CHARACTERISATION OF THE EVENTS INVOLVED IN THE RESOLUTION OF ACUTE DUCK HEPATITIS B VIRUS INFECTION

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by

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Dedication

This thesis is dedicated to my parents

Georges Youssef Reaiche

and

Arelis Mercedes Amaro de Reaiche

Abstract

The human hepatitis B virus (HBV) is the prototype member of the Hepadnaviridae family of viruses. Various other hepadnaviruses are used as models to study human HBV infections as all Hepadnaviridae family members have similar virus structure and replication strategies. The studies performed and described in this thesis were carried out using duck hepatitis B virus (DHBV) infection of Pekin ducks as a model system. Hepadnavirus infections can have either an acute or a chronic outcome. The factors that contribute to these outcomes include the immune response, the age of the host at the time of infection as well as size of viral inoculum. The overall aim of this project was to gain a detailed understanding of the mechanisms involved in clearance of virus and resolution of acute DHBV infections.

As a first step, molecular and immunohistochemical detection methods for a range of cellular markers in ducks had to be developed as assays were not readily available. Quantitative reverse transcription PCR assays (qRT-PCR) were developed for the detection of mRNA of the duck T-lymphocyte markers, CD3, CD4, CD8, duck cytokines, IFN-α, IFN-γ, TNF-α and the duck housekeeping genes, β-actin and GAPDH. Immunohistochemistry was developed for the detection of duck CD4 + and CD8 + on T cells and for the detection of proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation. These methods were then widely used throughout the project.

The innate immune response during HBV infections is not well understood. Toll-like receptors (TLR) are a family of pattern recognition receptors that form part of the innate immune response and are involved in the recognition of bacterial, fungal and viral

pathogens. The only TLR that have been reported to recognise viral pathogens are TLR-2, TLR-3, TLR-4, TLR-7 and TLR-9. The possible role of TLR during hepadnavirus infections had not been well characterized to date. In this project cDNA sequences for duck TLR-2, TLR-4 and TLR-7 were identified and characterised and qRT-PCR assays were developed for their detection. Changes in duck TLR-2, TLR-4 and TLR-7 mRNA expression during hepadnavirus infection were identified following DHBV infection of primary duck hepatocytes (PDH) *in vitro*. The results showed increased levels of expression of duck TLR early during infection indicating an involvement of TLR and the innate immune response during DHBV infection. During the *in vivo* DHBV infection studies performed to date TLR mRNA expression remained unchanged.

As previously mentioned hepadnavirus infection can have an acute or chronic outcome. We aimed to understand the mechanisms involved during the resolution of acute DHBV infection and to elucidate specific factors contributing to the successful resolution of infection. During acute infections immune markers were monitored by qRT-PCR and histological analysis of fixed liver sections was performed. Liver sections were analysed to detect liver inflammation, the number and size of Kupffer cells, hepatocyte apoptosis and changes in hepatocyte proliferation throughout the course of acute DHBV infection in 6-week-old ducks. By determining the percentage of DHBV-positive hepatocytes two patterns of clearance of acute DHBV infection were observed; early clearance of infected hepatocytes occurring before day 14 post infection (p.i.), and late clearance occurring after day 14, but before or on day 31 p.i. This viral clearance was seen to occur in a cell by cell pattern. Higher levels of hepatocyte proliferation and apoptotic hepatocytes were detected during the clearance phase (on day 14 p.i.) of the late clearance group. Periodic acid schiff-diastase (PAS-D) staining was used to show significant increases in both cell number and size of Kupffer cells. Levels of IFN-γ

mRNA increased significantly over the uninfected age-matched control ducks on day 14 p.i. Levels of CD3, CD4 and CD8 mRNA expression also increased over the uninfected controls on days 14 and 31 p.i. In summary, we found that resolution of acute DHBV infection occurred on a cell by cell pattern of clearance, it was accompanied by increases in hepatocyte proliferation, apoptotic hepatocytes and activated Kupffer cells, indicating that T lymphocytes and cell death play important roles in the rapid clearance of DHBV infection.

Following resolution of acute hepadnaviral infections residual viral DNA has been found to persist. Residual HBV DNA in humans can result in reactivation of HBV infection following liver transplantation or immunosuppressive drug treatment. This leads to possible pathogenic outcomes thus the need for further investigations. Previous studies performed in the duck model have shown that the major form of residual DNA is present as covalently closed circular DNA (cccDNA). We aimed to understand how this residual cccDNA was being maintained and if replication was involved in the process. Following resolution of infection in ducks, levels of residual DHBV DNA were monitored by quantitative PCR (qPCR). Ducks were treated with the Bristol-Myers Squibb nucleoside analogue Entecavir (ETV) in order to suppress any possible replication that might be maintaining levels of residual cccDNA. In DHBV-infected but non-ETV treated ducks, the levels of residual DHBV DNA decreased gradually when measured on days 60, 221 and 316 p.i. The observed decrease in residual DHBV DNA occurred in parallel with decreases in the rate of hepatocyte proliferation measured by PCNA staining. This finding suggests that levels of residual DHBV DNA and hepatocyte proliferation are linked and we hypothesise that hepatocyte turnover is involved in the clearance of residual DHBV DNA. ETV treatment did not have an effect

on the levels of residual DHBV DNA which suggests that it is present in a subset of long-lived hepatocytes that do not support virus replication.

