Targeting of AUF1 to vascular endothelial cells as a novel anti-aging therapy

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Abstract

Background Inhibition of aging of vascular endothelial cells (VECs) may delay aging and prolong life. The goal of this study was to prepare anti-CD31 monoclonal antibody conjugated PEG-modified liposomes containing the AU-rich region connecting factor 1 (AUF1) gene (CD31-PILs-AUF1) and to explore the effects of targeting CD31-PILs-AUF1 to aging VECs. Methods The mean particle sizes of various PEGylated immunoliposomes (PILs) were measured using a Zetasizer Nano ZS. Gel retardation assay was used to confirm whether PILs had encapsulated the AUF1 plasmid successfully. Fluorescence microscopy and flow cytometry were used to quantify binding of CD31-PILs-AUF1 to target cells. Flow cytometry was also used to analyze the cell cycles of aging bEnd3 cells treated with CD31-PILs-AUF1. We also developed an aging mouse model by treating mice with D-galactose. Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The malondialdehyde (MDA) and the superoxide dismutase (SOD) levels were detected by commercial kits. Hematoxylin-eosin (HE) staining was used to determine whether treatment with CD31-PILs-AUF1 was toxic to the mice. Results CD31-PILs-AUF1 specifically could targeted bEnd3 VECs and increased the percentage of cells in the S and G2/M phases of aging bEnd3 cells. ELISA showed that content of the IL-6 and TNF-α decreased in CD31-PILs-AUF1 group. The level of SOD increased, whereas MDA decreased in the CD31-PILs-AUF1 group. Additionally, CD31-PILs-AUF1 was not toxic to the mice. Conclusion CD31-PILs-AUF1 targets VECs and may delay their senescence.

Keywords: Aging; AU-rich region connecting factor 1; PEGylated immunoliposomes; Vascular endothelial cells

1 Introduction

As human society moves into an era heavily populated with aged individuals, health and longevity has become a concern. Currently, aging and anti-aging research has become a focus worldwide. Living standards and quality of life will continue to improve in the 21st century as scientific countermeasures to aging progress. With increasing age and cell degeneration, vascular endothelial cells (VECs) renew very slowly and show manifestations of aging. Long-term stimulation by pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), leads to chronic and low-grade microinflammation of VECs, which leads to age-related degenerative diseases. Many studies have shown close correlations between inflammation and DNA damage and between cell senescence and aging. Animal experiments have found that inhibition of VEC inflammation could delay aging and prolong life, thus it is imperative that we further investigate inhibition of VEC senescence.

Studies have shown that the AU-rich region connecting factor 1 (AUF1) gene controls the inflammatory response and maintains chromosome integrity by activating telomerase to repair the ends of chromosomes, thus AUF1 reduces inflammation and prevents rapid aging. By delivering AUF1 to VECs, we may be able to weaken the inflammatory cytokine response to ribonucleoproteins. Platelet endothelial cell adhesion molecule-1, which is also called cluster of differentiation 31 (CD31), is a member of the immunoglobulin superfamily and is expressed on endothelial cells, platelets, macrophages, and neutrophils and is involved in inflammatory angiogenesis. Inflammation, cell adhesion, and migration of endothelial cells play im-
portant roles in inflammatory angiogenesis, thus it is feasible to target CD31 to modulate VECs.

Recent studies have shown the potential application of nanoimmunoliposome drug carriers.\(^7\)\(^8\) PEGylated immunoliposomes (PILs) are modified with polyethylene glycol (PEG), which limits the particle size to approximately 100 nm. This carrier system can surround and protect peptide antigen and DNA molecules from non-specific phagocytosis.

This study aimed to design and build an anti-CD31 monoclonal antibody conjugated to a PEG-modified liposome containing the human AUF1 gene (CD31-PILs-AUF1) and to evaluate its ability to specifically bind aging VECs to achieve AUF1-targeted enrichment and delay senescence of VECs.

