Biochemical and immunological roles of heat shock proteins in human cancer

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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 the preparation of this thesis, and all sources used, have been acknowledged accordingly.

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Christopher Shipp

"To those human beings who are of any concern to me I wish suffering, desolation, sickness, ill-treatment, indignities - I wish that they should not remain unfamiliar with profound self-contempt, the torture of self-mistrust, the wretchedness of the vanquished"

Friedrich Nietzsche

Although probably not an accurate description of the process of obtaining most PhDs, the failures, difficulties and low periods are an important part of the journey and should be savoured and learnt from as much as the successes.

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Abstract

Found in every cell of every organism, heat shock proteins (hsps) participate in a wide range of cellular processes and primarily function as molecular chaperones that mediate the activity of other cellular proteins. Hsps are required for a range of fundamental mechanisms used by cancer cells and they have consequently been identified as valid targets in the treatment of cancer. It was the aim of this thesis to further investigate these roles in breast cancer and melanoma using novel approaches from a biochemical and immunological perspective.

In a preliminary study, breast cancer tissues (n = 30) were demonstrated by Western immunoblotting to widely express hsps 90 and 70. Two-dimensional gel electrophoresis indicated that a number of proteins were differentially expressed in tumour and healthy breast tissue from the same patient. These results suggest that a number of possibly unidentified proteins may play important roles in breast cancer and thus have use as therapeutic targets or biomarkers. The role of hsp90 and associated client proteins in breast cancer was further investigated by non-denaturing immunoprecipitation followed by elution with geldanamycin, a specific inhibitor of hsp90. Geldanamycin-sensitive hsp90 client proteins were observed in seven of 11 protein extracts from breast cancer patients and one healthy individual. Immunoprecipitation, Western immunoblotting and LC-MS identified hsps 40, 56/FKBP52, 60, 70, 105 and lumican as potential hsp90 client proteins. These proteins may thus assist breast cancer progression alongside hsp90. In one patient sample, a cancer-specific group of proteins was identified, while in all experiments geldanamycin resistance was observed. The results of this study may have relevance for the future of breast cancer research and clinical treatment.

Cell surface proteins involved in cell adhesion, apoptosis, antigen presentation as well as hsps were investigated for their role in melanoma metastasis. In addition, the influence of co-culture with stromal cells was investigated as a potential model system for *in vivo* growth conditions. Matched sets of primary-derived versus metastasis-derived melanoma cell lines were cultured and screened for the expression of CD44, CD54, CD95, CD155, MHC I, hsps 90, 70, 60, 40 and 32. In some instances, differential protein expression was observed in metastasis-derived cell lines as compared to lines derived from primary tumours, and although a number of consistent changes in protein expression were observed within the cell lines, these were not exclusively associated with primary or metastatic tumour origin.

Hypoxia is a well established characteristic of cancer cells. Paradoxically, *in vitro* studies on cancer cell lines are routinely performed under hyperoxic conditions. In this study, melanoma cell lines were cultured under high (20 % O_2, n = 42) and low (2 % O_2, n = 18) oxygen tension and monitored for the expression of hsps 90, 70, 60, 40 and 32. Total higher expression of hsps 90, 70 and 60 correlated with improved viability in low ($P < 0.05$) but not high oxygen tension. Relative hsp expression was consistent across the cohort of cell lines and the expression levels of hsps 90, 70, 60 and 40 correlated with one another ($P = 0.0001$), but not with hsp32. Expression of hsp90 was associated with cell line adhesion to collagen type IV and laminin ($P < 0.05$). Expression of hsp90 and hsp40 correlated with Breslow depth of the primary tumour from which these metastatic tumour cell lines were derived ($P < 0.04$), however, hsp expression was not correlated with other clinical parameters including Clark level or patient survival. Interestingly, all hsps were identified on the cell surface and these proteins may thus play roles in tissue invasion, metastasis and immunological recognition.

Using Western immunoblotting to examine the expression of hsp90, hsp70, hsp60, hsp40 and hsp32 in uncultured metastatic melanomas (n = 32) demonstrated that these proteins are widely expressed in melanoma tumour tissue. Correlating the expression of these hsps with patient clinical parameters showed that increased hsp90 ($P < 0.02$) and hsp40 ($P < 0.03$) expression was correlated with progression to advanced tumour stage (stage III to stage IV), higher hsp90 expression correlated with reduced patient follow-up time ($P < 0.04$) (survival since removal of the metastatic tumour that was therein examined) and hsp70 expression was associated with sex of the patient ($P < 0.05$). On the other hand, expression of the other hsps was not associated with any recorded patient clinical parameters. Fluorescence microscopy of whole melanoma tissues using the MelanA antigen as a specific marker for melanoma cells demonstrated increased expression of hsps 90, 70, 60, 40 and 32 in MelanA-positive cells compared to adjacent MelanA-negative (non-melanoma) cells. These data contribute to the proposal that hsps are valid therapeutic targets in the treatment of melanoma.

