Expression and function of osteopontin variants in HCV-related liver disease and hepatocellular carcinoma

Renée Jade Phillips, B. Biotech (Hons)

THE DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

THE SCHOOL OF MOLECULAR AND BIOMEDICAL SCIENCE

THE UNIVERSITY OF ADELAIDE

A dissertation submitted to The University of Adelaide

In candidature for the degree of

Doctor of Philosophy in the Faculty of Science

April 2010

Table of Contents

Abstract

Osteopontin (OPN) is a highly secreted multi-functional sialoprotein that is widely expressed in tissues, blood and urine. It is involved in a number of normal physiological functions, but is also significantly elevated in a number of cancers. While OPN is significantly expressed in hepatocellular carcinoma (HCC) little is known as to its role and if it is expressed in the pre-cancerous hepatitis C virus (HCV) infected liver.

In this thesis we show that OPN is expressed in the liver and in HCC as three variants, the full-length protein OPN-A and two splice variants OPN-B and OPN-C. Through production of stable Huh-7 cells expressing the OPN variants, we show for the first time that all variants increase proliferation of a range of cultured hepatoma cell lines in a paracrine manner through interactions with the cell surface OPN receptor CD44. Similarly, OPN-A (and to a lesser extent OPN-B and –C) accelerated Huh-7 derived tumor growth in a nude mouse model. We also show for the first time expression of all three OPN variants in the non-diseased liver as it was previously thought that splicing was a feature specific for tumor cells.

Clinically, OPN is known to be highly expressed in HCC, however, its expression in chronic hepatitis C is not well documented. In this thesis we show that OPN mRNA expression is elevated in the HCV-infected liver with a trend towards increased expression as liver disease progresses. Consistent with an increase in mRNA, serum OPN levels were also increased in the HCV-infected liver although we could find no correlation with degree of liver disease. However, our sample size was small and this section of the thesis needs repeating with a larger HCV-infected patient cohort. Furthermore, we show that elevated OPN expression is not specific to the HCV-infected liver as OPN is also elevated in the HBV-infected and alcoholic liver

suggesting that HCV does not drive OPN expression but is more likely as a result of the inflammatory process in the viral infected liver. Interestingly we also show that there is a shift of OPN expression from bile duct epithelial cells in the non-diseased liver to the hepatocyte in the HCV-infected liver which raises the question as to the role of OPN in hepatocyte transformation to facilitate the development of HCC. Our evaluation of serum OPN expression also suggests that OPN has potential as both a diagnostic and potentially prognostic biomarker for not only HCC (arising from HBV and HCV infections and alcohol abuse) but also the earlier stages of HCV-related liver disease.

This work for the first time characterises the expression of all OPN variants in the liver including HCC and may be useful for identifying targeted OPN-based therapeutic approaches for HCC and other cancers. Furthermore it also suggests that monitoring OPN in chronic hepatitis C may be useful in monitoring liver disease progression and early detection of HCC.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Renée Jade Phillips and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Renée Jade Phillips, B. Biotech (Hons)

April, 2010

Presentations and publications related to this thesis

Publications:

Renée J. Phillips, Karla J. Helbig, Kylie van der Hoek, Devanshi Seth and Michael R. Beard (2010). Osteopontin increases hepatocellular carcinoma cell growth through interactions with CD44. *Submitted (Hepatology).*

Conference Presentations (presenter in bold type):

Renée J. Phillips, Karla J. Helbig, Andrew R. Ruszkiewicz and **Michael R. Beard**. Osteopontin is significantly expressed in advanced HCV-related liver disease and can accelerate Huh-7 growth *in vitro* and in a nude mouse model. *4th Hong Kong-Shanghai International Liver Congress*, Hong Kong, 2008.

Renée J. Phillips, Karla J. Helbig, Andrew R. Ruszkiewicz and Michael R. Beard. Osteopontin is significantly expressed in advanced HCV-related liver disease and can accelerate Huh-7 growth *in vitro* and in a nude mouse model. *42nd Annual Meeting of the European Association for the Study of the Liver*. Barcelona, Spain, 2007 (poster presentation).

Renée J. Phillips, Karla J. Helbig, Andrew R. Ruszkiewicz and Michael R. Beard. Osteopontin is expressed in advanced HCV-related liver disease and can accelerate hepatocyte growth *in vitro*. *13th International Meeting on Hepatitis C Virus and Related Viruses*. Cairns, Australia, 2006.

