Targeting Therapy of Choroidal Neovascularization by Use of Polypeptide- and PEDF-loaded Immunoliposomes under Ultrasound Exposure

Tao LI (李 涛)*1, Ming ZHANG (张 铭)2, Yong HAN (韩 勇)3, Hong ZHANG (张 虹)1, Lingjuan XU (徐玲娟)1, Yan XIANG (向 艳)1
1Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
2Reproductive Medicine Center, Zhongnan Hospital of Wuhan University, Wuhan 430071, China
3Pharmaceutical Preparation Section, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

Summary: Pigment epithelium derived factor (PEDF) has been proven to be an effective drug for the treatment of choroidal neovascularization (CNV). However, the lack of ideal administration route is the biggest bottleneck preventing PEDF from wider clinical use. In this study, we developed a novel PEDF-carrying system which employed immuno-nano-liposomes (INLs) under ultrasound exposure. PEDF-loaded INLs were prepared by conjugating nanoliposomes to the peptide ATWLPPR specifically targeting the receptor-2 for vascular endothelial growth factor (VEGFR-2) and reversely encapsulating PEDF. RF/6A cells were incubated with PEDF-loaded INLs. CNV models of BN rats were injected with PEDF-loaded INLs. MTT assay was used to evaluate the cytotoxicity of the INLs on RF/6A cells. Flow cytometry was conducted to detect the apoptotic rate of cells. Laser scanning confocal microscopy was employed to observe the binding and transmitting process of PEDF-loaded INLs and to calculate the area of CNV in the rat model. The results showed that the PEDF-loaded INLs could exclusively bind to CNV but not to the normal choroidal vessels. The CNV area was significantly decreased in PEDF treatment groups in comparison with control group (P<0.05). Moreover, PEDF-loaded INLs exposed under ultrasound were more efficient in reducing the CNV area (P<0.05). It was concluded that INLs in combination with ultrasonic exposure can transmit PEDF into cytoplasm with high specificity and efficiency, which strengthens the inhibitory effects of PEDF on CNV and reduces its side effects. PEDF-loaded INLs possibly represent a new treatment paradigm for patients with ocular neovascularization.

Key words: choroidal neovascularization; pigment epithelium derived factor; ultrasound; nanometer; immunoliposome

Choroidal neovascularization (CNV) is the basic pathologic change of many intraocular diseases[1]. The therapy of CNV-related disorders constitutes an important component of modern ophthalmologic care. Recently, many achievements have been made in the medical therapy of CNV, such as development of new drugs and exploration of transgenic therapy aiming at inhibiting angiogenesis[2]. However, it is considered that an ideal therapy should not only inhibit angiogenesis but also destroy the already existing neovascularization. Pigment epithelium derived factor (PEDF), which can selectively induce the apoptosis of the epithelium of neovascularization without disturbance of normal vessels and resist the effects of angiogenic factors, is regarded as a promising drug[3], whereas, lack of an appropriate executive way prevents the wider use of PEDF in clinical practice. In this study, we explored a novel PEDF-loaded immuno-nano-liposome (INL) complex which can target and inhibit the neovascularization with high specificity, efficiency and effectiveness.

1 MATERIALS AND METHODS

1.1 Preparation of PEDF-loaded INLs
PEDF-loaded INLs were prepared as previously reported[4]. Briefly, nanoliposomes were conjugated to the peptide ATWLPPR which could specifically bind to VEGFR-2. Then PEDF was reversely encapsulated into the INLs. MTT assay was used to evaluate the cytotoxicity of the INLs on RF/6A cells. Flow cytometry was conducted to detect the apoptotic rate of cells. Laser scanning confocal microscopy was employed to observe the binding and transmitting process of PEDF-loaded INLs and to calculate the area of CNV in the rat model. The results showed that the PEDF-loaded INLs could exclusively bind to CNV but not to the normal choroidal vessels. The CNV area was significantly decreased in PEDF treatment groups in comparison with control group (P<0.05). Moreover, PEDF-loaded INLs exposed under ultrasound were more efficient in reducing the CNV area (P<0.05). It was concluded that INLs in combination with ultrasonic exposure can transmit PEDF into cytoplasm with high specificity and efficiency, which strengthens the inhibitory effects of PEDF on CNV and reduces its side effects. PEDF-loaded INLs possibly represent a new treatment paradigm for patients with ocular neovascularization.

1.2 Effects of INLs on Choroidal Microvascular Endothelial Cells
1.2.1 Cell Culture Rhesus macaque choroids-retinal endothelial cells (RF/6A, VEGFR-2 positive[5]) were purchased from Shanghai Institute of Cells, China and
cultured in a humidified atmosphere of 5% CO₂ in air at 37°C in DMEM medium (HyClone, Germany) containing 10% FBS (HyClone, Germany) and 100 U/mL penicillin/streptomycin.

1.2.2 Binding Activity and Cellular Uptake of INLs After culture with PEDF-loaded INLs for 30 min, 1, 2, 3 h respectively, RF/6A cells were washed with PBS for three times and were observed under a laser scanning confocal microscope (FV500 OLYMPUS, Japan). The binding activity and the cellular uptake of PEDF-loaded INLs were evaluated.

