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硕 士 学 位 论 文

人类免疫缺陷病毒 1 型包膜蛋白 gp140 在
哺乳动物细胞中的高效表达、性质鉴定及
中和单抗的筛选

Expression and Characterization of Human
Immunodeficiency Virus-1 Membrane Protein gp140 from
Mammalian Cells and Generation of Neutralizing
Monoclonal Antibodies

杨超

指导教师姓名: 顾颖副教授

专业名称: 卫生毒理学

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摘要

艾滋病 (Acquired Immune Deficiency Syndrome, AIDS) 是由人类免疫缺陷病毒 (Human Immunodeficiency Virus, HIV) 感染引起的获得性免疫缺陷综合征。艾滋在世界范围内流行, 其中主要是 1 型 HIV 病毒给人类健康带来严重威胁。据世界卫生组织 (World Health Organization, WHO) 报告, 自从 HIV 被发现以来, 被感染者已逾 7 千万人, 2015 年底全球仍有 3 千 6 百多万艾滋患者, 15 岁至 49 岁成人 HIV 感染率高达 0.8%^[1]。2012 至 2014 年连续三年, 我国艾滋病发病超过 4 万人/年^[2]。目前高活性抗逆转录病毒治疗 (Highly Active Antiretroviral Therapy, HAART) 已经可以抑制艾滋感染者体内病毒的复制, 延长患者寿命并使其可以像健康人一样正常生活。然而抗逆转录药物需要每天服用, 37.74% 的服药患者出现消化道反应、肝功能异常等副作用^[3], 而且 HAART 治疗不能从根源上斩断艾滋的流行, 疫苗的研发正是当务之急。HIV-1 包膜糖蛋白 (envelope glycoprotein, Env) gp160 在成熟过程中水解为 gp120 (surface glycoprotein, SU) 和 gp41 (transmembrane protein, TM), 这两个蛋白单体通过非共价键结合为异源二聚体, 三个二聚体组装成成熟的三聚体刺突铆定在病毒包膜上。病毒入侵宿主细胞时, gp120 上的 CD4 结合位点识别 CD4 细胞表面受体分子, 引发 Env 变构靠近细胞膜从而发生膜融合, 因此包膜蛋白是阻止病毒感染的重要切入点, 也是疫苗研究的重要靶点^[4]。

广谱中和单抗 N6, VRC01, b12, 2F5 等抗体分别针对 gp120 上的 CD4 结合位点和 gp41 上的近膜外区 (Membrane Proximal External Region, MPER), 能够实现对多种型别病毒的高效中和^[5,6,7,8]。然而根据这些抗体表位设计的疫苗仍未取得理想的效果。RV144 是第一个被证实具有降低 HIV-1 感染效果的疫苗。实验中, 表达 E 亚型 Env 和 B 亚型 Gag (group specific antigen)、Pro (protease) 的金丝雀痘病毒载体为初免, B/E 亚型 gp120 和铝佐剂加强免疫, 三年半后受试者仍保持 31.2% 的疫苗保护率^[9]。体内具有高水平阻断和结合抗体, 可能对艾滋感染有保护作用^[9]。另有文献报道, 通过对 BG505 毒株的 Env 进行改造, 可以实现体外的可溶性表达, 得到近似天然的蛋白三聚体^[10]。重组包膜蛋白 BG505 SOSIP.664 和 B41 SOSIP.664 均可在兔子体内诱导得到较强的广谱中和抗体^[11]。