2 Materials and methods

2.1 Materials

Cholesterol (Chol), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG\(_{2000}\)), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG\(_{2000\text{-MAL}}\)) were obtained from Avanti polar lipids (Alabaster, AL, USA). The AUF1 plasmid was constructed by Shanghai Shenggong Biological Engineering Technology & Services (Shanghai, China). The anti-CD31 monoclonal antibody were provided by Abcam (Cambridge, MA, USA). The phycoerythrin(PE)-labeled anti-CD31 monoclonal antibody was obtained from eBiosciences (San Diego, CA, USA). IL-6, TNF-\(\alpha\) Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Dakewe Co. (Shenzhen, China). Total superoxide dismutase assay kit with NBT and lipid peroxidation malondialdehyde (MDA) assay kit were purchased from Beyotime Biotechnology (Shanghai, China). A 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). D-Galactose and 4% polyoxymethylene were purchased from Sigma (St. Louis, MO, USA). F-12K medium, DMEM medium, and 10% fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Cell lines and animals

The mouse brain endothelial cell line bEnd3 and baby hamster kidney cell line BSR (BHK-21) were purchased from the cell bank of the Committee for Type Culture Collection of the Chinese Academy of Sciences. The cells grew in a humidified incubator at 37 °C with 5% CO\(_2\). The bEnd3 cells were cultured in F-12K medium and the BSR cells were cultured in DMEM medium. Both media were supplemented with 10% FBS (Hyclone) and 100 U/mL penicillin-streptomycin. bEnd3 cells passed more than 50 generations as aging cells.

Male Kunming mice aged 6–10 weeks were purchased from the Experimental Animal Center of Guangxi Medical University. All of the experimental procedures complied with the requirements of the National Animal Guidelines and all of the protocols were approved by the Ethics Committee of Guangxi Medical University.

2.3 PILs preparation

PILs were prepared with Chol, POPC, and DSPE-PEG 2000 at a 53: 40: 5 molar ratio. The lipids were dissolved in chloroform, transferred to a round-bottom flask, and a lipid film was formed by evaporating the chloroform for 2–3 h. The lipid film was hydrated in 1 mL of Tris-Hcl (pH 7.0, 10 mmol/L) and vortexed immediately to ensure that it was completely dissolved. Homogenous liposomes were prepared by manually extruding them through polycarbonate membrane filters with 200 nm, 100 nm, and 50 nm pores. The lipids were extruded 20 times for each filter beginning with the largest pore-size filter and ending with the smallest pore-size filter.

2.4 AUF1 plasmid encapsulation in PILs and conjugation of PILs with anti-CD31 monoclonal antibody

1 mL of PILs and 200 \(\mu\)g of AUF1 plasmid were mixed in 70% ethanol with 4 mm CaCl\(_2\). This prepared mixture was incubated in liquid nitrogen for 5 min, at 37 °C for 2 min, and at room temperature for 4 min, and this freeze-thaw-balance cycle was repeated five times. This solution was then manually extruded by passing it through polycarbonate membrane filters with 200 nm, 100 nm, and 50 nm pores. This solution was extruded 20 times for each filter beginning with the largest pore-size filter and ending with the smallest pore-size filter. PILs or PILs-AUF1 were incubated with anti-CD31 monoclonal antibody for 2 h. Gel retardation assay was used to confirm whether liposomes had encapsulated the AUF1 plasmid successfully. The mean particle sizes of various PILs were determined by Zetasizer Nano ZS (Malvern). All of the measurements were performed on three independent samples and data were analyzed with Malvern Zetasizer Software vs 6.2.

