Differential Leukocyte Heat Shock Protein Expression is Modulated in Health and Disease

By

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Biological, Biomedical and Molecular Sciences, Faculty of the Sciences, University of New England, Armidale, Australia.

March, 2006

This Thesis is dedicated to Warren, Stephanie, Philip, Elliott and Callum.

Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that to the best of my knowledge any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Linda Agnew

Acknowledgements

Nelson Mandela once said, "After climbing a great hill, one only finds that there are many more hills to climb". Many people have helped me to reach the top of my PhD 'hill' through their prayers, friendship, support and in many practical ways, and to all of my family, friends and colleagues I am truly grateful.

To my supervisor, Professor Kenneth Watson, I extend heartfelt thanks for caring not just about this PhD project, but more importantly, for caring about me personally. I have appreciated the many ways in which you have provided generous support throughout my candidature. As my mentor, you have encouraged me to think critically, you were always available when I needed advice and you provided the freedom for me to take responsibility for 'driving' this project. To my co-supervisor, Associate Professor Graham Jones, thank you for your support. I particularly appreciated your willingness to provide intellectual input when required.

The early stage of this project received input from Dr Rosemary Ffrench, formerly of the Westfield Research Laboratories, Sydney Children's Hospital, Randwick, and Dr Mark Kelly, formerly of the Albion Street Centre, Surry Hills, to whom I am grateful for their advice and support.

To Jega Serangapany, Dr Julian Gold, medical and nursing staff of the Albion Street Centre, Surry Hills, I extend my thanks for taking an interest in this project and for assistance with the recruitment and collection of blood samples from the HIV-positive patients who participated in these studies.

To Dr John Zaunders, Dr Anthony Kelleher, staff and students of the Centre for Immunology, St Vincent's Hospital, Sydney, I extend sincere appreciation for your assistance with the multicolour flow cytometry experiments conducted on HIV-positive samples. The time spent in your laboratory was very rewarding, and I particularly appreciated the way in which you welcomed me and provided a friendly and supportive work environment. To Preeyaporn Srasuebkul of the National Centre for HIV Epidemiology and Research, thank you for the statistical support you provided.

To Dr Jim McFarlane, Dr Kate Kauter and Kym Rae of the Physiology Department, University of New England, thank you for your contribution to the studies on hormonal modulation of heat shock proteins.

To Dr Ian Colditz of CSIRO Livestock Industries, Chiswick, thank you for your assistance with flow cytometry and for your contribution to the studies on hormonal modulation of heat shock proteins.

To Dr Anita Matthias of the MediHerb Research Laboratory, University of Queensland, thank you for performing the HPLC analysis of alkylamides for the Echinacea studies.

To Neil Horton and staff of the Pathology Department, Armidale and New England Hospital, thank you for your assistance with blood sample collection and haematological analysis of samples for the Echinacea study. This work was fitted into the extremely busy schedule of your diagnostic laboratory and for that I am grateful.

To the academic staff, technical staff, administrative staff, and fellow postgraduate students of the School of Biological, Biomedical & Molecular Sciences, University of New England, working within this School has given me an appreciation for the benefits of a multi-disciplinary approach to research. I will also take the 'institution' of Friday cake day with me wherever I go! In particular, I would like to thank Rosslyn Busby and Craig Lawlor for technical assistance. I would also like to thank Sharon Styles for administrative support. To Sharon Guffogg, I appreciated your assistance with the Echinacea studies and special thanks for the lighter moments in our office, having someone to share frustrations with helped keep me sane.

This project would not have been possible without the many volunteers who generously donated blood samples. To all who participated in these studies I extend my appreciation.

Financial support for this PhD candidature was provided by an Australian Postgraduate Award. Additional financial support was received from the AIDS Trust of Australia, MediHerb Pty Ltd and a University of New England Internal Research Grant .The work conducted at The Centre for Immunology, Sydney was funded by a Keith and Dorothy Mackay, short-term attachment travel scholarship.

To my husband Warren, daughter Stephanie, sons Philip, Elliott and Callum and my mother, Wendy, I could not have completed this PhD without your ongoing understanding and unconditional love and support.

Abstract

Heat shock proteins (hsps) are a group of highly conserved proteins that represent between 2% and 15% of total cellular protein and are expressed by every living organism. The main functions of hsps are to regulate apoptosis and to act as intracellular molecular chaperones that facilitate protein folding and assembly. Some hsps are highly immunogenic and elicit humoral, cytotoxic T-lymphocyte (CTL) and natural killer (NK) cell responses against viruses, tumors and infectious diseases.

