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Efficient siRNA delivery and tumor accumulation mediated by ionically cross-linked folic acid–poly(ethylene glycol)–chitosan oligosaccharide lactate nanoparticles: For the potential targeted ovarian cancer gene therapy

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ABSTRACT

For effective ovarian cancer gene therapy, systemic administrated tumor-targeting siRNA/folic acid–poly(ethylene glycol)–chitosan oligosaccharide lactate (FA–PEG–COL) nanoparticles is vital for delivery to cancer site(s). siRNA/FA–PEG–COL nanoparticles were prepared by ionic gelation for effective FA receptor-expressing ovarian cancer cells transfection and *in vivo* accumulation. The chemical structure of FA–PEG–COL conjugate was characterized by MALDI-TOF-MS, FT-IR and ^{1}H NMR. The average size of siRNA/FA–PEG–COL nanoparticles was approximately 200 nm, and the surface charge was +8.4 mV compared to +30.5 mV with siRNA/COL nanoparticles. FA–PEG–COL nanoparticles demonstrated superior compatibility with erythrocytes in terms of degree of aggregation and haemolytic activity and also effects on cell viability was lower when compared with COL nanoparticles. FA grafting significantly facilitated the uptake of nanoparticles via receptor mediated endocytosis as demonstrated by flow cytometry. The *in vitro* transfection and gene knockdown efficiency of HIF-1α were superior to COL nanoparticles (76–62%, respectively) and was comparable to Lipofectamine 2000 (79%) as demonstrated by RT-qPCR and Western blot. Gene knockdown at the molecular level translated into effective inhibition of proliferation *in vitro*. Accumulation efficiency of FA–PEG–COL nanoparticles was investigated in BALB/c mice bearing OVK18 #2 tumor xenograft using *in vivo* imaging. The active targeting FA–PEG–COL nanoparticles showed significantly greater accumulation than the passive targeting COL nanoparticles. Based on the results obtained, siRNA/FA–PEG–COL nanoparticles show much potential for effective ovarian cancer treatment via gene therapy.

1. Introduction

Ovarian cancer has the highest mortality rate and is among the most common of female malignancies in Western countries (Tagawa et al., 2012). Ovarian cancer is often undetectable in its early stages and therefore diagnosis usually occurs when surgical treatment is no longer an effective option (Hua et al., 2009). Moreover, ovarian cancer cells are known to develop resistance to standard chemotherapeutic treatments (Tagawa et al., 2012).

Gene silencing via RNA interferences (RNAi) mediated by short interfering RNA (siRNA) has enormous therapeutic potential for the treatment of cancer. Many genes associated with regulation of proliferation and angiogenesis are mutated in cancer resulting in uncontrolled proliferation and these genes are a potential target for the gene silencing therapy (Dykxhoorn and Lieberman, 2006; Cho et al., 2008; Davis et al., 2010). Hypoxic inducible factor-1α (HIF-1α) is often overexpressed in cancers including ovarian cancer

Abbreviations: ANOVA, analysis of variance; BCA, bicinchoninic acid; COL, chitosan oligosaccharide lactate; cps, centipoise; CV, coefficient of variation; DCC, dicyclohexylcarbodiimide; DCl, deuterium chloride; DEPC, diethyl pyrocarbonate; DLS, dynamic light scattering; DMEM, Dulbecco's modified eagle's medium; ECL, enhanced chemiluminescence; EDC, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide; EPR, enhanced permeability and retention effect; EtBr, ethidium bromide; FA, folic acid; FA–PEG–COL, folic acid–poly(ethylene glycol)–chitosan oligosaccharide lactate; FBS, fetal bovine serum; FT-IR, Fourier transformed infrared spectroscopy; HIF-1α, hypoxic inducible factor-1 alpha; HRP, horseradish peroxidise; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MWCO, molecular weight cut off; NH_2–PEG–COOH, α-aminopropyl-ω-carboxypentyloxy-polyxyethylene·hydrochloride; NHS, N-hydroxysuccinimide; NIRF, near-infrared fluorescence; NPs, nanoparticles; NTC, no template control; PDI, polydispersity index; PVDF, polyvinylidene fluoride; RBCs, red blood cells; RGD peptide, arginine–glycine–aspartic acid peptide; RT-qPCR, real-time quantitative polymerase chain reaction; TBE, Tris/borate/EDTA; TBS-T, Tris-buffered saline with Tween; TPP, tripolyphosphate.

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and it is associated with tumor aggressiveness, angiogenesis, cell migration, proliferation, survival, glucose metabolism, metastasis and drug resistance through oncogene gain-of-function and tumor suppressor gene loss-of-function (Semenza, 2001; Yeo et al., 2004). Aside from induction via hypoxic conditions associated with tumor microenvironment, genetic alterations in cancer cells can induce HIF-1α expression via a non-hypoxic stimulation, leading to cancer progression (Hirota and Semenza, 2006). We here predicted that HIF-1α suppression via siRNA technique would provide effective tumoricidal outcome in human ovarian cancer cells. In fact, siRNA knockdown of the HIF-1α gene has been reported to inhibit angiogenesis and tumor cellular energy production resulting in growth suppression (Ziello et al., 2007; Chiavarina et al., 2010).

However, the use of siRNA gene therapy is hindered because achieving sufficient concentration of siRNA at the tumor site(s) is difficult. siRNA has a high degradation rate in serum due to its physical characteristics, a rapid elimination by the renal pathway and a low permeability across cellular membranes (Miyagishi et al., 2004; Sioud and Sorensen, 2003; Sorensen and Sioud, 2003). One way to enhance the delivery of siRNA to the site of action is a development of a suitable delivery platform with characteristics that enables biocompatibility, a high loading capacity, protection of siRNA during transport and a high targeting ability (Creusat et al., 2012).

