Preparation of Poly Lactic-co-Glycolic Acid/Pigment Epithelium-Derived Factor Emulsions and Their Inhibition on Vascular Endothelial Growth Factor Gene Expression

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Pigment epithelium-derived factor (PEDF) is encapsulated in poly lactic-co-glycolic acid (PLGA) polymer to prepare PLGA/PEDF emulsions for high efficient inhibition on vascular endothelial growth factor (VEGF) gene expression. Analyses by Fourier transform infrared spectrum (FT-IR), transmission electron microscopy (TEM) and dynamic light scattering (DLS) proved the formation of the PLGA/PEDF emulsions. Furthermore, CCK-8 assay was used to detect the cytotoxicity of the PLGA/PEDF emulsions to human umbilical vein endothelial cells (HUVEC). Finally, the VEGF levels in the HUVEC cell are tested by enzyme-linked immunosorbent assay at different conditions, such as different concentrations of PLGA/PEDF emulsion, the culture time and the hypoxia treatment. The results showed that the obtained PLGA/PEDF emulsions were good agents for controlled release and inhibit effectively VEGF expression under hypoxia. Therefore, proliferation of HUVEC cells was inhibited by PLGA/PEDF emulsions, and the cell viability of HUVEC cells decreased gradually with increase of concentrations of PLGA/PEDF emulsions.

Keywords pigment epithelium-derived factor, vascular endothelial growth factor level, hypoxia, cytotoxicity

Introduction

Pathological retinal neovascularization (RNV) is a leading cause of irreversible serious vision damage, which can result in many diseases such as retinopathy of prematurity (ROP), age-related macular degeneration (AMD), retinal vein occlusion (RVO), diabetic retinopathy (DR), persistent hyperplastic primary vitreous (PHPV), proliferative vitreoretinopathy (PVR), and so on. Therefore, it is very important to inhibit the growth of RNV for treating these diseases. Furthermore, with the depth exploration of RNV formation mechanism, the molecular biology researches on promoting expression of RNV including on vascular endothelial growth factor (VEGF) have been reported. In view of the important role of VEGF in vascular ophthalmopathy, the development of anti VEGF drugs has been focused gradually.

In past years, many medicaments such as inhibitors of VEGF are applied to treat RNV. The past anti-VEGF therapies require some frequent repetitions of administration with uncertain visual acuity recovery, which is unsuitable to all patients. Therefore, there is an urgent need to develop treatment modalities. Among them, pigment epithelium-derived factor (PEDF) is the most functionally important in RNV. PEDF is a kind of protein with relative molecular mass of 50,000, which can be used as new vessels inhibiting factor. In these days, there are many researches on PEDF for anti angiogenesis effect, antioxidation and vascular permeability resistance effect. Additionally, there are also reports on its inhibitory effect of inflammation. For example, Gao et al. revealed the angiogenesis inhibited effect of PEDF, which adjusted the growth of RNV. Stellmach et al. reported that PEDF could promote the apoptosis of the vascular endothelial cells, which could stop the vascular endothelial cells responding to the signal for hypoxia. Thus, it is still a challenge to use PEDF as a drug for anti VEGF.

Poly lactic-co-glycolic acid (PLGA) micro--nanostructures, have excited tremendous interests due to their excellent biocompatibility, efficient transport to the target and the changeable biodegradability, molecular weight and chemical structure by varying composition (lactide/glycolide ratio). Therefore, they have been...
widely used in pharmaceutical and medical engineering materials, such as skin transplantation, wound closure, drug carrier, and so on. In this work, PEDF is encapsulated in PLGA polymer to prepare PLGA/PEDF emulsions for high efficient inhibition on VEGF gene expression. The obtained emulsions can be utilized to inhibit VEGF levels in the human umbilical vein endothelial cell (HUVEC) cell culture medium by ELISA with different conditions of the concentrations of PLGA/PEDF emulsions, the culture time, the hypoxia treatment time, and oxygen concentration under hypoxia. The research provides a potential chance applying PEDF to treat RNV.

