Nanoparticles co-encapsulating hVEGF and hAng-1 can induce mitotic and antiapoptotic effect on vascular endothelial cells

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ABSTRACT

The study demonstrates the combined effect of the angiogenic proteins, hVEGF and hAng-1, coencapsulated in human serum albumin (HSA) nanoparticles (NPs), towards Human Umbilical Vein Endothelial Cells (HUVECs) and evaluation of the potential application of this delivery system towards therapeutic angiogenesis. The size of the NPs cross-linked with glutaraldehyde varied between 101.0± 0.9nm and the zeta potential was found to be -18± 2.9 mV. The NPs were incubated for 2 weeks to study the release profiles of the proteins and it was observed that 49±1.3 % of hAng-1 and 59±2.1 % of hVEGF had been released from the NPs. In addition, HUVEC proliferation and percent apoptosis in response to the proteins released from the HSA NPs was observed. Both the angiogenic proteins, hVEGF and hAng-1were biologically active and the combined application demonstrated a significantly high proliferative and anti-apoptotic effect on HUVECs.

Keywords: Encapsulation, nanoparticles, growth factors, angiogenesis, regenerative medicine

1 INTRODUCTION

Programmed cell death or apoptosis is required in multicellular organisms for normal development where unwanted cells are eliminated during physiological and certain pathological conditions [1]. Dysregulation of endothelial cell apoptosis has a major adverse effect in the embryo leading to haemorrhage and finally embryonal death. Further, endothelial cell apoptosis may lead to vessel regression by inhibiting angiogenesis. Thus preventing endothelial cell apoptosis, especially in the patients suffering from ischemic diseases, could be a potential therapeutic route towards the improvement of angiogenesis and vasculogenesis.

Vascular endothelial growth factor (hVEGF) plays a crucial role in the protection of these endothelial cells against apoptosis [2] and its application in the promotion of therapeutic angiogenesis has been widely shown [3, 4]. Controversy exists over whether the presence of hVEGF alone could achieve functional and mature vessels. Thus, Angiopoietin-1 (hAng-1) is being investigated to determine its ability to stabilize and assist blood vessels maturation [5] and in fact inhibit endothelial cell apoptosis [6]. An efficient strategy for therapeutic angiogenesis could be administration of a combination of hAng-1 with hVEGF. Proteins have very limited in vivo half-lives and need to be administered through multiple injections to achieve the desired therapeutic effect. Appropriate delivery vehicles need to be developed for these unstable macromolecules to protect them from degradation in the biological environment. By encapsulating the proteins, the physicochemical properties of the delivery system could enable a more controlled and continuous protein release over time. The objective of this study was to investigate the feasibility of entrapping the growth factors, hVEGF and hAng-1, in Human Serum Albumin (HSA) nanoparticles (NPs) cross-linked with glutaraldehyde to allow potential therapeutic angiogenesis.

2 MATERIALS AND METHODS

Preparation and Characterization of Nanoparticles

HSA NPs were prepared using pH-coacervation technique according to previously published work [7]. 100mg of HSA protein was added to 2ml 10mM NaCl solution at room temperature under constant stirring in a glass beaker. 0.1N NaOH was added to bring the pH to 8. 14µl of 0.5mg/mL of hAng-1, 1µl of 0.5mg/mL of hVEGF and 1µl of of 0.5mg/mL of BSA were added to Endothelial cell medium (ECM) (with or without 5% FBS) and this aqueous phase was then desolvated with the addition of 4ml of

ethanol at 1ml/min to form the NPs. The resulting NPs were then stabilized by coating with 40µl of 5% glutaraldehyde. The loading efficiency of the proteins was determined in terms of the amount of hAng-1 and hVEGF transformed into NPs before coating using the respective ELISA kits. The particle size, zeta potential and polydispersity index (PDI) of the NPs was measured using a Zeta Potential Analyzer. Scanning Electron Microcopy (SEM) was used to study the morphological characteristics of the NPs. Transmission Electron Microscopy (TEM) was used to obtain the size characterization.

Nanoparticle cytotoxicity analysis

In order to observe the in vitro cytotoxicity of the various concentrations of glutaraldehyde on endothelial cells, CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used [7]. The HUVECs were washed with PBS and incubated for 96 hours with NPs cross-linked with 40µl of 1.5%, 3%, 4.5%, 6% and 7.5% and control NPs without glutaraldehyde cross-linking.

Release of co-encapsulated hAng-1 and hVEGF from nanoparticles

The protein loaded NPs were incubated at 37°C with 5ml of ECM with 1% penicillin/streptomycin under constant shaking. The amount of hAng-1 and hVEGF protein released into the supernatant was determined using the respective protein ELISA kits.