Recently the delivery system for gene therapy has moved from viral vectors to synthetic and natural cationic polymers because viral vectors have potential to evoke immunogenic responses and can be hazardous during preparation (Liang et al., 2009). A representative cationic polysaccharide is a natural substance, chitosan (Liang et al., 2009; Chan et al., 2007; Salmaso et al., 2004). Chitosan forms ionic interactions with siRNA via a high net positive charge of its amino group. Moreover, nano-metric particles are easily formed by crosslinking chitosan with a counter ion such as tripolyphosphates (TPP), which particle provides a protection against degradation of loaded siRNA (Park et al., 2010).

There are problems in the use of chitosan for gene delivery. First, excessive positive charge that remains on the surface of the nanoparticles after formulation brings about interaction with red blood cells (RBCs), opsonization and activation of immune system resulting in elimination of them. PEGylation has been reported to reduce their surface charge and thus prolong their circulating half-life (Hua et al., 2009; Dykxhoorn and Lieberman, 2006; Cho et al., 2008; Park et al., 2010). Second, achieving a sufficient concentration of siRNA at the tumor site in a timely manner is difficult, as systemically administrated chitosan nanoparticles are only passively delivered there via the enhanced permeability and retention (EPR) effect (Chan et al., 2007). Third, uptake of chitosan nanoparticles by non-specific endocytosis in tumor cells results in a low siRNA transfection efficiency. To address these problems, an active targeting system that can also aid the uptake of nanoparticles is required.

Although targeting ligands, such as RGD peptide (Park et al., 2010), epitope of Herceptin (Park et al., 2010) and fibroblast growth factor (Kalli et al., 2008), have been developed for the enhancement of targeting ability of delivery systems, high cost and handling issues are often associated with such compounds (Parker et al., 2005). In the present study we have emplyed folic acid (FA) as a targeting ligand because FA is harmless on normal cells, little immunogenic, inexpensive and stable under both *in vitro* and *in vivo* conditions (Creda-Cristerna et al., 2011; Yang et al., 2010) and furthermore the expression level of the FA receptor is high in ovarian cancer cells (Kalli et al., 2008; Parker et al., 2005; Werner et al., 2011), which promotes the receptor-mediated endocytosis of nanoparticles.

The present study shows the potential utility of an siRNA delivery system with FA–PEG–COL nanoparticles encapsulating an HIF-1α siRNA for a targeted ovarian cancer gene therapy.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Chitosan oligosaccharide lactate (COL) (3–5 kDa), sodium tripolyphosphate (TPP), folic acid ⩾ 97%, diethyl pyrocarbonate ⩾ 98% (DEPC) and fluorescein isothiocyanate (FITC) were purchased from Sigma Aldrich (St. Louis, MO). α-Aminopropyl-ω-carboxypentyloxy-polyxyethylene·hydrochloride (NH_2–PEG–COOH) was purchased from NOF Corporation (Tokyo, Japan). Dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), 35% deuterium chloride solution in deuterium oxide 99.5% and DNA Step Ladder (10–100 bp) were purchased from Wako Pure chemical industries Ltd (Osaka, Japan). N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) was purchased from Acros organics. Alexa Fluor 647 carboxylic acid succinimidyl ester was purchased from Molecular probes (Eugene, OR), Silencer select validated siRNAs targeting against human HIF-1α (x2); Sense-strand; CCUCAGUGUGGGUAUAAGATT and CCAUAUAGAGAUACUCAAATT, Anti-sense strand; UCUUAUACCCACACUDAGGTT and UUUGAGUAUCUCUAUAUGGTT and Silencer Select Negative Control #1 siRNA were purchased from Ambion (Japan K.K., Tokyo, Japan), Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA).

2.1.2. Cells and animals

Human ovarian endometriod carcinoma OVK18#2 cells were purchased from RIKEN cell bank (Ibaraki, Japan), and were cultured in RPMI 1640 medium (Wako chemical industries Ltd., Osaka, Japan) or FA-free RPMI 1640 (Gibco by Life technologies, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, NY, USA). Mouse macrophage RAW 264.7 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Wako chemical industries Ltd., Osaka, Japan) supplemented with 10% FBS and was a gift from Dr. Kotani, Kochi Glycobiology Research Centre, Kochi Medical School, Kochi University, Japan. BALBc nude/nude mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Animals were kept under pathogen-free conditions and exposed to 12 h light/dark cycle. All animal experiments were performed in accordance with guidelines approved by the ethics committee of Kochi Medical School, Kochi University. Four weeks prior to experimentations, animals were fed an alfalfa-free chow.

2.2. Methods

2.2.1. Synthesis of folic acid–poly ethylene glycol (FA–PEG) conjugate

The conjugation of FA to NH_2–PEG–COOH was adapted from a previously reported method with minor modifications (Liang et al., 2009; Chan et al., 2007; Salmaso et al., 2004; Park et al., 2010). Briefly, FA dissolved in dehydrated dimethyl sulfoxide (DMSO) was reacted with DCC and NHS under nitrogen atmosphere in the dark at ambient temperature for 18 h to form an activated FA (FA/NHS/DCC molar ratio, 1:5:5), the solution was filtered through a 0.2 μm Teflon syringe filter to remove insoluble dicyclohexylurea side product. NH_2–PEG–COOH was added to the activated FA solution at a molar ratio of 10:1 (FA:PEG). The reaction was allowed to proceed under nitrogen atmosphere in the dark at ambient temperature for 8 h. The reactant was then precipitated with excess acetone and the precipitate was dialyzed against Milli-Q water (Spectra/Por 6; MWCO, 1000) for 5 days. The precipitate was centrifuged and freeze-dried (DC 801, Yamato Scientific Co., Ltd., Tokyo, Japan).