2.5 Binding of CD31-PILs-AUF1

BSR and bEnd3 cells were used to testify the targeting of
CD31-PILs-AUF1 to VECs. Although bEnd3 cells express high levels of CD31, BSR cells do not express CD31. Cells were seeded in separate 6-well dishes for 12 h before initiating the uptake experiments. The PILs were added to the wells at a concentration of 20 nm per 5×10⁴ cells and they were incubated for 2 h at 37°C in media supplemented with 10% FBS and 1% penicillin-streptomycin. Unbound PILs were removed by washing 3 times with 0.1 M PBS pH 7.0. Cells were fixed in 4% polyoxymethylene for 20 min and nuclei were stained with DAPI for 5 min. Fluorescence images were obtained by fluorescence microscopy (Nikon DS-Ri1, Japan).

Flow cytometry (Beckman Coulter Epics XL, USA) was used to quantify the binding of CD31-PILs-AUF1. PILs were added to cells at a concentration of 20 nM per 5×10⁴ cells and they were incubated for 2 h at 37°C in media supplemented with 10% FBS and 1% penicillin-streptomycin. After the 2 h incubation, the cells were trypsinized and unbound PILs were removed by washing 3 times with 0.1 M PBS pH 7.0. Binding of CD31-PILs-AUF1 to cells was evaluated using Flowjo Software v7.6.

2.6 Cell cycle assay

Aging bEnd3 cells were treated with PBS, PILs, CD31-PILs, PILs-AUF1, or CD31-PILs-AUF1 in F-12K medium containing 10% FBS and 10 g/L D-galactose for 7 days, then cells were washed with PBS, resuspended in 300 μL of PBS, treated with the Cell Cycle Assay Kit, and analyzed by flow cytometry (Beckman Coulter Epics XL, USA). Data were analyzed using EXPO32 ADC Analysis software.

2.7 ELISA assay

Aging bEnd3 cells were treated with PBS, PILs, CD31-PILs, PILs-AUF1, or CD31-PILs-AUF1 in F-12K medium containing 10% FBS and 10 g/L D-galactose for seven days, then cells supernatant was collected and detected by IL-6, TNF-α cytokines commercial kits following the manufacturer’s recommendations.

2.8 Animal experiment

Thirty mice were randomly separated into five groups. The mice were injected subcutaneously with D-galactose at 0.1 g/kg for 45 days. After injection for D-galactose three weeks the five groups were injected subcutaneously with (1) 0.1 mL of PBS (vehicle control), (2) 0.1 mL of PILs, (3) 0.1 mL of PILs-AUF1, (4) 0.1 mL of CD31-PILs, or (5) 0.1 mL of PILs-CD31-AUF1 for four weeks once a week. The mice were sacrificed on the last day of treatment, and their sera were assayed using MDA and superoxide dismutase (SOD) assay kits. The assays were performed according to the manufacturer’s instructions. Sections of major tissues, including heart, lung, liver, spleen, and kidney, were immersed in 10% formaldehyde, dehydrated, and paraffin-embedded. Serial sections (4 nm) were cut and stained with Hematoxylin-eosin (HE).

2.9 Statistical analysis

Datas were presented as mean ± SD and the differences among groups were analyzed by analysis of variance (ANOVA) using GraphPad Prism 6.0 software. A P-value of < 0.05 was considered statistically significant.

3 Results

3.1 Physical properties of PILs

We characterized PILs by laser particle size analyzer. As shown in Figure 1A, the particle size distribution of PILs was fairly narrow indicating relatively uniform particles. The average diameters of PILs, CD31-PILs, PILs-AUF1, and CD31-PILs-AUF1 particles were 81.71 nm, 93.69 nm, 129.02 nm, and 149.87 nm, respectively. Successful encapsulation of AUF1 plasmid DNA into PILs and CD31-PILs was confirmed by gel retardation assay (Figure 1B).

3.2 CD31-PILs-AUF1 specifically binds to bEnd3 cells in vitro

Binding of the different PILs to bEnd3 and BSR cells was evaluated by fluorescent microscopy and flow cytometry. CD31-PILs-AUF1 bound efficiently to bEnd3 cells, but not to BSR cells (Figure 2A). The findings from flow cytometry analyses revealed that CD31-PILs-AUF1 and CD31-PILs bound specifically to bEnd3 cells, but not to the control BSR cells (Figure 2B).

