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Novel delivery of Chlorin e6 using anti-EGFR antibody tagged virosomes for fluorescence diagnosis of oral cancer in a hamster cheek pouch model

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Chemical compounds:
Chlorin e6 (PubChem CID: 5357355)
7,12-Dimethylbenz[a]anthracene (PubChem CID: 6001)
Dimethyl sulfoxide (PubChem CID: 679)
Hypnorm (PubChem CID: 171220)
Dormicum (PubChem CID: 4192)
Hematoxylin (PubChem CID: 320930)
Eosin (PubChem CID: 11048)

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ABSTRACT

Purpose: Overexpression of epidermal growth factor receptor (EGFR) is observed in oral squamous cell carcinoma (OSCC) and is associated with increased proliferation, metastasis and therapeutic resistance. We aim to develop a novel drug delivery system comprised of a photosensitizer Chlorin e6 (Ce6) that is encapsulated in a viral envelope and tagged with anti-EGFR antibody to target OSCC.
Methods: Ce6 was encapsulated in both virosomes (Ce6-Vir) and virosomes tagged with anti-EGFR antibody (Ce6-Vir-EGFR′). *In vitro* studies were conducted to assess the cellular uptake and bioavailability of the photosensitizer in OSCC cells. Ce6 alone or in constructs was then administered in a hamster cheek pouch model and fluorescence imaging and spectroscopy was performed.
Results: In vitro results showed that the uptake of Ce6-Vir-EGFR′ was lower than that for Ce6-Vir and Ce6 possibly due to its large size. Nevertheless, *in vivo* results showed significant tumor specificity of Ce6-Vir-EGFR′ compared to Ce6. The tumor to normal mucosa ratio showed that Ce6-Vir-EGFR′ can successfully target OSCC lesions and therefore shows potential for use in fluorescence diagnosis of OSCC.
Conclusions: Both the virosome-Ce6 constructs were internalized by OSCC cells and successfully used for fluorescence imaging. Tagging with anti-EGFR antibody further improved the targeting ability toward OSCC.

1. Introduction

Photodynamic techniques such as fluorescence diagnosis (FD) and photodynamic therapy (PDT) have been undergoing intensive clinical investigations as diagnosis and adjuvant treatment approaches for various types of malignancies including brain tumors, cutaneous malignancies and head and neck tumors (Dolmans et al., 2003; Kostron, 2010). Fluorescence diagnosis which often goes parallel with PDT due to the similarities in biochemistry, aids to visualize the location of the tumors as well as the margins between tumors and normal tissues (Ladner et al., 2001). It involves the use of light-activatable drugs or photosensitizers which emit strong fluorescence signals under irradiation with light of specific wavelengths (Rosenthal et al., 2007). Since most photosensitizers do not have tumor targeting properties, one promising approach is to improve the selectivity of the photosensitizer to tumor cells (Parihar et al., 2013). The use of photosensitizers encapsulated into transportation vehicles coupled to molecules such as folic acid, steroids, growth factors and antibodies against specific cell surface molecule have shown significant improvement in the tumor selectivity properties of drugs (Parihar et al., 2013).

In this study, Chlorin e6 (Ce6), a chlorophyll derivative was used as the photosensitizer as it has an amphiphilic structure that allows easy penetration through cell membranes, thus ensuring effective accumulation inside cells (Rotomskis et al., 2013), while virosomes were used as the delivery system. Ce6 is a natural product that is extracted from live chlorella, *Chlorella ellipsoidea* (Moon et al., 2009). It is a promising photosensitizer characterized by a high photosensitizing efficacy and rapid elimination from the body (Jeong et al., 2011). It can be used for both FD and PDT as it can be excited for fluorescence emission at 405 nm and singlet oxygen production at 665 nm. Virosomes are

Abbreviations: OSCC, oral squamous cell carcinoma; Ce6, Chlorin e6; Ce6-Vir, Ce6 encapsulated in virosome; Ce6-Vir-EGFR′, Ce6 encapsulated in virosome tagged with anti-EGFR antibody.

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spherical, unilamellar vesicles with a mean diameter of approximately 150 nm (Mengiardi et al., 1995). They consist of reconstituted viral envelopes without viral genetic material of the source virus and can act as carriers for target proteins and photosensitizers (Mischler & Metcalfe, 2002). Virosomes with a neutral phospholipid bilayer can be used as a specific and non-specific drug carrier system. They attach to target cells via hemagglutinin (HA) but in the case of specific virosomes, they attach using the antibody fragment anchored in their lipid bilayer (Mastrobattista et al., 2002). Subsequently, the virosomes are taken up by the cell by receptor-mediated endocytosis (Waelti & Glück, 1998).

Overexpression of epidermal growth factor receptor (EGFR) is associated with aggressive tumor behavior including increased proliferation, metastasis, and therapeutic resistance in squamous cell carcinoma of the oral cavity and oropharynx (Yu et al., 2012; Shaib & Kono, 2012). Therefore, we developed a novel virosomal envelope as a targeted delivery vehicle of Ce6 using an anti-EGFR antibody to target EGFR overexpressing oral squamous cell carcinoma (OSCC) lesions. We compared the *in vitro* and *in vivo* uptake kinetics of Ce6 and 2 different kinds of virosome-Ce6 constructs, Ce6 encapsulated in virosomes (Ce6-Vir) and Ce6 encapsulated in virosomes tagged with anti-EGFR antibody (Ce6-Vir-EGFR′). We aim to develop cancer-specific probes that will enable minimally invasive fluorescence diagnosis of oral cancer.