Experimental

Materials

Poly(lactide-co-glycolide) Resomer RG502 (PLGA-COOH, MW 20,000) was purchased from Ji’nan Daigang Biomaterial Co., Ltd. Amine-PEG-carboxymethyl (NH2-PEG-COOH, MW 3,400) was purchased from Shanghai Seebio Biotech. All chemical reagents were analytical grade or above and purchased from Sinopharm Chemical Reagent Co., Ltd. Triethylamine and dichloromethane were dried with calcium hydride before use. HCT116 cell line was purchased from cell bank of Chinese Academy of Sciences, and the cultivate reagents were purchased from Life Technologies (Gibco, USA). Cell counting kit-8 (CCK-8) was purchased from Beijing Fanbo Biochemicals Co., Ltd.

Preparation of PLGA/PEDF emulsions

10 mg of PLGA was dissolved into 200 µL of dichloromethane along with 100 µg of PEDF and 200 µL 15% Span80/dichloromethane (w/v). After well blended, the organic solution was then emulsified into 2 mL using an Ultra-turrax T10 (IKA) to form a pre-emulsion, and emulsification was performed in a 15 mL centrifuge tube placed over ice for 1 min. Then the pre-emulsion was sonicated with a vibrating metallic tip, JY 92-IIN (SCIENTZ, China), for 3 min over ice. It works for 3 s, then stops for 10 s, and repeats the process under 50% power (300 W). After further stirring with Ultra-turrax T10 (IKA) for 10 min (work for 1 min and stop for 1 min) over ice, organic solvents were then evaporated by magnetic stirring for about 3 h at room temperature.

Characterization

Particle size was measured by a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) based on dynamic light scattering (DLS). The 1H NMR spectra were recorded on a Bruker AM-400 spectrometer in CDCl3 solution at room temperature. Transmission electron microscopy (TEM) images were recorded on a JEOL-2100F instrument using an accelerating voltage of 200 kV. Fourier transform infrared spectroscopies (FT-IR) of PLGA and PLGA/PEDF emulsions were investigated on a Nicolet 6700 FT-IR spectrometer.

Cell cytotoxicity

Cells were cultured in 37.5 cm2 flasks in Dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics at a humidified, 37 °C and 5% CO2 incubator. The cytotoxic effects of PLGA/PEDF emulsions were determined with the CCK 8 assay. Briefly, HUVEC cells were seeded at a density of 5000 cells per well in 96-well plates, respectively. After 48 h, the medium was replaced with 100 µL medium with different concentration of PLGA/PEDF emulsions, but control cells were incubated with DMEM medium alone. After incubation for 24 and 48 h, the supernatant was removed and 10 µL CCK-8 solutions were added to each well of the plate (total medium 100 µL/well). Then cells were incubated with at 37 °C. After incubation for 4 h, absorbance was measured using iMark Microplate Reader (Bio-Rad, USA) at 450 nm as measure wavelength.

PEDF in vitro release

200 µg of PLGA/PEDF emulsion was immersed in 10 mL phosphate buffered saline (PBS, pH 7.4), and then transferred into a centrifuge tube containing 2 mL of PBS incubating at 37 °C. The speed of centrifuge rotation was 150 rpm. At predetermined time intervals (1—24 h), 1 mL of supernatant was collected from the centrifuge tube. The removed solution was replaced with an equal volume of fresh PBS. The enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer’s instructions to detect the supernatant’s PEDF content.

Quantification of VEGF by ELISA

The concentrations of VEGF in the cell culture medium were measured using ELISA according to their manufacturers’ instructions.

