UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF AN IN VITRO CELL CULTURE SYSTEM FOR HUMAN HEPATITIS B VIRUS

WANG SUK MEI

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ESTABLISHMENT OF AN *IN VITRO* CELL CULTURE SYSTEM FOR HUMAN HEPATITIS B VIRUS

By

WANG SUK MEI

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Medicine and Health Sciences Universiti Putra Malaysia

July 2001

Specially dedicated to,

My beloved parents , brothers, sisters, husband and friends

for their invaluable love, support, patience and understanding…….

Abstract of thesis presented to the Senate Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

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WANG SUK MEI

July 2001

Chairman : Associate Professor Dr. Seow Heng Fong

Faculty : Medicine and Health Sciences

The major obstacle in the study of HBV has been the inability to infect either animal model system *in vivo* or continuous cell lines *in vitro* due to strict virus-host range and preferential attachment of the HBV envelope proteins onto the plasma membrane of human hepatocytes. The direct involvement of human annexin-V, a calcium dependent phospholipid - binding protein in the initial step of HBV infection has been reported. Over the last decade, various culture conditions with exogenous soluble factors have also been exploited to enhance HBV infection *in vitro*.

The aim of this study was to establish an *in vitro* cell culture system that would continuously produce sufficient HBV by episomal replication. To facilitate the penetration and internalization of HBV, the expression of annexin V (AV) was

enhanced using dexamethasone and by transfection of two liver cell lines, Chang liver and HepG2 cells. In addition, culture conditions containing (i) glucose supplement and dimethylsulphoxide (DMSO) (ii) glucose supplement and liver extract, (iii) glucose supplement, DMSO and liver extract, (iv) glucose supplement only and (v) human interleukin-6 (hIL-6) without glucose supplement, were used to determine which condition was the best for HBV infection *in vitro*.

The results indicated that 10^{-6} M dexamethasone did not significantly upregulate annexin V mRNA expression in both Chang and HepG2 cell lines and Chang cells had higher basal levels of annexin V mRNA. The Chang cell line was more susceptible to HBV infection than HepG2, before and after transfection with human annexin V (hAV), as well as in most of the culture conditions. Only culture conditions containing: (i) glucose supplement, DMSO and liver extract and (ii) glucose supplement and DMSO, inhibited the expression of viral protein probably either at transcription or translation level, without affecting the appearance of viral covalently closed circular (ccc) DNA. Glucose supplement in the presence of liver extract, and hIL-6 (without glucose supplement) supported HBV replication and enhanced viral protein expression.

The establishment of an *in vitro* cell culture system for HBV infection is very important because it is an essential tool for the development of new antiviral strategies against hepatitis B virus infection and for studying the molecular events in viral replication.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENUBUHAN SISTEM SEL KULTUR SECARA *IN VITRO* UNTUK VIRUS HEPATITIS B

Oleh

WANG SUK MEI

Julai 2001

Pengerusi : Profesor Madya Dr. Seow Heng Fong

Fakulti : Perubatan dan Sains Kesihatan

Penghindaran yang utama dalam penyelidikan HBV ialah ketidakupayaan menjangkiti sistem model haiwan secara *in vivo* ataupun jujukan sel berterusan secara *in vitro* disebabkan oleh lingkaran yang sempit bagi interaksi di antara virus dengan perumah serta kecenderungan perlekatan protein membran luar HBV pada membran plasma hepatosit manusia. Pembabitan annexin-V manusia, sesuatu protein-fosfolipid perlekatan yang bergantung kepada kalsium secara langsung dalam langkah awal infeksi HBV telah dilaporkan. Di sepanjang abad yang lalu, pelbagai keadaan kultur dengan faktor-faktor luaran yang terlarut telah digunakan untuk meningkatkan infeksi HBV secara *in vitro*.

Objektif utama penyelidikan ini ialah untuk menubuhkan sebuah sistem kultur sel secara *in vitro* yang akan dapat menghasilkan HBV yang mencukupi secara bereplikasi episomal. Untuk membantu penembusan dan kemasukan HBV,

ekspresi annexin-V telah dipertingkatkan dengan menggunakan dexamethasone dan transfeksi dua jujukan sel hati, sel Chang dan HepG2. Di samping itu, keadaan kultur yang mengandungi (i) glukos tambahan dan dimetilsulfoksida (DMSO), (ii) glukos tambahan dan ekstrat hati, (iii) glukos tambahan, DMSO dan ekstrak hati, (iv) glukos tambahan sahaja serta (v) interleukin 6 manusia tanpa glukos tambahan, digunakan untuk memastikan yang mana keadaan adalah paling baik untuk infeksi HBV secara *in vitro*.