In the first study, twenty male patients with HIV disease and fifteen age-matched controls were recruited. Lymphocytes were isolated and incubated at either 37^0C for 1 h or heat shocked at 42.5^0C for 1 h. Lymphocytes were then allowed to recover at 37^0C for 3 h and hsp expression was measured using both western immunoblots and 1D-SDS-PAGE (β-actin used as internal control). After a mild heat shock (from 37°C to 42.5°C for 1 hr) lymphocytes displayed an augmented synthesis of hsp 110, hsp 90 and hsp 70, relative to actin, in all individuals regardless of HIV-status. There were apparent differences in levels of expression of newly synthesized hsps between the HIV-positive and HIV-negative groups. Within this cohort these differences were not correlated with $CD4^+$ count, viral load, dietary supplement use, smoking or the use of highly active antiretroviral therapy (HAART). The significance of altered hsp expression remains to be determined. However, given the recent reports on the role of these proteins in cross-presentation of antigens, α-defensin internalization and pro-inflammatory cytokine production, further investigation is merited.

Flow cytometric methods have demonstrated that hsp70 is constitutively expressed in human leukocytes but that the level of expression varies considerably between different cell types. Specifically, monocytes express significantly more hsp70 than any other leukocyte subtype. These findings were confirmed and extended to develop a scientifically robust system for measuring hsp70 expression in leukocyte sub-populations that only required small volumes (1 – 2 ml) of whole blood. This methodology was then employed to compare hsp70 expression patterns in HIV-positive and HIV-negative cohorts. The results of this study demonstrated significant differences ($p<0.05$) in hsp70 expression at both basal and heat shock levels, in a number of leukocyte sub-populations, between HIV-positive ($n = 42$) and HIV-negative ($n = 11$) individuals. These differences were apparent in cytotoxic T cells (CD8+), helper T cells (CD4+) and monocytes (CD14+). There were no apparent differences in natural killer cell (CD56+) expression of hsp70. A common predictive factor for altered hsp70 expression was viral load suggesting that hsp70 expression may be a reflection of host-virus interactions resulting in alterations to the cellular stress response. Furthermore, preliminary findings indicated that leukocyte hsp70 expression may differ with disease progression/status as indicated by the patterns of hsp70 expression observed in the HIV-positive sub-groups examined (seroconverters, long-term non-progressors and Hepatitis C co-infected individuals). Further studies will be required before a definitive statement can be made, however, if the current findings are confirmed, leukocyte hsp70 expression may be a useful clinical marker for HIV-disease progression.

The immunomodulatory effects of oral dosing with an Echinacea preparation were investigated in a small scale clinical trial ($n = 11$). Expression of leukocyte heat shock protein 70 (hsp70), serum chemistry, haematological values and plasma alkylamide

concentrations were evaluated in eleven healthy individuals (26 to 61 years of age) at baseline (day 1) and on day 15 after consuming two commercially blended Echinacea tablets (containing both *Echinacea angustifolia* and *Echinacea purpurea* root) per day for fourteen days. Plasma alkylamide levels were determined one hour after ingestion of one Echinacea tablet and concentrations were found to be 12 ± 2 ng equiv/ml plasma. Echinacea supplementation significantly enhanced the fold increase from 2.2 ± 0.4 to 3.3 ± 0.7 ($p = 0.03$) in leukocyte hsp70 expression after a mild heat shock. Serum chemistry and haematological values for subjects after Echinacea supplementation did not vary significantly from baseline levels with the exception of white cell counts which increased from 6.6 ± 0.4 to 7.2 ± 0.3 x 10^9 ($p = 0.04$). Differential white cell counts displayed modest increases after Echinacea supplementation although only the lymphocyte sub-population approached significance ($p = 0.06$). The enhanced hsp70 stress response found was indicative of an improved immune response given that increases in hsp70 expression following cellular stress may play a critical role in antigen presentation, lymphocyte effector function and cytokine induction. This pilot study therefore suggested that supplementation with Echinacea may invoke an immune response through altered expression of hsp70 and increased white cell counts.

The effects of gender, menstrual cycle phase and oral contraceptive use on leukocyte hsp70 expression were examined in a small-scale study involving 11 female and 5 male participants. The results of these studies demonstrated that female leukocyte hsp70 expression displayed gender differences and in females, menstrual cycle phase differences and alterations as a result of oral contraceptive use were observed. These studies also demonstrated that serum leptin levels displayed gender dimorphism, serum cortisol levels were higher in females using oral contraceptives, and that serum prolactin levels displayed menstrual cycle variation in females and showed gender dimorphism. This is the first report of changes in leukocyte hsp70 expression in response to gender, menstrual cycle phase and oral contraceptive use indicating interactions between the immune response and stress response pathways.