Cell culture during hypoxia treatment

HUVEC cells were seeded at a density of 5000 cells per well in 96-well plates. After 48 h, the medium was replaced with 100 µL DMEM without FBS, then 100 µL liquid paraffin was added to each well of the plate except the control group. Because of its low density and highly hydrophobic nature, liquid paraffin formed a film over the medium. As a consequence, the medium was separated from the normoxic atmosphere by the covering liquid paraffin film, which provided a hypoxic environment to the cell culture. After incubation under hypoxia condition for 4 and 8 h under the condition of 1% or 10% O2 in three gas incubator, liquid paraffin and DMEM were removed and 100 µL medium with 10% FBS and different concentration of PLGA/PEDF emulsion was added.
Confocal imaging of cells

Confocal imaging of cells was performed using a Leica laser scanning confocal microscope (Wetzlar, Germany). HUVEC cells (1 × 10⁶ cells/mL) were incubated with PLGA/PEDF emulsion for 2 h for confocal imaging, fixed with 4% paraformaldehyde for 30 min. All cells were washed twice with PBS before confocal imaging.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was done using Student’s t-test. Differences were considered significant at P < 0.05.

Results and Discussion

The morphology and structure of PLGA/PEDF emulsions were characterized. Figure 1a shows that PLGA/PEDF emulsions with core in gray and shell in dark are uniform nanospheres with an average diameter of about 150 nm. Furthermore, the average hydrodynamic size of the PLGA and PLGA/PEDF emulsions in deionized water is about 153 and 200 nm, respectively, which reveals the PEDF addition has a little increase on the size of the emulsion (Figure 1b). The zeta potential values before and after introducing PEDF are −20.5 and −5.2 mV, respectively, which suggests that PEDF is encapsulated into PLGA polymer successfully (Supporting Information). Additionally, the addition of PLGA/PEDF emulsion is further evidenced by FT-IR spectra (Figure 1c). In the spectra of PLGA emulsions, the band at 1100 and 1160 cm⁻¹ results from the absorption of C—C and C—O. In addition, the bands at 1756 cm⁻¹ is attributed to the absorption of C=O. Furthermore, in the spectra of PLGA/PEDF emulsion, compared to the spectra of PLGA emulsion, the typical N—H and C=C peaks of PEDF at 2800 and 2000 cm⁻¹ can be obviously found in the spectra of PLGA/PEDF emulsion besides the typical peaks of PLGA emulsion, which suggests that PEDF has been encapsulated in PLGA emulsion successfully.

Moreover, UV-vis spectra also prove that the addition of PEDF in PLGA emulsion. As shown in Figure 1d, the typical UV-vis peaks of PEDF at 295 nm can be obviously found in the spectra of PLGA/PEDF emulsion compared to the spectrum of PLGA emulsion. The above results show that the expected product is prepared successfully in our experiment.

Figure 2 further shows the release behavior of PEDF from the PLGA/PEDF emulsion in PBS buffers (pH 7.4), which is related to the natural environment. The final release is about 50.9% at 24 h, demonstrating that the PEDF inside the PLGA/PEDF emulsion could be released slowly. The curve of PEDF release showed sustained release behavior and the PEDF was progressively released by desorption and diffusion to the PBS solution. However, the final release increases to 76.3% at 24 h owing to the PLGA/PEDF emulsions could be decomposed to release PEDF under acidic conditions. The result indicates that the PLGA/PEDF emulsions have favorable release properties.

