主論文の要旨

Plasma-activated medium suppresses choroidal neovascularization in mice: a new therapeutic concept for age-related macular degeneration

プラズマ賦活液による脈絡膜新生血管抑制：
加齢黄斑変性の新規治療法開発

名古屋大学大学院医学系研究科　細胞情報医学専攻
頭頸部・感覚器外科学講座　眼科学分野

（指導：寺﨑 浩子 教授）

叶 福相

【**Introduction**】

    Choroidal neovascularization (CNV) is the main pathogenesis of wet age-related macular degeneration (AMD), which leads to severe vision loss in many aged patients in most advanced countries. CNV compromises vision via hemorrhage and retinal detachment on account of pathological neovascularization penetrating the retina.

    Plasma medicine represents the medical application of ionized gas "plasma" that is typically studied in the field of physical science. In recent years, nonequilibrium atmospheric pressure plasma (NEAPP, also known as cold plasma or non-thermal plasma) therapy has been investigated as a new medical tool especially in cancer researches. Because angiogenesis, an important concept in cancer research, is also the main pathogenesis of wet AMD, we hypothesized that plasma-activated medium (PAM) could exert an anti-angiogenic effect on blood vessel growth in the eye.

【**Methods**】

    PAM was generated by an experimental NEAPP system from ultrapure phosphate-buffered saline (PBS) for *in vivo* experiments and from recommended culture medium for *in vitro* experiments. *In vitro*, the effect of PAM on vascularization was assessed on the basis of human retinal endothelial cell (HREC) tube formation. In mice, laser photocoagulation was performed to induce CNV (laser-CNV), followed by intravitreal injection of PAM with different concentrations. Seven days after the injection, CNV volumes were measured , and CNVs were visualized using tetramethylrhodamine isothiocyanate (TRITC)-conjugated isolectin-B4 (iB4) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. Maximum dose of N-Acetylcysteine (NAC) within the safe range (presumed final concentration of 10 mM) was used to examine the role of reactive oxygen species (ROS) in PAM-induced CNV suppression. Mouse retinal flat mounting and vessel staining with TRITC-conjugated iB4 were performed seven days after intravitreal injection of PAM. Fundus imaging, retinal histology examination, and electroretinography (ERG) were also performed to evaluate PAM-induced retinal toxicity. Mann-Whitney *U* test and Kruskal-Wallis test with *post-hoc* Steel's test were used for statistical analysis. Differences were considered to be statistically significant at $P < 0.05$.

【**Results**】

    First, we examined the effect of PAM treatment on vessel tube formation *in vitro*. After 4 h of incubation with PAM, the ability of HRECs to form vascular tubes *in vitro* was clearly inhibited compared with that in cells treated with control medium. The observed number of branching points showed that different concentrations of PAM (4%, 10%, and 25%) dose dependently blocked tube formation ($P = 2.2 \times 10^{-9}$; Figure 1).

    We also examined the ability of PAM to reduce angiogenesis *in vivo*. As a representative *in vivo* ocular angiogenesis model, laser-CNV was induced in wild-type mice and different volumes of PAM (0.5, 1.0, and 2.0 μL) were intravitreally administered. The CNV volumes in the eyes injected with PAM were dose dependently reduced compared with the control eyes ($P = 0.0015$; Figure 2). These results indicate

that PAM has the potential to suppress ocular angiogenesis both *in vitro* and *in vivo*.

Next, we explored the mechanism by which PAM reduced CNV in mice. In the eyes injected with the control PBS, CNV occurred in the subretinal space, penetrating the retinal pigment epithelium (RPE; Figure 3D). iB4 staining confirmed CNV according to the presence of endothelial cells (Figure 3A). In contrast, the eyes injected with PAM exhibited much less CNV (Figure 3E and 3H), and co-staining with fluorescein isothiocyanate (FITC)-TUNEL showed that most of the endothelial cells involved in CNV were apoptotic (Figure 3F and 3G). These results indicate that the reduction in CNV observed with PAM treatment was due to vascular endothelial cell apoptosis.

Reactive oxygen species (ROS) are a key factor to explain the mechanism underlying the anti-tumor effects of PAM. Therefore, we speculated that PAM-mediated CNV suppression is dependent on ROS generated by PAM. Following previous reports that used N-acetylcysteine (NAC) to neutralize ROS, we also examined if PAM-mediated CNV suppression is canceled by NAC (Figure 4). In our *in vivo* experiments, the CNV volume in eyes injected with PAM and/or NAC was reduced compared with that in control eyes ($P = 5.8 \times 10^{-4}$). The CNV volumes in eyes injected with both NAC and PAM were still significantly less than those in the control eyes ($P = 0.024$), which indicates that the maximum dose of NAC within the safe range only partly inhibited the PAM-induced reduction in laser-CNV.

Then we examined whether PAM treatment not only inhibits neovasculature but also causes regression of pre-existing regular retinal vessels. The percentages of specific retinal regions occupied by retinal vessels that were stained by TRITC-conjugated iB4 did not differ significantly between eyes injected with PAM and those injected with control PBS ($P = 0.093$; Figure 5), indicating that PAM did not induce vessel regression among pre-existing regular retinal vessels.

We also evaluated the retinal toxicity of the intravitrealy injected PAM (Figure 6). All examinations were performed 7 days after PAM injection. PAM-injected eyes did not show retinal degeneration in the fundus. H&E-stained cryosections of PAM-injected eyes also showed no histological change. In addition, ERG showed that a-waves (photoreceptor function) and b-waves (inner retinal function) from PAM-injected eyes were not reduced compared with those in counterpart eyes injected with control PBS (a-wave, $P = 0.85$; b-wave, $P = 0.97$). These results indicate that intravitreal injection of PAM reduced laser-CNV without causing retinal toxicity.

【**Discussion**】

In this study, we demonstrated that PAM reduced mouse CNV and thus has therapeutic potential for treating wet AMD. It was previously reported that proliferation, migration, and tube formation of arterial endothelial cells were enhanced upon exposure to NEAPP via ROS-induced fibroblast growth factor-2 release. Other previous reports also revealed that PAM suppresses tumor cell proliferation by upregulating ROS secretion in target cells, and this effect of PAM is nullified by co-administration of NAC. These previous findings indicated the possibility that although ROS reactions have bilateral effects on angiogenesis, PAM-generated ROS can act as anti-angiogenesis factors in case of CNV. Our results suggest that PAM inhibits ocular angiogenesis without causing retinal toxicity, at least in part, via a

mechanism independent of ROS activity.

The discrepancies between previous results and those of the current study with respect to the effects of NEAPP and NEAPP-activated medium on CNV may be due, in part, to the conditions under which the medium was exposed to NEAPP, including the duration of treatment, distance between the medium and NEAPP source, and the NEAPP-generating device itself. Because PAM has great therapeutic potential for use in human, further studies are needed to address several important problems: the precise mechanism by which PAM suppresses CNV, beyond ROS induction, must be elucidated; more thorough evaluations of the retinal toxicity and other side effects of PAM are needed; a specific scale for PAM dosing is required to standardize PAM treatments.

【Conclusion】

Our findings indicate the potential of PAM as a novel therapeutic agent for suppressing CNV, which contributes proof-of-principle evidence for using PAM in treating ocular diseases.