2. Materials and methods

2.1. Photosensitizer and virosome constructs

Constructs comprising Ce6 encapsulated in virosomes (Ce6-Vir) and virosomes tagged with anti-EGFR antibody (Ce6-Vir-EGFR′) were customized and prepared by Pevion Biotech AG, Switzerland (Fig. 1) following methods previously described in detail (Amacker et al., 2005). Briefly, influenza viruses of the X-31 and A/Sing (A/Singapore/6/86) strains were propagated and purified. Chimeric immunopotentiating reconstituted influenza virosomes (CIRIVs) with HA from the two viral strains were formed. To obtain Ce6-Vir-EGFR′, αEGFR monoclonal antibody (MAB1095) was conjugated to the CIRIVs. Chlorine6 was obtained from Frontier Scientific, USA and encapsulated in the Ce6-Vir and Ce6-Vir-EGFR′ constructs at a concentration of 0.4 mg/ml for both constructs. Construct sizes were determined using dynamic light scattering (DLS).

2.2. Fluorescence spectroscopy of Ce6 in solution

A 2 mM stock solution of Ce6 (Frontier Scientific, USA) was prepared by dissolving Ce6 in anhydrous dimethyl sulfoxide (DMSO). The stock solution was serially diluted using PBS to reach concentrations ranging from 2.5 μM to 1 mM. Fluorescence spectroscopy was performed using a spectrophotometer (PhotoniTech (Asia) Pte Ltd., Singapore) with light excitation at 405 nm and the emission spectra of Ce6 were recorded from 600 to 700 nm. The highest fluorescence intensity value (at a single wavelength) between 660 and 670 nm was picked and plotted.

2.3. Cell culture

Human OSCC cells (CAL-27) were purchased from American Type Culture Collection (ATCC), USA. CAL-27 cells were grown in RPMI 1640 medium, containing 10% standard fetal bovine serum (HyClone, USA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 292 μg/ml L-glutamine (Gibco, Life Technologies, USA) in an atmosphere of 5% CO_2 and at 37 °C.

2.4. Cell viability test

2500 cells were seeded in each well of 96-well plates (Nunc, Denmark) and incubated overnight for cell adhesion. On the following day, the medium in each well was removed and replaced with serum free medium containing pure Ce6, Ce6-Vir and Ce6-Vir-EGFR′ of different concentrations (5 μM and 10 μM). Subsequently the plates were incubated over 72 h. At each designated time point, the culture medium with Ce6 or virosome-Ce6 constructs from each well were removed and replaced with MTS solution. The MTS solution was prepared by dissolving 1 ml of CellTiter 96® AQueous One Solution Reagent (Promega, USA) into 5 ml of 4.5 g/l glucose containing PBS. The plates were incubated for 3 h and read by Tecan Safire2 Microplate Reader (Tecan, Switzerland). The quantity of formazan product was measured by the absorbance at 490 nm. The intensity of the absorbance value is proportional to the viable cell number.

2.5. Drug uptake assay

120,000 cells were seeded in each well of 12-well plates (Nunc, Denmark) and incubated overnight for cell adhesion. On the following day, the medium in each well was removed and replaced with serum free medium containing pure Ce6, Ce6-Vir and Ce6-Vir-EGFR′ of different concentrations (5 μM and 10 μM). Subsequently the plates were incubated over 48 h. At each designated time point, the culture medium with Ce6 or virosome-Ce6 constructs from each well were removed and washed 3 times with PBS. The cells were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) and then tested for the presence of Ce6. A customized spectrofluorometer (PhotoniTech, Singapore) was used to record the Ce6 fluorescence emission spectra from 600 to 700 nm under 400 nm excitation. Control cell solutions without Ce6 were also measured to identify any auto fluorescence from cells. Experiments were conducted under low ambient lighting.

2.6. Hamster cheek pouch model

Twenty Syrian Golden hamsters (*Mesocricetus auratus*, male, 6–12 weeks old, 100–150 g) were purchased from Charles River Lab, USA for the experiments. The hamsters were randomly placed in 4 different groups, housed in 4 cages under controlled environment conditions

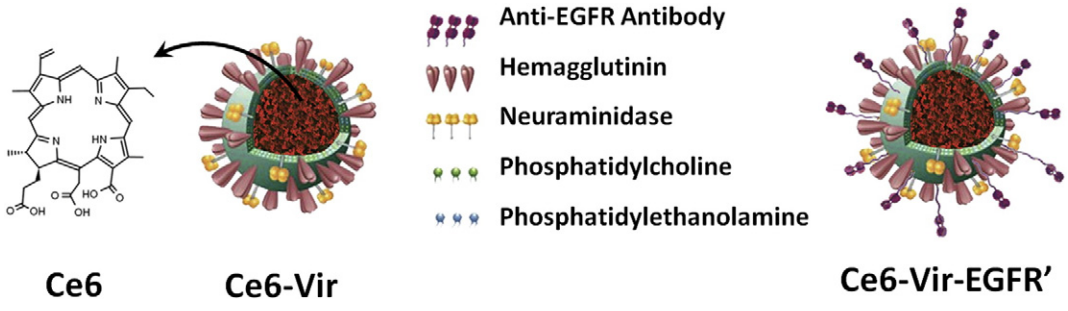

Fig. 1. Schematic representation of single Ce6 molecule (Ce6), Ce6 encapsulated in virosomes (Ce6-Vir) and Ce6 encapsulated in virosomes tagged with anti-EGFR antibodies (Ce6-Vir-EGFR′).