这些报道对艾滋疫苗的进一步研究提供了新的方向和信心。

本研究的目的是通过优化 HIV-1 gp140 编码基因的密码子和克隆设计,从而实现 gp140 蛋白在哺乳动物细胞 HEK293 中高效表达,并对纯化获得的蛋白进行抗原性质鉴定。本研究选择 B 亚型 HIV-1 NL4-3 基因序列为模板进行 gp140 克隆构建,通过密码子优化、信号肽替换、增加柔性连接、三聚体辅助折叠序列等方法优化设计,确定了最终的重组克隆 SOSIP (G4S)₂ 663CO-T₄his 用于蛋白表达和性质鉴定。此外我们验证了 HIV-1 转录反式激活因子 (Transactivator of Transcription, Tat) 共转 HEK293T 细胞对 gp140 蛋白表达的促进作用,并摸索得到最佳的 Tat/gp140 共转比例。镍柱纯化后,每升培养基可获得 0.5 mg gp140 蛋白,目的蛋白纯度约为 70%。通过 ELISA 鉴定发现该重组蛋白具有良好的抗原活性,电镜下呈现均匀的三聚体结构。通过弗氏佐剂与目的蛋白混合免疫 Balb/c 小鼠,随着免疫次数增加小鼠血清中和能力逐渐提高,并最终筛选得到 10 株针对 NL4-3 毒株的中和单抗,其中两株具有型间交叉中和能力。本研究对 B 亚型 HIV-1 NL4-3 gp140 进行改造、优化转染条件,获得了 gp140 三聚体在体外的高效表达,并对其抗原性和免疫原性进行了鉴定,探索了 HEK293 系统表达 gp140 作为重组蛋白疫苗的可行性,为 HIV-1 包膜蛋白结构研究、疫苗和药物设计奠定基础。

关键词: 1 型人类免疫缺陷病毒; 包膜糖蛋白 gp140; 真核表达; 免疫原性

Abstract

AIDS is an acquired immunodeficiency syndrome caused by HIV (Human Immunodeficiency Virus) infection. AIDS is prevalent in the world, and type 1 HIV virus mainly poses a serious threat to human health. As reported by World Health Organization, there are more than 70 million people who have been infected since the discovery of HIV-1. By the end of 2015, there were still more than 36 million AIDS patients worldwide. HIV-1 infection rate of 15 to 49 years old adults is as high as 0.8%. AIDS incidence of China was more than 40,000 people per year in 2012-2014. At present, highly active antiretroviral therapy (HAART) is able to inhibit the replication of the virus in people living with HIV and make a significant contribution to extending the life of patients. However, anti-transcription drugs need to be taken every day, and 37.74% of patients get side effects such as gastrointestinal reactions, abnormal liver function and others. HAART can not cut off the root of AIDS epidemic, while the vaccine research and development is the urgent affairs. HIV-1 envelope glycoprotein(Env) gp160 is hydrolyzed to gp120 (surface glycoprotein, SU) and gp41 (transmembrane protein, TM) during maturation, and these two proteins are assembled by noncovalent bonds into heterodimers. Then three dimers assemble into mature trimer and rivet on the virus envelope. When the virus invade into the host cells, the CD4 binding site on gp120 will recognize the CD4 cell surface receptor molecule, triggering the close to cell membrane and virus-cell fusion. For this reason, envelope protein is an important point for preventing viral infection and an important target for HIV vaccine.

The broadly monoclonal neutralizing antibodies N6, VRC01, b12, 2F5 and other antibodies can neutralize multiple types HIV-1, and are directed to the CD4 binding site on gp120 or the MPER region on gp41. However, vaccines designed according to these epitopes have not yet achieved the desired results. RV144 is the first vaccine with the effect of reducing HIV-1 infection. In this experiment, canarypox virus vector expressing subtype E Env and subtype B Gag is used for priming inoculation, subtype

B/E gp120 with aluminum adjuvant is used for boost inoculation. The vaccine protection rate maintain 31.2% after three and a half years. As reported, the body with a high level of blocking and binding antibodies may have the protective effect on HIV infection. Through the transformation of BG505 Env, it can be expressed soluble as approximate natural trimer *in vitro*. Recombinant envelope protein BG505 SOSIP.664 and B41 SOSIP.664 induced strong broad-spectrum neutralizing antibody in rabbits. These reports provide new directions and confidence in further research on HIV vaccines.

