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.

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

By

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
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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 dexamethasone 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 penimbunan 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:

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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 concurrently submitted for any other degree at UPM or other institutions.

WANG SUK MEI

Date: 23.7.2001
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LIST OF ABBREVIATIONS

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
CaCl₂ Calcium chloride
cAMP Cyclic adenosine monophosphate
ccc DNA Covalently closed circular DNA
CD Cluster designation
cDNA complementary DNA
ChHBV Chimpanzee hepatitis B virus
CO₂ carbon dioxide
CTLs Cytotoxic T lymphocytes
d day(s)
ddH₂O 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)
dsDNA 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
MgCl2 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
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>pHSA</td>
<td>Polymerised human serum albumin</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PreS</td>
<td>Hepatitis B PreS gene</td>
</tr>
<tr>
<td>PreS2</td>
<td>Hepatitis B PreS2 gene</td>
</tr>
<tr>
<td>rcDNA</td>
<td>Relaxed circular DNA</td>
</tr>
<tr>
<td>rhAV</td>
<td>recombinant human annexin V</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>RT</td>
<td>reverse transcription or reverse transcriptase</td>
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<tr>
<td>s</td>
<td>seconds</td>
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<tr>
<td>S</td>
<td>Surface or small protein</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficiency disease</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl phosphate</td>
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<tr>
<td>SFM</td>
<td>Serum free medium</td>
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<tr>
<td>SHBsAg</td>
<td>Small hepatitis B surface antigen</td>
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<tr>
<td>ss DNA</td>
<td>Single stranded DNA</td>
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<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus thermostable DNA</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per unit volume</td>
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<td>WHV</td>
<td>Woodchuck hepatitis B virus</td>
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CHAPTER 1

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 (Hian 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 (Yagumuna 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.