ENGINEERED CHO CELL LINE WITH COXSACKIE AND ADENOVIRUS RECEPTOR GENE ENHANCE THE SUSCEPTIBILITY OF ADENOVIRUS INFECTION

SAYANG BINTI BABA

UNIVERSITI TEKNOLOGI MALAYSIA

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SAYANG BINTI BABA

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Alhamdullilah…

Segala puji-pujian kehadrat Allah s.w.t

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ABSTRACT

Gene-based therapies promise the potential to target the explicit gene delivery and expression to target cell populations. Adenoviral vectors are presently being tested clinically as a new strategy for the treatment of cancer. However, an important determining factor for the successful entry of such adenoviruses into target cells is expression of the Coxsackie virus and Adenovirus Receptor (CAR) at the cell surface. This raises the possibility that those gene-based therapy face the greatest therapeutic challenge might be the least susceptible to infection with therapeutic adenoviruses. If effective strategies can be implemented to boost CAR expression and hence the presence of primary receptor at the cell surface, this could prove most useful to adenovirus-based gene transfer. Therefore, this study aimed to possibly boost the expression of CAR specifically on CAR-negative Chinese Hamster Ovary (CHO) cell lines and evaluate their biological function by examining their susceptibility to transduction and infection of Adenovirus type 5. Initially, CHO cell line were transfected with human CAR cDNA fragment tagged with red fluorescent protein (DsRed) and sequentially selected with G418 antibiotic to generate stable cell line containing CAR-DsRed. Several transfection reagents such as GeneJuice (Novagen), Lipofectamine^{TM} 2000 (Invitrogen) and Xtreme HP plasmid DNA (Roche) were used for transfection study using flow cytometry. In addition, the expression of CAR on CHO-CAR-DsRed cell was determined by inverted fluorescent microscope, qRT-PCR and western blotting and antibody-blocking assay to verify the role of CAR in CHO-CAR-DsRed cells. CHO-CAR-DsRed cells were further examined by infecting wild-type adenovirus type 5 (wt-Ad5) and transduction recombinant adenovirus type 5 tagged with enhanced green fluorescent protein (Ad5EGFP) and their susceptibility were observed by using cytopathic effect and Giemsa staining. Flow cytometry analysis, showed that transfection efficiency of Xtreme HP (39.25±1.00 MFI) was higher than Lipofectamine^{TM} 2000 (17.1±1.00 MFI) and GeneJuice (11.1±1.00 MFI). The stable CHO-CAR-DsRed cells were estimated at average of 68.9%±1.12 MFI by transduction of Ad5EGFP and supported by evidence of infectibility of wt-Ad5 from Giemsa staining. Moreover, blocking of CAR expression on CHO-CAR-DsRed showed negative susceptibility to Ad5EGFP. These findings suggested that the strategy could be implemented to augment CAR expression and enhance the presence of primary cell surface receptor, as this could ornament the cell's susceptibility to adenovirus infection and beneficial for adenovirus-based gene therapy.