The role of hsp90 in the immunological recognition of cancer cells was investigated by treating target melanoma cells with the hsp90 inhibitor geldanamycin and using T cell lines and T cell clones as effector cells. Geldanamycin treatment was observed to abrogate the recognition of melanoma cells by T cell lines. In contrast, under identical conditions, T cell clones were able to recognise geldanamycin-treated melanoma cells. These data allude to the complexity of the hsp90 molecular chaperone and may have consequences for the rarely considered immunological aspects of hsp90 inhibitors used clinically.

The present studies employed a diverse range of experimental approaches to examine the role of hsps, hsp-client proteins and cell surface proteins in a wide range of human cancer cells, including breast cancer, melanomas and related cell lines. A number of novel experimental approaches were developed which included screening of hsp expression in matched primary and metastatic tumour cell lines, the adoption of cancer cell line culture under conditions of low oxygen tension, immunofluorescence to identify hsp expression in melanoma and adjacent non-melanoma cells *in situ* and attempts to relate hsp expression to patient clinical parameters. The overall outcomes of these studies have clearly demonstrated a critical role for hsps in cancer pathogenesis and in the immune recognition of cancer cells. Moreover, these studies have provided fresh evidence that hsps provide a valid therapeutic target in the treatment of cancers. Nevertheless, many of the present results were equivocal and contradicted some previous studies. These observations simply confirm our limited understanding of the complexities of cancer pathogenesis and the hsp molecular chaperone system.

Publications arising from this thesis

Shipp, **C**., Watson, K., Jones, G.L. Associations of HSP90 Client Proteins in Human Breast Cancer. Anticancer Research 2011; 31(6): 2095-2101.

Shipp, **C**., Derhovanessian, E. and Pawelec, G. Effect of culture at low oxygen tension on the expression of heat shock proteins in a panel of melanoma cell lines. (Accepted by PLoS ONE)

Shipp, **C**., Weide, B., Derhovanessian, E. and Pawelec, G. Hsps are up-regulated in melanoma tissue and correlate with patient clinical parameters. (In preparation)

Copies of these manuscripts appear in Appendix I

Aspects of this thesis presented at conference proceedings

Shipp, **C**., Watson, K., and Jones G. L. Stress Proteins in Human Breast Cancer. P27. EMBO meeting on Cellular Signalling and Molecular Medicine. pp 114 – 115. Cavtat, Croatia. May 29th –June 4th (2008) (Poster)

Shipp, **C**., Watson, K., and Jones G. L. Stress protein interactions in human breast cancer. University of New England Faculty of Arts and Sciences Postgraduate Research Conference. University of New England, Armidale, Australia. Nov 14th – 15th (2008) (Lecture)

Shipp, **C**., Watson, K., and Jones, G. L. Hsps 70 and 105 associate with a group of hsp90 client proteins that are selectively found in human breast cancer. D5. International Symposium on Signal Transduction and Disease. pp 87-88. Aachen, Germany. September 27th – 30th (2009). (Poster)

Shipp, **C**., Watson, K., Jones, G. L. Hsp90 client proteins in human breast cancer. The 5th International Conference on The Hsp90 Chaperone Machine. pp 126. Les Diablerets, Switzerland. Sep 29th – Oct 3rd (2010) (Poster)

Shipp, **C**., Derhovanessian, E. and Pawelec, G. Biochemical and immunological roles of hsps in human melanoma. P87. EMBO meeting on The Biology of Molecular Chaperones. pp 152. Grundlsee, Austria. May 19th – 24th (2011) (Poster)

Copies of abstracts and posters appear in Appendix II

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Abbreviations

17-AAG	17-Allylamino-17-demethoxygeldanamycin
2D SDS-PAGE	2 Dimensional Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
APC	Antigen Presenting Cell
ASI	Age Standardised Index
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CD	Cluster of Differentiation
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHIP	C-terminus of Hsp70-Interacting Protein
DC	Dendritic Cell
ddH_2O	Distilled De-ionised Water
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EHS	Engelbreth-Holm-Swarm
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
EST	European Searchable Tumour Line
ESTDAB	European Searchable Tumour Line Database
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FI	Fluorescence Index
FITC	Fluorescein Isothiocyanate
FKBP52	FK506-Binding Protein 52
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HER2	Human Epidermal Growth Factor Receptor 2
HIF1α	Hypoxia-Inducible Factor 1 alpha
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase

HSE	Heat Shock Element
HSF	Heat Shock Factor
hsp	Heat Shock Protein
IFN	Interferon
IL	Interleukin
kDa	Kilodalton
LPS	Lipopolysaccharide
LC-MS	Liquid Chromatography-Mass Spectrometry
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NK	Natural Killer (cell)
OR	Oestrogen Receptor
PBMC	Peripheral Blood Mononuclear Cell
PBS-T	Phosphate Buffered Saline-Tween 20
PE	Phycoerythrin
pI	Isoelectric Point
PMSF	Phenylmethanesulfonyl Fluoride
PO	Pacific Orange
PPI	Peptidyl-prolyl Isomerase
PR	Progesterone Receptor
SDS	Sodium Dodecyl Sulphate
SMP	Skim Milk Powder
TAP	Transporter Associated with Antigen Processing
TPR	Tetratricopeptide Repeat
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate
TEMED	N,N,N′,N′-Tetramethylethylenediamine