Mathematical modelling was performed to predict the rate of hepatocyte proliferation required for the elimination of residual cccDNA. The mathematical modelling showed that the predicted rate of hepatocyte proliferation was consistent with the rate of hepatocyte proliferation measured by PCNA. Further mathematical modelling showed that residual cccDNA is most likely to survive mitosis and it decreases due to several rounds of hepatocyte proliferation required for its elimination.

Altogether, this project has elucidated mechanisms involved during the resolution of acute DHBV infection and also possible mechanisms by which residual DHBV DNA is maintained following resolution of infection. Detailed understanding of the virological and immunological events that occur during the resolution of an acute hepadnavirus infection would assist in the development of new therapeutic treatments for the cure of chronic HBV infections.

Declaration of Originality

This work contains no material, which has been submitted for the award of any other degree or diploma in the University of Adelaide or any other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due references has been made in the text.

I consent to this copy of my thesis being made available for circulation and photocopying for the purposes of study and research in accordance with the rules established by the University of Adelaide.

Georget Yacknisa Reaiche July 31st, 2008.

Publications, presentations and awards arising from the research presented in this thesis

Manuscripts in preparation:

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"And all things, whatsoever ye shall ask in prayer, believing, ye shall receive." (Mathew, 21:22)

Abbreviations

AEC	Animal Ethics Committee
ALT	alanine aminotransferase
Anti-DHBc	anti-duck hepatitis B virus core
Anti-DHBs	anti-duck hepatitis B virus surface
Anti-HBc	anti-hepatitis B virus core
Anti-HBe	anti-hepatitis B virus e
Anti-HBs	anti-hepatitis B virus surface
APC	antigen presenting cells
bp	base pair
BrdU	Bromodeoxyuridine
BSA	bovine serum albumin
cccDNA	covalently closed circular deoxyribonucleic acid
cds	coding DNA sequence
CHB	chronic hepatitis B
cm	centimetre
CM	culture medium
CMI	cell mediated immune (response)
CTL	cytotoxic T lymphocytes
CV	central vein
DAB	3,3-diaminobenzidine tetrahydrochloride
DAPI	4',6-Diamidino-2-Phenylindole
dATP	deoxyadenosine-5'-triphosphate
DC	dendritic cells
dCTP	deoxycytosine-5'-triphosphate

DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine-5'-triphosphate
DHBcAg	duck hepatitis B virus core antigen
DHBsAg	duck hepatitis B virus surface antigen
DHBV	duck hepatitis B virus
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
DR	direct repeat
dsDNA	double-stranded DNA
DW	deionized water
EAA	ethanol acetic acid (3:1)
E. coli	*Escherichia coli*
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EST	expressed sequence tags
ETV	Entecavir
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
H&E	haematoxylin and eosin
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBSS	Hanks balanced salt solution
HBV	hepatitis B virus
HCC	hepatocellular carcinoma

HCV	hepatitis C virus
HIV	human immunodeficiency virus
hr	hour
HRP	horseradish peroxidase
HSV	herpes simplex virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMVS	Institute of Medical and Veterinary Science
i.p.	intraperitoneal
i.v.	intravenous
kb	kilo base (pair)
kD	kilo Dalton
kg	kilogram
LB	Luria Broth
LPS	lipopolysaccharides
MHC	major histocompatibility complex
μg	micrograms
μl	microliters
μM	micromolar
mA	milliamps
mg	milligrams
min	minutes
ml	millilitre
mM	millimolar
mRNA	messenger RNA

m.o.i.	multiplicity of infection
MTA	material transfer agreement
NCBI	National Center for Biotechnology Information
NDS	normal duck serum
NDL	normal duck liver
NK	natural killer (cells)
nm	nanometres
NSS	normal sheep serum
nt	nucleotide
OD	optical density
OPD	*o*-phenylenediamine
ORF	open reading frame
PAMP	pathogen associated molecular patterns
PAS-D	periodic acid schiff- diastase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	PBS with 0.05% Tween 20
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDH	primary duck hepatocyte
PHA	phytohaemagglutinin
pg	pico grams
pgRNA	pre-genomic RNA
p.i.	post infection
pM	pico Mole
Pol	polymerase

poly I:C	polyinosinic and polycytidylic acids
PT	portal tracts
qPCR	quantitative PCR
qRT-PCR	quantitative reverse transcription PCR
rDHBcAg	recombinant DHBcAg
RI	replicative intermediates
RT	reverse transcription
RT	room temperature
RT-PCR	reverse transcription PCR
rcDNA	relaxed circular DNA
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulphate
sec	second(s)
ssDNA	single-stranded DNA
TAE	Tris acetate EDTA buffer
TLR	Toll-like receptor
Tm	melting temperature
TNF-α	tumor necrosis factor alpha
Tris	3,3,5,5-tetramethylbenzidine
vge	viral genome equivalent
VIDRL	Victorian Infectious Diseases Reference Laboratory
WHO	World Health Organisation
WHV	woodchuck hepatitis virus

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