Renée J. Phillips (McKay), Karla J. Helbig and Michael R. Beard. The role of osteopontin in hepatitis C virus-related liver disease and hepatocellular carcinoma. *Australian Society for Medical Research (ASMR) Medical Research Week Scientific Meeting (SA)*. Adelaide, SA, Australia, 2005.

Renée J. Phillips (McKay), Karla J. Helbig and Michael R. Beard. The role of osteopontin in hepatitis C virus-related liver disease and hepatocellular carcinoma. *2nd Australian Centre for Hepatitis Virology and HIV Virology National Scientific Meeting*. Terrigal, NSW, Australia, 2005.

Acknowledgements

Firstly I would like to thank my principal supervisor Assoc. Prof. Michael Beard and co-supervisor Dr. Allison Jilbert for providing unwavering support, guidance, advice and encouragement over the course of both my Honours year and my PhD.

Sincere thanks to all current and former members of the Hepatitis C Research Group who have assisted me both professionally and personally over the last few years. Special thanks go to Dr. Karla Helbig who initiated the work on osteopontin that formed the starting point of my project and for her constant advice and suggestions, Mrs. Ljiljana Semendric and Mrs. Gorjana Radisic who taught me most of what I know technically, and Dr. Kylie van der Hoek for her invaluable help with immunohistochemistry and histology. Thank you also to Nick, Erin, Evelyn, Eddie, Susan and all our past honours students for your friendship, technical expertise, proof reading and snack food.

To everyone from other laboratories who helped me with reagents, equipment and advice, I could not have done it without you. Thank you to my fellow PhD students who have helped keep my sanity in check, and who understand so well the life of a graduate student. I would also like to acknowledge the following individuals and organisations who donated patient specimens for this study: Dr. Hugh Harley (Royal Adelaide Hospital), Dr. Ed Gane (Auckland Hospital), Dr. Tin Nguyen (Royal Prince Alfred Hospital), the Institute of Medical and Veterinary Sciences and the Australian Red Cross Blood Service.

Finally I would like to thank my husband Brad for encouraging me to continue with my studies, and my family and friends who have been very patient in allowing me to be a student for the past 10 years.

List of Figures and Tables

Chapter 2

Chapter 3

Chapter 6

Abbreviations

6-FAM	6-carboxyfluorescein
A	adenosine
AFB_1	aflatoxin B1
AFLP	amplified fragment length polymorphism
AFP	alpha-fetoprotein
AFP-L3	lens culinaris agglutinin-reactive AFP
AFU	α-1-fucosidase
aka	also known as
ALT	alanine transaminase
ANOVA	analysis of variance
AP-1	activator protein 1
AP2	activating protein 2
AUC	area under the curve
bp	base pairs
BSA	bovine serum albumin
C	cytosine
°C	degrees centigrade
CA 125	cancer antigen 125
CDC	Centres for Disease Control
cDNA	complimentary DNA
CHC	chronic hepatitis C
cm	centimeter(s)
CO_2	carbon dioxide

DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine-5'-triphosphate
DCP	des-γ-carboxy prothrombin
dCTP	deoxycytosine-5'-triphosphate
DFS	disease-free survival
dGTP	deoxyguanosine-5'-triphosphate
dH_2O	deionised water
DMEM	Dulbecco's Modified Eagle Medium with HEPES
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Eta-1	early T-cell activation factor
EtOH	ethanol
F	fibrosis
FCS	foetal calf serum
FDA	Food and Drug Administration

FITC	fluorescien isothipcyanate
g/L	grams per litre
G	guanosine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEP	granulin-epithelin precursor
GFP	green fluorescent protein
GP73	golgi protein 73
GPC3	glypican 3
H&E	haematoxylin and eosin
HAI	histological activity index
HAV	hepatitis A virus
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HGF	hepatocyte growth factor
HIV	Human Immunodeficiency Virus
HRP	horseradish peroxidase
HSC	hepatic stellate cell
IDU	injecting drug use(rs)
IF	immunofluorescence
IFN-γ	interferon gamma
IFN-λ	interferon lambda
IgG	immunoglobulin G

IHC	immunohistochemistry
IL-x	interleukin x
IMVS	Institute of Medical and Veterinary Sciences
iNOS	inducible nitric oxide synthase
IRES	internal ribosome entry site
IRF-1	interferon regulatory factor-1
IU	international units
kDa	kilodalton
L	litre
LB	Luria Bertani broth
LDL	low density lipoprotein
μg	microgram(s)
μl	microlitre(s)
μm	micrometer(s)
μM	micromolar
μmol	micromole(s)
MAPK	mitogen-activated protein kinase
mAU	milli-absorbance units
mg	milligram(s)
MIA	melanoma inhibitory activity
ml	millilitre(s)
mm	millimeter(s)
mM	millimolar
MCS	multiple cloning site
mRNA	messenger RNA