Keputusan menunjukkan 10^{-6} M dexmethasone tidak meningkatkan ekspresi mRNA annexin-V secara bererti (significantly) di dalam kedua-dua jujukan sel HepG2 dan Chang, dan sel Chang mengandungi paras mRNA annexin V yang lebih tinggi. Jujukan sel Chang adalah lebih mudah dijangkiti oleh infeksi HBV daripada HepG2, sebelum dan selepas transfeksi dengan annexin V manusia, serta di dalam kebanyakan keadaan kultur. Hanya keadaan kultur yang mengandungi: (i) glukos tambahan, DMSO dan ekstrat hati dan (ii) glukos tambahan dan DMSO, telah dipamerkan dapat melarang ekspresi protein virus berkemungkinan pada langkah transkripsi atau translasi tanpa mengganggu penimbulan DNA bulat tertutup berkovalen (ccc) virus. Glukos tambahan dengan kehadiran ekstrat hati, dan IL-6 manusia (tanpa glukos tambahan) telah dipamerkan dapat menyokong replikasi HBV dan meningkatkan ekspresi protein virus.

Penubuhan sistem kultur sel secara in vitro bagi infeksi HBV adalah sangat penting sebab ianya merupakan alatan yang diperlukan untuk

memperkembangkan strategi antivirus yang baru bagi menentangkan infeksi HBV serta menyelidiki hal-hal molekular dalam replikasi virus.

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I certify that an Examination Committee met on 3rd July 2001 to conduct the final examination of Wang Suk Mei on her Master thesis entitled "Establishment of an *In Vitro* Cell Culture System for Human Hepatitis B Virus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981 The Committee recommends that the candidate be awarded the relevant degree Members of the Examination Committee are as follows

DATIN DR. FARIDA JAMAL
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

SEOW HENG FONG, Ph.D.
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

DR. LESLIE CHARLES LAI CHIN LOY
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

ABDUL MANAF ALI, Ph.D.
Associate Professor
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

MOHD GHAZALI MOHAYIDIN, Ph.D.
Professor/Deputy Dean of Graduate School
Universiti Putra Malaysia

Date 2 JUL 2001

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

AINI IDERIS, Ph.D.,
Professor
Dean of Graduate School
Universiti Putra Malaysia

Date: 09 AUG 2001

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrenly submitted for any other degree at UPM or other institutions.

WANG SUK MEI

Date: 23.7.2001

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ε	5' epsilon loop of HBV pregenome
μ	micro
AMP	Adenosine monophosphate
Apo H	Apolipoprotein H
AV	Annexin V
bp	base pair
BSA	Bovine serum albumin
C	Core protein
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
ccc DNA	Covalently closed circular DNA
CD	Cluster designation
cDNA	complementary DNA
ChHBV	Chimpanzee hepatitis B virus
CO_2	carbon dioxide
CTLs	Cytotoxic T lymphocytes
d	day(s)
ddH_2O	double-distilled water
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
DHBcAg	Duck hepatitis B core antigen
DHBsAg	Duck hepatitis B surface antigen
DHBV	Duck hepatitis B virus
dl	Double stranded linear
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP(s)	dideoxynucleotide triphosphate(s) (dATP, dTTP, dCTP and dGTP)
ds DNA	double stranded DNA
E. coli	Escherichia Coli
EDTA	diaminoethanetetra-acetic acid disodium salt
Enh	Enhancer
ER	endoplasmic reticulum
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
g	gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gp	glycoprotein
gp 180	Glycoprotein 180
GRE	glucocorticoids responding element
GRP94	Glucose regulated protein 94
GSHV	Ground squirrel hepatitis B virus

h	hour(s)
hAV	Human annexin V
HBcAg	hepatitis B core antigen
HBsAg	hepatitis B e antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	hydrochloric acid
HFF	Human foreskin fibroblasts cells
hGR	human glucocorticoid receptor
HHBV	Heron hepatitis B virus
hIL-6	Human interleukin 6
hIL-6Rα	human interleukin 6 receptor alpha
hIL-6Rβ	human interleukin 6 receptor beta
hPXR	human pregnane X receptor
Hsp70	Heat shock protein 70
IFN-γ	Interferon-gamma
IFN-α/β	Interferon-alpha or beta
IFN-α	Interferon-alpha
IL-1β	Interleukin-1 beta
IPTG	Isopropylthio-β-D-galactosidase
kb	kilobase
kDa	kilodaltons
L	large protein
LB	Luria-Bertani medium
M	Molar or middle protein
mA	milliamperes
MEM	Minimal essential medium
mg	milligram
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
min	minute(s)
ml	millitre
mM	millimolar
MMLV	Moloney Murine Leukaemia Virus
mRNA	messenger ribonucleic acid
n	nano
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-IL6	Nuclear factor interleukin 6
NLS	nuclear localization signal
NPC	Nonparenchymal cells
°C	degrees Centigrade
ORF	open reading frame
P/ Pol	Polymerase protein
p.i.	post-infection
PAGE	polyacrylamide gel electrophoresis