ABSTRAK

Terapi berasaskan gen menjanjikan potensi untuk sasaran penyampaian gen yang tepat dan ekspresi kepada sasaran populasi sel. Vektor Adenoviral kini sedang diuji secara klinikal sebagai strategi baru untuk rawatan kanser. Walaubagaimanapun, penentuan kejayaan Adenovirus masuk ke dalam sel sasaran adalah ekspresi "Coxsackie virus and Adenovirus Receptor" (CAR) di permukaan sel. Ini meningkatkan kemungkinan bahawasanya terapi berasaskan gen terapeutik menghadapi cabaran terbesar boleh jadi kurangnya penerimaan terhadap jangkitan adenoviruses terapeutik. Jika strategi yang berkesan boleh dilaksanakan bagi peningkatan ekspresi CAR dan dengan kehadiran reseptor utama di permukaan sel, sekaligus memberi pembuktian yang sangat berguna bagi terapi gen berasaskan Adenovirus. Oleh itu, matlamat kajian ini bagi berkemungkinan meningkatkan ekspresi CAR pada negatif CAR iaitu sel Ovari Hamster Cina (CHO) dan memeriksa kecenderungan mereka kepada transduksi dan jangkitan Adenovirus jenis 5. Pada mulanya, sel CHO transfeksi dengan jujukan CAR manusia cDNA dilabelkan dengan protein merah pendarfluor (DsRed) dan pemilihan berterusan dengan G418 antibiotik untuk menjana barisan sel stabil yang mengandungi CAR-DsRed. Beberapa reagen transfeksi seperti GeneJuice (Novagen), Lipofectamine^{TM} 2000 (Invitrogen) dan Xtreme HP plasmid DNA (Roche) reagen transfeksi digunakan bagi kajian sitometri aliran. Di samping itu, ekspresi CAR pada sel CHO-CAR-DsRed boleh ditentukan oleh mikroskop pendarfluor songsang, qRT-PCR, pewarnaan western dan esei penyekatan-antibodi untuk mengesahkan peranan CAR dalam sel CHO-CAR-DsRed. CHO-CAR-DsRed seterusnya dikaji dengan menjangkiti adenovirus kumpulan 5 jenis-liar (wt-Ad5) dan trasnduksi rekombinan Ad5 berlabel dengan peningkatan fluoresen protein berwarna hijau (Ad5EGFP) dan penerimaan jangkitan telah diperhatikan telah menggunakan kesan *cytopathic* (CPE) dan perwarnaan oleh Giemsa. Analisis sitometri aliran, memperlihatkan bahawa kebolehan transfeksi Xtreme HP (39.25 ± 1.00 MFI) adalah tertinggi berbanding kepada Lipofectamine^{TM} 2000 (17.1 ± 1.00 MFI) dan GeneJuice (11.1 ± 1.00MFI). Sel-sel CHO-CAR-DsRed yang stabil telah dijangkakan pada purata 68.9% ± 1.12 MFI oleh transduksi daripada Ad5EGFP dan disokong dengan pembuktian oleh jangkitan daripada jenis liar-Ad5 melalui pewarnaan Giemsa. Selain itu, penyekatan CAR menunjukkan pemerhatian yang negatif terhadap kecenderungan mana-mana Ad5EGFP. Kepentingan penemuan ini dapat memberi kesimpulan bahawa strategi ini boleh dilaksanakan untuk menghadirkan ekspresi CAR dan meningkatkan kehadiran reseptor primer di permukaan sel, justeru memudahkan penerimaan jangkitan adenovirus terhadap sel dan sekaligus bermanfaat untuk terapi gen berasaskan Adenovirus.

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LIST OF ABBREVIATIONS

SYMBOLS	DESCRIPTION
abs	absorbances
Ad	Adenovirus
Ads	Adenoviruses
Ad5	Adenovirus serotype 5
APS	Ammonium Persulfate
ATCC	Animal Tissue Culture Collection
BSA	Bovine Serum Albumin
bp	base pair
CAR	Coxsackie and adenovirus receptor
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
CO_2	Carbon dioxide gas
CPE	Cytophatic Effect
CTX	Cortical Thymocyte Xenopus
D1	Extracellular Domain 1
D2	Extracellular Domain 2
DNA	Deoxyribonucleic acid
DsRed	*Discoscoma* Red Fluorescence Protein
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetate
EGFP	Enhance green fluorescent protein
FBS	Fetal Bovine Serum
FPs	Fluorescence proteins
IF	Immunofluorescence
Ig	Immunoglobulin
IRES	internal ribosome entry site
g	gram

