect of vitreous injection of APRPG-liposomal SU5416 on CNV inhibition is necessary, the findings in this study suggest that APRPG-liposomal SU5416 is a strong candidate for development as an agent for the treatment of conditions such as exudative AMD by a single vitreous injection. The liposomal delivery of small compounds may be a new treatment modality for CNV.

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