3.3 Characterization of the cell cycles of aging bEnd3 cells with and without CD31-PILs-AUF1

A decline in the ability of a cell to proliferate is a biological sign of aging, and changes in the cell cycle may reflect the ability of a cell to proliferate. In aging bEnd3 cells, most of the cells stagnated in G1 phase, G2/M phase tended to disappear. After treatment with CD31-PILs-AUF1, the percentage of cells in the S and G2/M phases increased significantly. The percentage of cells in the G2/M phase in the CD31-PILs-AUF1 group was higher than in the other groups (Figure 3). These results indicated that CD31-PILs-AUF1 could promote the recovery proliferation of aging bEnd3 cells.
3.4 The levels of IL-6 and TNF-α of aging bEnd3 cells treatment with CD31-PILs-AUF1 declined.

The levels of inflammatory cytokines secreted by bEnd3 cells were evaluated by ELISA assay. The results showed that the levels of IL-6 and TNF-α increased in aging bEnd3 cells. After treatment with CD31-PILs-AUF1, the levels of IL-6 and TNF-α declined, compared with PBS group, PILs group, and CD31-PILs group respectively (Figure 4). But compared with control group, the levels of IL-6 and TNF-α were not significantly different.

3.5 MDA and SOD serum levels after treatment with CD31-PILs-AUF1

By detecting MDA and SOD levels in the sera of aging mice after treatment with various PILs, we found that SOD levels increased, whereas MDA levels decreased in the CD31-PILs-AUF1 group relative to PBS group, PILs group, and CD31-PILs group respectively (Figure 5). But compared with control group, the levels of MDA and SOD were not significantly different.

3.6 Toxicity of CD31-PILs-AUF1 in vivo

HE staining showed that the main organs of the mice were not significantly change upon treatment with CD31-PILs-AUF1 (Figure 6) indicating that CD31-PILs-AUF1 is not toxic in vivo.
4 Discussion

PILs have been shown to deliver molecules efficiently and with low immunogenicity, good biocompatibility and biodegradability, and no toxic side effects.\textsuperscript{[9,10]} To extend the time that a drug circulates in the body, liposomes can be PEGylated on their surfaces, which creates steric hindrances that prevent the flow of water molecules and DNA plasmids across the membranes of liposomes.\textsuperscript{[11–13]} PEGylation has been well documented to increase the half-life of liposomes \textit{in vivo}.\textsuperscript{[14]} The properties of our liposomes were in accordance with other studies that used liposomes for targeting purposes \textit{in vitro} and \textit{in vivo}.

By inhibiting VEC inflammation, aging may be delayed and life may be prolonged. However, there is no targeted therapy for the aging of vascular endothelial cells. To enhance the effects of anti-aging treatments, we constructed a drug delivery system using liposomes conjugated with anti-CD31 monoclonal antibody (CD31-PILs) because anti-CD31 monoclonal antibody targets VECS. This CD31-PILs delivery system was able to encapsulate the AUF1 plasmid and to deliver it to bEnd3 VECs.

A decline in cell proliferation ability is one of the biological signs of aging, and cell cycle changes can reflect the