MW	molecular weight
MWM	molecular weight marker
n/a	not applicable
n/avail	not available
NAFLD	non-alcoholic fatty liver disease
NANBH	non-A non-B hepatitis
NASH	non-alcoholic steatosis
NCI	National Cancer Institute
n.d.	not determined/not detected
ng	nanogram(s)
NIH	National Institute of Health
NHL	normal human liver
NKT	natural killer T
nm	nanometer(s)
nM	nanomolar
NML	normal mouse liver
NTC	no template control
OPN	osteopontin
ORF	open reading frame
OS	overall survival
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	PBS-Tween 20
PCR	polymerase chain reaction
PDGF	platelet derived growth factor

peg-IFN-α	pegylated interferon alpha
PEI	percutaneous ethanol injection
pg	picogram(s)
PIVKA-II	protein induced by vitamin K absence
pmol	picomole(s)
pro-MMP-2	pro-matrix metalloprotease-2
PSA	prostate specific antigen
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROC	receiver operating characteristics
RPLPO	ribosomal protein, large, P0
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
SCCA	squamous cell carcinoma antigen
SDS	sodium dodecyl sulfate
SIBLING	small integrin-binding ligand N-linked glycoprotein
siRNA	short interfering RNA
SNP	single nucleotide polymorphism
SPP1	secreted phosphoprotein 1
STAT-C	specifically targeted antiviral therapy for hepatitis C
SVR	sustained virological response
T	thymidine
TAE	Tris/acetic acid/EDTA
TEMED	N,N,N',N'-tetramethylethylethylenediamine

TNF-α	tumor necrosis factor alpha
Tris	3,3,5,5-tetramethylbenzidine
U/L	units per litre
uPA	urokinase plasminogen activator
US	United States of America
UTR	untranslated region
UV	ultraviolet
VDRE	vitamin D responsive element
VEGF	vascular endothelial growth factor
v/v	volume per volume
WB	western blotting
WHO	World Health Organisation
w/v	weight per volume

Materials Providers

Ambion	Austin, Texas, USA
Amersham Pharmacia Biotech (GE Healthcare)	Piscataway, New Jersey, USA
AppliChem	Darmstadt, Germany
Applied Biosystems	Foster City, California, USA
BD Biosciences	Franklin Lakes, New Jersey, USA
BDH (Merck)	Poole, UK
Becton Dickinson Labware	Franklin Lakes, New Jersey, USA
BioRad Laboratories	Hercules, California, USA
Biotium Inc.	Hayward, California, USA
Canon	Tokyo, Japan
Cell Signaling	Danvers, Massachusetts, USA
Chemicon (Millipore)	Billerica, Massachusetts, USA
Corning Costar®	Lowell, Massachusetts, USA
CSL	Parkville, VIC, Australia
Dako	Glostrup, Denmark
Diploma (Fonterra Brands)	Mt. Waverley, VIC, Australia
Dynatech Laboratories	Chantilly, Virginia, USA
GeneWorks	Hindmarsh, SA, Australia
Gibco BRL (Invitrogen)	Carlsbad, California, USA
Greiner Bio-one	Frickenhausen, Germany
Invitrogen	Carslbad, California, USA
Kodak	Rochester, New York, USA
Livingstone	Rosebery, NSW, Australia

Menzel-Glaser®	Braunschweig, Germany
Mo Bio Laboratories	Carlsbad, California, USA
Molecular Probes	Eugene, Oregon, USA
Nalgene	Rochester, New York, USA
NeoMarkers (LabVision)	Fremont, California, USA
New England Biolabs	Ipswich, Massachusetts, USA
Nunc (ThermoFisher Scientific)	Roskilde, Denmark
Olympus	Tokyo, Japan
Pharmacia (Pfizer)	New York, New York, USA
Promega	Madison, Wisconsin, USA
Qiagen	Hilden, Germany
Roche	Basel, Switzerland
R&D Systems	Minneapolis, Minnesota, USA
Rockland	Gilbertsville, Pennsylvania, USA
Santa Cruz Biotechnology	Santa Cruz, California, USA
Sigma	St. Louis, Missouri, USA
SPSS Inc.	Chicago, Illinois, USA
Techno-Plas	St. Marys, SA, Australia
Trace Biosciences	Castle Hill, NSW, Australia