To evaluate the cytotoxicity characteristics of PLGA/PEDF emulsions, CCK-8 assays were performed. As shown in Figure 3, the effect of varying concentrations (0—25 µg/mL) of PLGA/PEDF emulsions on the viability of HUVEC cells after exposure for 24 and 48 h was investigated. The cell viability of PLGA/PEDF emulsion on HUVEC cells decreases obviously. There is significant difference in cell viability with the increasing concentration of PLGA/PEDF emulsion on HUVEC cells decreases obviously. There is significant difference in cell viability with the increasing concentration of PLGA/PEDF emulsion from 0—25 µg/mL compared to the control group after exposure for 24 and 48 h, which shows a slight decline. About 59% and 34% cell viabilities are maintained even up to a relatively high dose of 25 µg/mL after exposure for 24 and 48 h, respectively. The reason is according to the inhibition of PEDF on VEGF gene expression in HUVEC cells.
In order to clarify the reason for the decreasing HUVEC cell viability during PLGA/PEDF emulsion treatment, ELISA analysis was performed to examine the VEGF level in cells. As shown in Figure 3, different VEGF levels are evaluated at the condition of different culture time and concentration of PLGA/PEDF emulsion. The initial VEGF content is about 220 pg/mL without PLGA/PEDF emulsions, which is according to the previous report (Figure 4a). With increase of PLGA/PEDF emulsions, the VEGF levels in HUVEC cell decreases gradually. With 25 µg/mL PLGA/PEDF emulsion at 24 h culture, their VEGF content is about 162 pg/mL. The result shows that PEDF released from PLGA/PEDF emulsion can inhibit VEGF expression and then reduce cell proliferation, which is according to Figure 3b. Furthermore, the VEGF levels at different culture time are detected (Figure 4b). With increase of culture time, the VEGF levels in HUVEC cell also decreases gradually. With 24 h culture at 25 µg/mL PLGA/PEDF emulsions, their VEGF content is about 121 pg/mL, which results from the controlled release of PEDF in PLGA/PEDF emulsions. The results reveal that the obtained PLGA/PEDF emulsions are good agents for controlled release and inhibiting VEGF.

Based on the previous reports, hypoxia is a kind of stress factor, which not only affects the transcription expression of tumor related factors but also provides a route for the occurrence of new blood vessels.[14] Furthermore, PEDF can preferably regulate VEGF expression under the condition of hypoxia. Therefore, the VEGF levels are further performed under different oxygen concentration at 25 µg/mL PLGA/PEDF emulsion. As shown in Figure 5a, low oxygen concentration can activate the VEGF level in HUVEC cell. When the oxygen concentration is 1%, the VEGF level in HUVEC cell is improved to 385 pg/mL from 162 pg/mL. Moreover, the inhibition rate of PEDF on VEGF increases from 30% under normoxia to 39% under 1% oxygen concentration. Additionally, with increase of hypoxia treatment time, both the VEGF levels in HUVEC cell and the inhibition rate of PEDF on VEGF increases gradually (Figure 5b). These results reveal that the obtained PLGA/PEDF emulsion effectively inhibit VEGF expression under hypoxia.

To examine the feasibility of the obtained PLGA/PEDF emulsions for in vitro growth inhibition on HUVEC cells, their cell cytotoxicity and uptake on HUVEC cells were investigated. As shown in Figure 6a, the growth inhibitions were observed after the cell incubation with PLGA/PEDF emulsion and free PEDF. Furthermore, the IC50 values are 14.2 µg/mL.
for PEDF in PLGA/PEDF emulsion and 18.3 µg/mL for free PEDF, which also indicated that PLGA/PEDF emulsion increased the \textit{in vitro} growth inhibitory effect. Furthermore, the confocal image reveals the result of HUVEC cells incubated with PLGA/PEDF emulsion for 48 h. The result shows that HUVEC cells incubated with PLGA/PEDF emulsion (Figure 6b) is abundant than that with PEDF (Figure 6c).

Figure 6 (a) Inhibitory effects of PLGA/PEDF emulsion and free PEDF on HUVEC cells as examined by CCK-8 assay, and laser scanning confocal microscopy images of HUVEC cells incubated with (b) PLGA/PEDF emulsion and (c) free PEDF.

Conclusions

PEDF has encapsulated in PLGA polymer to prepare PLGA/PEDF emulsions for testing the VEGF inhibition level in HUVEC cells. The VEGF inhibition experiment of PLGA/PEDF emulsions by ELISA are performed under the conditions of the concentration of PLGA/PEDF emulsion, the culture time, the hypoxia treatment time and oxygen concentration under hypoxia. The results show the efficient inhibition on VEGF expression, which is according to the good inhibition on HUVEC cell proliferation by CCK-8 assay. Moreover, the IC\textsubscript{50} values on HUVEC cell are 14.2 µg/mL for PEDF in PLGA/PEDF emulsion and 18.3 µg/mL for free PEDF, which indicated that PLGA/PEDF emulsions increased the \textit{in vitro} growth inhibitory effect. The research provides a potential chance applying PEDF to treat RNV.

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