Figure 2. Binding of PILs to Bend3 and BSR cells. (A): Fluorescence microscopy images of bEnd3 and BSR cells treated with PE-labeled CD31-PILs-AUF1(red). Nuclei were labeled with DAPI (blue), (400X); (B): Flow cytometry was used to quantify binding of various PILs to bEnd3 and BSR cells. The binding curves of CD31-PILs and CD31-PILs-AUF1 to bEnd3 cells significantly shifted. AUF1: AU-rich region connecting factor 1; CD31: cluster of differentiation 31; DAPI: 6-diamidino-2-phenylindole dihydrochloride; PE: phycoerythrin; PILS: PEGylated immunoliposomes.
Figure 3. Cell cycles of aging bEnd3 cells upon treatment with various PILs. After treatment with CD31-PILs-AUF1, the percentages of cells in G1 phase was 80.8%, S phase was 4.9%, G2 phase was 8.9%. Compare with other groups, the percentages of cells in the G2 phase of CD31-PILs-AUF1 group significantly increased, while reduced in G1 phase. AUF1: AU-rich region connecting factor 1; CD31: cluster of differentiation 31; PILs: PEGylated immunoliposomes.

Figure 4. The IL-6 and TNF-α levels of aging bEnd3 cells treatment with various PILs. ELISA was used to detect cytokines IL-6, TNF-α secreted by young bEnd3 cells and aging bEnd3 cells treatment with various PILs. The asterisks indicated significant differences between the CD31-PILs-AUF1 group and other groups as follows: *P < 0.05, **P < 0.01, ***P < 0.001. AUF1: AU-rich region connecting factor 1; CD31: cluster of differentiation 31; IL-6: interleukin-6; ns: no significant; PILs: PEGylated immunoliposomes; TNF-α: tumor necrosis factor-α.
Figure 5. MDA and SOD levels in sera after mice were treated with various PILs. MDA and SOD levels in sera were evaluated in young mice and aging mice treatment with various PILs. The asterisks indicated significant differences between the CD31-PILs-AUF1 group and other groups as follows: "P < 0.05, ""P < 0.01, """"P < 0.001, AUF1: AU-rich region connecting factor 1; CD31: cluster of differentiation 31; MDA: malondialdehyde; ns: no significant; PILS: PEGylated immunoliposomes; SOD: superoxide dismutase.

ability of a cell to proliferate. Analysis of cell cycle distributions showed that after treatment with CD31-PILs-AUF1, the percentages of cells in the G2/M phases significantly increased, while the percentages reduced in G0/G1 phase. These data are consistent with previous reports that AUF1 plays roles in anti-aging and in maintaining cell proliferation, thus, delivery of the AUF1 plasmid may play a role in anti-aging.

Our findings are consistent with earlier work showing increased IL-6 expression in old rats compared with young rats and after anti-inflammatory treatment, inflammation related factors are reduced and symptoms of aging can be improved.\cite{15-17} Whether the effects of these cytokines are mediated through the generation of intracellular reactive oxygen species, or through another defined cell-signaling mechanism, is further being studied.

To verify the effect of CD31-PILs-AUF1 in vivo, we developed an aging mouse model using D-galactose.\cite{18,19} The result show D-galactose accelerates aging in rodents by inducing oxidative stress by increasing the MDA level and reducing SOD activity. This is consistent with previous reports, indicating the success of the aging mouse model.\cite{20} MDA content decreased and the SOD content increased in mice treated with CD31-PILs-AUF1 indicating that CD31-PILs-AUF1 may delay the senescence induced by D-galactose. In conclusion, we have developed an effective PILs strategy to deliver the AUF1 plasmid to a specific target, and this system may be useful for the development of new anti-aging drugs.

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Figure 6. Toxicity of CD31-PILs-AUF1 in vivo. HE staining showed that the heart, liver, spleen, kidney of mice treated with CD31-PILs-AUF1 and without treatment. AUF1: AU-rich region connecting factor 1; CD31: cluster of differentiation 31; HE: hematoxylin-eosin; PILS: PEGylated immunoliposomes.
References

1 Chung HY, Cesari M, Anton S, et al. Molecular inflammation: underpinnings of aging and age-related diseases. *Ageing Res Rev* 2009; 8: 18–30.

2 Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature* 2010; 464: 520–528.

3 Andrews NP, Fujii H, Goronzy JJ, et al. Telomeres and immunological diseases of aging. *Gerontology* 2010; 56: 390–403.