GFP	Green fluorescent protein
hAd5	human Adenovirus serotype 5
hCAR	human CAR
HEK	Human embryonic kidney
HER	Human embryonic retinoblastoma
kb	kilobase
kDa	kilo-Dalton
L	Litre
LB	Luria Broth
M	marker
µg	microgram
µL	microlitre
µm	micrometer
µM	microMolar
mA	miliAmpere
mg	miligram
mL	mililiter
MOI	Multiplicity of Infection
nm	nanometer
OD	optical density
P	Population
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline with 0.05% Tween 20
PCR	Polymerase Chain Reaction
pCMV	Cephalomycarditis virus plasmid
PMSF	Phenylmethylsulfonate
Q	Quadrant
qRT-PCR	quantitative Real-Time PCR
rAd	recombinant Adenovirus
rAd5	recombinant Adenovirus type 5
RGD	Arg-Gly-Asp peptide motif
rpm	revolution per minute
S.O.C	Super Optimal broth with Catabolite repression

SYBR	Synergy Brands, Inc. (stock symbol)
TAE	Tris-acetate-EDTA
TEMED	N,N,N’N’-Tetramethylenediamine
UV	ultraviolet
V	voltage
WB	Western Blot
v/v	volume by volume
~	approximately
^{o}C	Degree centrigrade
%	percentage

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Adenoviruses (Ads) vectors have been widely employed for therapeutic gene delivery for clinical trials (Räty *et al.*, 2008; James *et al.*, 2009; and Griesenbach *et al.*, 2010). The most extensively studied and commonly used Adenovirus (Ad) serotype for gene delivery applications is human Adenovirus serotype 5 (hAd5). Promptly, from year 1989 to 2011, extensive pre-clinical and clinical studies using adenovirus-based vectors have been explored and conducted or are currently in progress (http://www.wiley.com/legacy/wileychi/genmed/clinical). Updated in June 2012, nearly 23.3% of 1843 gene therapy clinical trials were approved worldwide by utilizing the Ad-vector based. For example, it has been the first choice for the treatment of cytic fibrosis (Kovesdi *et al.*, 1997; Vorburger and Hunt, 2002 and Price *et al.*, 2007). Adenoviral vectors were also have been manipulated for use in gene therapy applications in glioma, prostate, hepatocellular, head and neck, gastric and oral leukoplakia cancers (Immonen *et al.*, 2004; Li *et al.*, 2007; Shirakawa *et al.*, 2007; Khalighinejad *et al.*, 2008 and Li *et al.*, 2009).

Adenoviruses (Ads) have gained much attention due to their favourable features for gene therapy practices such as the ease to produce high titers with high purity and its ability to transduction a variety of proliferating and quiescent (inactive) cells. Ads also possess a large capacity of DNA cassette and high expression of transgenes. Furthermore, the main issue is the safety concern, adenovirus DNA is not incorporating into host genome (Russell, 2009) and their low pathogenicity for

humans (Glasgow *et al.*, 2004). In addition, recombinants Ads are stable structurally, retains stability *in vivo* (Kanerva and Hemminki, 2005) such as transduction of Ad5Cox2Lluc and Ad5Cox2LlucUL into human fibroblasts (HS173We) and no mutations reported (McCelland *et al.*, 2007).

Further study had revealed CAR as a prime docking site of all subgroups adenovirus except for subgroup B (Roelvink *et al.*, 1998). The entry of Ads into target cell surface involves two diverse, chronological steps. An interaction between knob of the protruding fiber protein of particle of Ads with extracellular domain of CAR on the target cell surface leading toward internalisation via the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with secondary host-cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