Publications arising from this Thesis

Agnew LL, Kelly M, Howard J, Jeganathan S, Batterham M, Ffrench RA, Gold J, Watson K. Altered lymphocyte heat shock protein 70 expression in patients with HIV disease. *AIDS,* 2003; **17(13):** 1985-1988.
A copy of this paper appears in Appendix III.

Agnew LL, Guffogg SP, Matthias AM, Lehmann RP, Bone K, Watson K. Echinacea intake induces an immune response through altered expression of leukocyte hsp70, increased white cell counts and improved erythrocyte antioxidant defences. *Journal of Clinical Pharmacy and Therapeutics* 2005; **30:** 363-369.
A copy of this paper appears in Appendix III.

Agnew LL. And Watson K. Stress Proteins as Biomarkers of Oxidative Stress. *Current Protocols in Toxicology* 2006; Supplement 28, **17.8:** 1 – 25.
A copy of this paper appears in Appendix III.

Agnew LL, Zaunders J, Serangapany J, Kelleher A, Gold J, Watson K. HIV-positive individuals display altered hsp70 expression in a number of leukocyte subtypes. In preparation

Agnew LL, Colditz IG, Rae K, Kauter K, McFarlane JR, Watson K. Leukocyte hsp70 expression is modulated by gender, menstrual cycle phase and oral contraceptives. In preparation

Aspects of Thesis presented at conference proceedings

Poster "Altered Expression of Heat Shock Proteins (Hsp70, Hsp90, Hsp110) in Lymphocytes from HIV-positive Individuals" Presented at the Australasian Society for HIV Medicine Inc, 15th Annual Conference, Cairns, 2003.

Poster "HIV-positive individuals have altered hsp70 expression in a number of leukocyte subtypes" at the Gordon Research Conference Stress Proteins in Health, Development and Disease, Newport, Rhode Island, USA, 2005. Also presented at the School of Biological, Biomedical & Molecular Sciences, Postgraduate Conference, University of New England, Armidale Australia, 2006

Poster "Effect of Echinacea on human immune responses *in vivo*" Presented at the 53rd Annual Congress of the Society for Medicinal Plant Research, Florence, Italy, 2005.

Abstracts and copies of these posters are presented in Appendix II.

Abbreviations

AAPH	2,2'-azobis-(2-amidinopropane) dihydrochloride
ACTH	adrenocorticotropic hormone
ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immune deficiency syndrome
AMPS	ammonium peroxodisulphate
APC	Allophycocyanin
APC	antigen presenting cell
APC-Cy7	Allophycocyanin-Cy7
ASC	Albion Street Centre
BSA	bovine serum albumin
CAM	complementary and alternative medicine
CHD	cardiovascular heart disease
CFI	Centre for Immunology, St Vincent's Hospital, Sydney
CNS	central nervous system
CO	carbon monoxide
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DNPH	2,4-dinitrophenylhydrazine
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	flow cytometry and cell sorting
FCS	fetal calf serum
FITC	Fluorescein
FSH	follicle stimulating hormone
GnRH	gonadotrophin-releasing hormone
grp	glucose regulated protein
GSH	glutathione
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HO-1	heme oxygnase

HPA	hypothalamic-pituitary-adrenal axis
HRT	hormone replacement therapy
HSE	heat shock element
HSF	heat shock factor
hsp	heat shock protein
HTLV	human T-lymphocytotropic virus
IFN-γ	interferon-gamma
IL	interleukin
kDa	kilodalton
LAV	lymphadenopathy associated virus
LH	luteinizing hormone
LPS	lipopolysaccharide
LTNP	long-term non-progressor
MAPK	mitogen-activated protein kinases
MDA	maliondialdehyde
MHC	major histocompatibility complex
MS	multiple sclerosis
MUFA	monounsaturated fatty acids
NAC	N-acetylcysteine
NK	natural killer cells
NF-κB	nuclear factor- κB
NNRTI	non-nucleoside reverse transcriptase inhibitor
NO	nitric oxide
NRTI	nucleoside reverse transcriptase inhibitor
OC	oral contraceptive
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy7
PerCP	Peridinin Chlorophyll Protein
PI	protease inhibitor
PLWHA	people living with HIV/AIDS
PMSF	phenylmethylsulphonyl fluoride

PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SEM	standard error of the mean
SFA	saturated fatty acid
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus
STD	sexually transmissible disease
STI	structured treatment interruption
TAS	total antioxidant status
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TCR	T cell receptor
TEMED	N,N,N',N',-tetramethylenediamine
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
WRL	Westfield Research Laboratories

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