4 Browner WS, Kahn AJ, Ziv E, et al. The genetics of human longevity. *Am J Med* 2004; 117: 851–860.

5 Hasegawa Y, Saito T, Ogihara T, et al. Blockade of the nuclear factor-kappaB pathway in the endothelium prevents insulin resistance and prolongs life spans. *Circulation* 2012; 125: 1122–1133.

6 Pont AR, Sadri N, Hsiao SJ, et al. mRNA decay factor AUF1 maintains normal aging, telomere maintenance, and suppression of senescence by activation of telomerase transcription. *Mol Cell* 2012; 47: 5–15.

7 Weng KC, Noble CO, Papahadjopoulos-Sternberg B, et al. Targeted tumor cell internalization and imaging of multifunctional quantum dot-conjugated immunoliposomes in vitro and in vivo. *Nano Lett* 2008; 8: 2851–2857.

8 Li T, Zhang M, Han Y, et al. Targeting therapy of choroidal neovascularization by use of polypeptide- and PEDF-loaded immunoliposomes under ultrasound exposure. *J Huazhong Univ Sci Technolog Med Sci* 2010; 30: 798–803.

9 Gao J, Yu Y, Zhang Y, et al. EGFR-specific PEGylated immunoliposomes for active siRNA delivery in hepatocellular carcinoma. *Biomaterials* 2012; 33: 270–282.

10 Wicki A, Rochlitz C, Orleth A, et al. Targeting tumor-associated endothelial cells: anti-VEGF2 immunoliposomes mediate tumor vessel disruption and inhibit tumor growth. *Clin Cancer Res* 2012; 18: 454–464.

11 Giddam AK, Zaman M, Skwarczynski M, et al. Liposome-based delivery system for vaccine candidates: constructing an effective formulation. *J Nanomedicine* (Lond) 2012; 7: 1877–1893.

12 Wang Z, Yu Y, Dai W, et al. A specific peptide ligand-modified lipid nanoparticle carrier for the inhibition of tumor metastasis growth. *Biomaterials* 2013; 34: 756–764.

13 Zhang X, Guo S, Fan R, et al. Dual-functional liposome for tumor targeting and overcoming multidrug resistance in hepatocellular carcinoma cells. *Biomaterials* 2012; 33: 7103–7114.

14 Gupta B, Torchilin VP. Monoclonal antibody 2C5-modified doxorubicin-loaded liposomes with significantly enhanced therapeutic activity against intracranial human brain U-87 MG tumor xenografts in nude mice. *Cancer Immunol Immunother* 2007; 56: 1215–1223.

15 Donato AJ, Black AD, Jablonski KL, et al. Aging is associated with greater nuclear NF-κB, reduced IκBα, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. *Aging Cell* 2008; 7: 805–812.

16 Belmin J, Bernard C, Cormann B, et al. Increased production of tumor necrosis factor and interleukin-6 by arterial wall of aged rats. *Am J Physiol* 1995; 268: H2288–H2293.

17 Csisza A, Ungvari Z, Koller A, et al. Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging. *Physiol Genomics* 2004; 17: 21–30.

18 Cui X, Zuo P, Zhang Q, et al. Chronic systemic D-galactose exposure induces memory loss, neurodegeneration, and oxidative damage in mice: protective effects of R-alpha-lipoic acid. *J Neurosci Res* 2006; 84: 647–654.

19 Huang CC, Chiang WD, Huang WC, et al. Hepatoprotective Effects of Swimming Exercise against D-Galactose-Induced Senescence Rat Model. *Evid Based Complement Alternat Med* 2013; 2013: 275431.

20 Lu J, Wu D, Hu B, et al. Chronic administration of troxerutin protects mouse brain against D-galactose-induced impairment of cholinergic system. *Neurobiol Learn Mem* 2010; 93: 157–164.