However, it has been suggested that expression of Coxsackie and Adenovirus Receptor (CAR) is a rate-limiting factor for infectivity of adenoviral vectors; and subsequently shown to cause poor transduction of adenoviral vectors gene-delivery in clinical trials (Bauerschmitz *et al.*, 2002 and Kanerva and Hemmiki, 2004). Pre-clinical studies revealed that CAR down-regulation on primary human tumor cells such as ovarian, cervical, prostate, head and neck, bladder, melanoma, glioma cancer and others may be a major hurdle for efficient Ad gene therapy utility (Breidenbach *et al.*, 2004; Rein *et al.*, 2004; Kanerva *et al.*, 2002). Consequently, various approaches have been evaluated to modify or circumvent CAR deficiency, including genetic capsid modifications or retargeting complexes (Zhang and Bergelson, 2005; and Sharma *et al.*, 2009). Scientists had also explored the possibility of finding new additional receptor for Ad other than its prime receptor (Short *et al.*, 2006; Fleischli *et al.*, 2007; Tuve *et al.*, 2008).

By introducing CAR *ex vivo* or *in vitro* to little or no CAR expression host's cell prior to adenovirus-based gene transfer may overcome the problem. Thus, it is crucial to investigate the possibility of cloning and expressing human CAR-receptor onto CAR-negative CHO cell lines that was tagged with *Discoscoma* Red Fluorescent Protein (DsRed). Then, the transduction with recombinant adenovirus that expresses green fluorescence protein (EGFP) could be performed in order to evaluate the efficacy of the engineered CAR gene. The outcome could be used as a useful model to boost the presence of CAR expression on host's cell surface and enhance the adenovirus-based transfer efficacy.

1.2 PROBLEM STATEMENT OF RESEARCH

The research was conducted to insure the successfulness of viral gene therapy based on adenoviral vector utilities into host's cells. Therefore, an examination of structure and co-localization of receptor had been done intently due to fact that the absence of CAR expression in target cells hindered susceptibility of Ads (Wang *et al*., 2007). Since the presence of CAR is a crucial factor to determine the efficacy of adenoviruses' susceptibility into host's cells. Thus, the absence or sparse of CAR expression on the cell surface will hinder the use of Ad based vector. If the presence of CAR can be boosted, subsequently the efficacy of Ad transduction can be enhanced and achieved.

1.3 RESEARCH OBJECTIVES

The objectives are:

1) To construct full-length of human Coxsackie and Adenovirus Receptor (pCAR) into pDsRed Monomer-N1 expression vector (DsRed)

2) To optimise transfection of CAR-DsRed into CHO cell line (CAR-negative) and evaluate of CAR-DsRed protein expression

3) To evaluate the infectibility of stable engineered CHO-CAR-DsRed cell line with wild-type Adenovirus serotype 5 and recombinant Adenovirus serotype 5 expressing enhance green fluorescence protein (Ad5-EGFP)

1.4 SCOPE OF RESEARCH

The scope of study for the first phase involves the construction of cDNA full-length CAR (pCAR) receptor fusion with pDsRed-Monomer-N1 vector with respective molecular cloning techniques. The second section focused on the transfection of CAR-DsRed onto negative-CAR mammalian cell lines namely Chinese Hamster Ovary (CHO) cells via several commercial chemical transfections such as GeneJuice (Novagen), Lipofectamine^{TM} 2000 (Invitrogen) and Xtreme HP Plasmid DNA Transfection Reagent (Roche) according to the respective manufacturer's protocol. Engineered stable CHO-CAR-DsRed cells were evaluated for CAR expression by using indirect immunofluorescence, quantitative Real-Time PCR (qRT-PCR), protein analysis such as western blotting and antibody-blocking assay were performed. Besides that, for the biological functions of engineered CHO-CAR-DsRed towards recombinant Adenovirus expressing enhance green fluorescent

(Ad5EGFP) was assessed by using flow cytometry. On the other hand, qualitative assessments of cytopathic effects were conducted by Giemsa staining and live imaging microscopic.

1.5 SIGNIFICANCE OF STUDY

This study focused on genetically engineered negative mammalian cell lines expressing stable CAR. Thus, the outcome of this study would infer the strategy to boost CAR expression and enhance the presence of primary receptor on cell surfaces. This could be useful tool to improve adenovirus-based gene transfer to CAR-deficient cells.

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