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pg	picogram
pHSA	Polymerised human serum albumin
PKA	Protein kinase A
PKC	Protein kinase C
PreS	Hepatitis B PreS gene
PreS2	Hepatitis B PreS2 gene
rc DNA	Relaxed circular DNA
rhAV	recombinant human annexin V
RNA	Ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription or reverse transcriptase
s	seconds
S	Surface or small protein
SCID	severe combined immunodeficiency disease
SDS	Sodium dodecyl phosphate
SFM	Serum free medium
SHBsAg	Small hepatitis B surface antigen
ss DNA	Single stranded DNA
TAE	Tris acetate EDTA buffer
Taq	Thermus aquaticus thermostable DNA
TEMED	N,N,N',N'-tetramethylethylethylenediamine
TNF-α	Tumour necrosis factor alpha
v/v	volume per unit volume
WHV	Woodchuck hepatitis B virus

CHAPTER I

INTRODUCTION

1.1 General View

Hepatitis B virus (HBV), a small DNA virus that replicates via reverse transcription, causes acute and/or chronic hepatitis and chronically infected individuals are at high risk for developing hepatocellular carcinoma (HCC), one of the commonest malignancies in the world (Okuda, 2000). It has been estimated that more than 2 billion people today have been infected with the hepatitis B virus (HBV) (De Meyer *et al.*, 1997) and about 350 million are hepatitis B surface antigen (HBsAg) carriers, of whom over 250 000 die annually from hepatitis B associated liver disease (Lok, 2000).

The standard treatment with interferon-alpha (IFN-α) and other cytokines such as thymosin α-1 are not adequate to interfere with HBV replication as HBV covalently closed circular (ccc) DNA is not eliminated (Locarnini and Birch, 1999). Only 10% to 20% of chronically HBV infected patients with reduction in viral load (Andreas *et al.*, 1999) and limitation of dose-dependent side effects (Carithers, 1998) are observed during interferon-alpha therapy. Newer antiviral therapies using nucleoside analogues such as lamivudine, famciclovir, adefovir and lobucavir are unsatisfactory because short-term therapy is insufficient to clear viral infection but virus rebounds rapidly upon drug withdrawal, and long-term

monotherapy has been associated with resistant mutants due to the mutations in the HBV polymerase (Lai and Yuen, 2000).

Only humans, chimpanzees, gibbons, certain macaca are so far known to be susceptible to HBV infection (Walter *et al.*, 1996). Recently tree shrews (tupaias) have also been shown to be susceptible to HBV infection but with low efficiency (Ren and Nassal, 2001). The predominant site of clinical pathology for hepatitis B virus is the liver. The restricted host and tissue susceptibility of HBV infection suggests the existence of host and liver-specific viral regulatory elements for HBV replication. However, the major obstacle in the study of HBV has been the lack of an *in vitro* propagation system and the lack of animal model systems due to strict virus-host range, restraining the overall progress towards the understanding of the mechanisms that dictate the biological and tissue-tropic aspects of the HBV infection cycle.

Many aspects of HBV biology including HBV life cycle have been unravelled by studying related hepadnaviruses, such as the duck hepatitis B virus which is capable of *in vitro* infection, and the woodchuck hepatitis B virus which allows for the *in vivo* study in an animal model system. However, woodchuck hepatitis B virus and duck hepatitis B virus, are either difficult to keep or significantly different from HBV. For example, avian hepatitis B virus does not encode the X gene, the duck HBV receptor (Tong *et al.*, 1999) and coreceptor (Li *et al.*, 1999) is probably different, and the transcriptional events of woodchuck hepatitis B virus is distantly related to HBV. Convenient small animal models are

not available because rats and mice are not infectable by HBV However, cross-species replication of HBV has been obtained in hepatocytes of transgenic mice (Guidotti *et al*, 1995) and trimera mice (Ilan *et al*, 1999)

Within the last two decades, most of the *in vitro* studies of HBV replication have been performed by transfecting HBV genome into human hepatoblastoma cell line HepG2 (Sells *et al*, 1987), Huh-7 (Yaginuma *et al*, 1987) and rat hepatoma cell lines (Shih *et al*, 1989) The most widely used are the HepG2 2 2 15 cell line (Sells *et al*, 1987) derived from HepG2 hepatoblastoma cell line and HB611 derived from the HuH6 hepatoma cell line However, there are several drawbacks which preclude the use of these cell lines in studying some aspects of HBV biology These drawbacks are (1) Transfection with HBV genome into the cell uses constructs containing strong heterologous promoters Viral production in this system is mainly the result of chromosomal replication and does not mimic the natural infection *in vivo* in which HBV gene expression is driven solely by endogenous HBV promoters (2) HBV expressing cell lines contain multiple copies of integrated HBV DNA which is not an obligatory part of the HBV lifecycle and is not required for HBV replication HBV genomes are not integrated routinely but are maintained as a pool of episomal ccc DNA molecules in the nucleus of infected cells *in vivo* (3) HBV gene expression and replication cannot be regulated and are restricted to the fixed number of integrated HBV genomes in stable HBV expressing cell lines