This study aimed to construct the recombinant clone of HIV-1 envelope glycoprotein gp140, seek efficient expression methods in HEK293 and characterize the recombinant protein. Construct design based on HIV-1 NL4-3 gp140 included codon optimization, signal peptide replacement, flexible linker involvement and foldon utilization, and at last we got the clone of SOSIP (G₄S)₂ 663CO-T4his. Besides, the helpful impact was confirmed that co-transfection with HIV-1 transactivator of transcription (Tat) can enhance the expression of gp140, and the best plasmid ratio of Tat/gp140 was founded. After purified by Ni NTA chromatography, about 0.5 mg recombinant gp140 with a purify of about 70% could be gotten per liter medium. Afterwards, the antigenicity of this recombinant gp140 was confirmed by ELISA. In electron microscopy image, the protein was observed as trimer. Purified gp140 was emulsified with freund's adjuvant to subcutaneously inoculate Balb/c mice, and the neutralizing capacity of mice serum increased gradually. At last, ten neutralizing antibodies was screened, and two of them could neutralize corss subtypes of HIV-1. In this research, the subtype B HIV-1 virus NL4-3 gp140 was cloned and engineered, transfection methods was optimized, to express envelope protein trimer efficiently *in vitro* and assess its antigenic activity and immunogenicity. Besides, we explore the feasibility of using HEK293 cells to express gp140 as a recombinant protein vaccine, and to provide the foundation for the study of HIV-1 envelope protein structure, vaccine and drug design.

Key words: Human immunodeficiency virus-1; Envelope glycoprotein gp140;

Eukaryotic expression; Immunogenicity

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缩略词

缩写	英文全称	中文全称
aa	amino acid	氨基酸
AIDS	Acquired Immune Deficiency Syndrome	获得性免疫缺陷综合征
Amp	Ampicillin	氨苄青霉素
bnAbs	broadly neutralizing antibodies	广谱中和抗体
bp	base pair	碱基对
BSA	bovine serum albumin	牛血清白蛋白
CA	capsid protein	衣壳蛋白
CD4bs	CD4 binding site	CD4 结合位点
CryoEM	cryo-electron microscopy	低温电子显微镜
DMSO	dimethyl sulfoxide	二甲基亚砜
DNA	deoxyribonucleic acid	脱氧核糖核酸
<i>E. coli</i>	<i>Escherichia coli</i>	大肠埃希菌
ELISA	enzyme-linked immunosorbent assay	酶联免疫吸附试验
Env	envelope glycoprotein	包膜糖蛋白
ES	special designed for embryonic stem cells	针对胚胎干细胞的牛血清
FBS	fetal bovine serum	胎牛血清
FCM	flow cytometry	流式细胞分析仪
Gag	group specific antigen	主要结构蛋白
GAM	goat anti-mouse	羊抗鼠
GFP	green fluorescence protein	绿色荧光蛋白
H & T	hypoxanthine & thymidine	次黄嘌呤和胸腺嘧啶核苷
HA	Hemagglutinin	血凝素
HAART	Highly Active Antiretroviral Therapy	高活性抗逆转录病毒治疗
HEK293	human embryo kidney cells 293	293 人胚肾细胞
HIV	Human Immunodeficiency Virus	人类免疫缺陷病毒

HRP	horseradish peroxidase	辣根过氧化物酶
Ig	Immunoglobulin	免疫球蛋白
IN	Integrase	整合酶
Kb	kilo base	千碱基
kD/kDa	kilo Daltons	千道尔顿 (分子量)
LTR	long terminal repeat	长末端重复序列
MA	matrix protein	基质蛋白
mAb	Monoclonal antibody	单克隆抗体
MHC	major histocompatibility complex	主要组织相容性复合物
MPER	membrane proximal external region	近膜胞外区
mRNA	messenger RNA	信使 RNA
MW	molecular weight	分子量
NC	nucleocapsid protein	核衣壳蛋白
Nef	negative factor	负调控因子
NFL	native flexibly linked	天然柔性连接
PCR	polymerase chain reaction	聚合酶链式反应
PEG	polyethylene glycol	聚乙二醇
PEI	polyethyleni mine	聚乙烯亚胺
PH	hydrogen ion concentration	氢离子浓度指数
PNGS	potential N-glycosylation sites	潜在 N 链糖基化位点
Pol	Polymerase	聚合酶
Pro	Protease	蛋白酶
Rev	regulator of expression of virion	病毒表达调控
Rev	regulator of expression of virion	病毒表达调控
RNA	ribonucleic acid	核糖核酸
RT	reverse transcriptase	反转录酶
SIN	Sindbis Virus	辛德毕斯病毒
SIV	Simian Immunodeficiency Virus	猿免疫缺陷病毒
SU	surface glycoprotein	表面糖蛋白

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