1.2.3 Cytotoxicity Test of INLs The cytotoxicity of INLs was evaluated by MTT assay. Briefly, RF/6A cells were cultured in a 96-well plate (2×10³/mL, 250 μL/well) overnight. The supernatant was discarded. Solutions of free PEDF, PEDF-loaded liposomes, and PEDF-loaded INLs at different concentrations (20, 40, 80, and 160 mg/L) were prepared and added to the cell pellets (250 μL/well) in sextuplicate with each concentration. After incubation at 37°C for 4 h, the supernatant was removed by centrifugation. The cells were washed once, covered with the culture medium (250 μL/well) and incubated at 37°C for 48 h. Then 30-μL MTT solution (2 g/L, Sigma, USA) was added to each well. Four h later, the supernatant was discarded and 100-μL DMSO (Sigma, USA) was added to each well. At last, the 96-well plate was oscillated for 30 min and measured at 540 nm on a microplate reader. Empty liposomes containing no drug were used as control. Incubation time was calculated according to the following formula: The inhibition rate = (1–As40 value of the treated group / As40 value of the control group) × 100%.

1.2.4 Detection of Apoptosis by Flow Cytometry Free PEDF, PEDF-loaded liposomes, or PEDF-loaded INLs was added respectively to the culture medium of RF/6A with a final concentration of 80 mg/L. After incubation for 24 h, RF/6A cells were collected and washed once with the culture medium. Then the precipitation was resuspended with 100 μL of Annexin V labeling buffer (Immunotech, USA) and incubated at room temperature for 15 min. The cells were collected and washed again, followed by the addition of SA-Flous buffer. After incubation at 4°C for 20 min, the cells were washed and detected by flow cytometry (BD Co., USA). Empty liposomes served as control.

1.2.5 Effects of Ultrasonic Energy on INLs After incubation with 80-mg/L INLs, RF/6A cells were exposed to ultrasound (0.5 W/cm²) for 20 s. Then the culture plate was immediately put into the 5% CO₂ incubator and incubated at 37°C. The uptake of INLs, inhibition rate and apoptosis rate of cells were evaluated as mentioned above.

1.3 Effect of PEDF-loaded INLs under Ultrasound Exposure on CNV

1.3.1 Establishment of CNV Rat Model Forty-eight BN rats were randomly selected for the establishment of CNV models and the other three BN rats were taken as normal control. The experimental protocol was as follows: After anesthetization with intraperitoneal injection of ketamine (100 mg/kg), the rats were given 0.5% tropicamide to dilate the pupils. Then the semiconductor laser at 532 nm was administered to the right eyes of the rats by using slit lamp microscopy and three-mirror (contact lens) funduscopy. Photocoagulation (525 mW in power, 50 μm in diameter, 0.05 s in time) was performed at 8 to 10 points in the round area apart from the optic disc by two to three PD till Bruch membrane was broken which were represented by the appearance of bubbles. Seven days after the photocoagulation, the BN rats were injected intraperitoneally with 10% fluorescein sodium (1 μL/kg) and were immediately observed by retinal vascular imaging camera. The continuous results were recorded.

1.3.2 Specific Binding of PEDF-loaded INLs to CNV Fourteen days after the laser photocoagulation, three randomly selected rats with CNV were injected with PEDF-loaded INLs (10 μg of PEDF per kilogram of body weight) through the tail vein. The rats were anesthetized to receive operation 30 min later as follows. First, the rats were fixed in a supine position. Then, the bilateral carotid arteries were rapidly exposed. Then, the proximal parts of the carotid arteries were ligated and 10-μL heparin saline injected into the distal end. Last, the eyeballs were enucleated and the anterior segments and retinas removed to sample the tissues of choroid with sclera wall. Frozen sections of the choroid-sclera-complex was stained with propidium iodide (PI, 500 mg/L) at 37°C for 10 min and washed with PBS for three times in the dark. The stained sections were observed under a laser confocal microscope (excitation wavelength of 480 nm and absorption wavelength of 505 nm for FITC, excitation wavelength of 578 nm and absorption wavelength of 603 nm for PI).

1.3.3 Determination of the CNV Area Seven days after the photocoagulation, the remaining 45 CNV rat models were randomly divided into 8 groups: group A, in which three rats served as blank control; group B (n=6), in which 10-μL PEDF (0.2 g/L) was injected once into the right vitreous cavity; group C (n=6), in which 10-μL PEDF (10 μg/kg) was injected into the tail vein, once a day, for seven days; group D (n=6), 10-μL PEDF (10 μg/kg) was injected into the tail vein, and 30 min later ultrasonic irradiation of 0.5 W/cm² was exerted to the eyes for 20 s, once a day, for seven days; group E (n=6), in which PEDF-loaded liposomes (10 μg PEDF per kilogram of body weight) were injected through the tail vein, once a day, for seven days; group F (n=6), in which the same injection was done as that in the group E, except that the eyeballs of the rats in the group F were subjected to the ultrasonic irradiation of 0.5 W/cm² 30 min later, once a day, for seven days; group G (n=6), in which PEDF-loaded INLs (10 μg of PEDF per kilogram of body weight) was injected through the tail vein, once a day, for seven days; group H (n=6), in which the same injection was done as that in the group E except that 30 min after the injection, ultrasonic irradiation of 0.5 W/cm² was exerted to rat eyeballs, once a day, for seven days. Fourteen days after the photocoagulation, similar operation to what had described in 1.3.2 was made in the rats except that perfusion of fluorescein isothiocyanate-dextran (FITC-dextran, Sigma, USA) was given right after injection of heparin saline. The eyeballs were removed immediately and fixed in 4% paraformaldehyde solution for 10 min. The choroids with sclera wall were collected after removal of the anterior segments and retinas. Then four radial incisions were made and the