HUVEC proliferation assay

To evaluate the retention of the bioactivity of the proteins released from the NPs, proliferative studies using Human umbilical vein endothelial cells (HUVECs) were carried out. CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to assess HUVEC cell proliferation treated with NP supernatant [8]. 24hrs post seeing, HUVECs were incubated for 96 hours with 0.1ml of NP supernatant, NP supernatant with excess hVEGF antibody and NP supernatant with excess hAng-1 antibody released from the NPs into the ECM on day 2, 8 and 14. ELISA of the respective proteins was used to determine the concentration of hAng-1 and hVEGF in the NP supernatant. The relative HUVEC viability was determined by MTS assay.

HUVEC apoptosis assay

NPs were prepared as per previous particle preparation protocol but the proteins were dissolved in serum-free ECM (without 5% FBS) instead of ECM with 5% FBS before being added to the HSA solution. Percent apoptosis was determined.

3 RESULTS AND DISCUSSION

Characterization of nanoparticles

NPs formed had a size ranging between 101.0± 0.9 nm. The TEM and SEM micrographs of the NPs illustrated in **Figure 1** demonstrated that the particles are properly dispersed, spherical and had smooth surfaces. The protein NPs were negatively charged and the zeta potential varied between -18± 2.9 mV at pH 7.0. The polydispersity index was 0.3.

Cytotoxicity of the NPs to endothelial cells increased as the concentration of glutaraldehyde was increased during particle formation. HSA-NPs with no glutaraldehyde coating demonstrated a higher than 90% cell viability for 96 hours. In our experiments, 40µl of 5% (w/v) glutaraldehyde was chosen as the optimal concentration for the NP preparation.

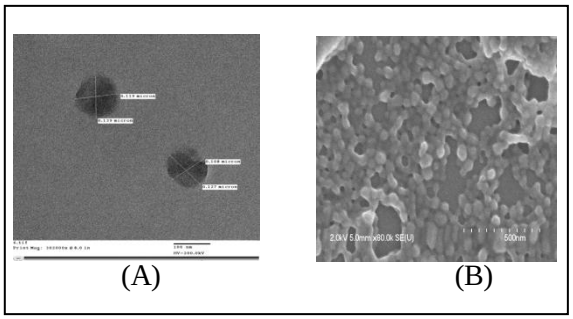

Figure 1: TEM and SEM micrographs of the NPs demonstrate that the particles are properly dispersed, spherical and had smooth surfaces

Loading efficiency and release profiles of proteins from NPs

The loading efficiency of hAng-1 and hVEGF during NP fabrication was 49% and 55% respectively. Cumulative release of of hAng-1 and hVEGF was observed for 2 weeks to determine protein release from the NPs in the culture medium. The NPs demonstrated an initial burst release amounts (approximately $617 \pm 100\, ng$ and $88 \pm 10\, ng$), with lower steady release amounts (approximately 171.5 ng and 13.75 ng) respectively for hAng-1 and hVEGF.

Bioactivity of released proteins

The application of this delivery system for therapeutic angiogenesis hVEGF in combination with hAng-1 significantly increases HUVEC proliferation. Our results show that the least cell proliferation occurs with NP supernatant containing hAng-1 (VEGF antibody added), moderate proliferation with NP supernatant containing hVEGF (Ang-1 antibody added), and finally the highest proliferation is seen in the case of NP supernatant containing both hVEGF and hAng-1 combined. Negligible proliferation was observed were the control NP supernatant containing BSA was used. The maximum HUVEC growth (89.62%) was seen in the case of NP supernatant containing both hAng-1 and hVEGF, proliferation was 67.79% in the case of NP supernatant containing hVEGF (Ang-1 antibody added), and finally a much lower proliferation (5.87%) when using NP supernatant containing hAng-1(VEGF antibody added) was observed.

Anti-apoptotic effect of proteins

Combined anti-apoptotic effect of the proteins on the seeded HUVECs was studied. A percent apoptosis above 90% was observed when HUVECs were incubated with serum free control media and NP supernatant containing BSA. From day 2 to day 14, the percent apoptosis due to NP supernatant containing hAng-1 (hVEGF antibody added) decreased from 31.5% to 27.06% and the due to NP supernatant containing hVEGF (hAng-1 antibody added) decreased from 29.6% to 27.74%. Compared to control, the cell apoptosis decreased almost by 50% with the application of supernatant from NPs loaded with both the proteins The combined antiapoptotic effect of the NP supernatant containing hAng-1 and hVEGF decreased from 16.44% to 10.42% for day 2 to 14.

4 CONCLUSION

The potential of using the angiogenic proteins, hVEGF and hAng-1, coencapsulated in albumin nanoparticles towards therapeutic angiogenesis has been demonstrated. Our results illustrate that the NP system provided controlled and extended protein release leading to increased combined effect of the released angiogenic proteins towards proliferation and anti-apoptosis of HUVECs.

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