# Chapter 8: Summary and Future Directions

At the commencement of this PhD candidature (January 2003), there were no reports of hsp expression in HIV-positive individuals. Preliminary investigations during the author's Honours candidature had indicated that lymphocyte hsp70 expression was altered in HIV-positive individuals. The aim of the work presented in this Thesis, therefore, was to confirm and extend those preliminary findings and to develop and apply an experimental system to investigate the modulation of hsp70 expression in HIV-infection.

The impetus for using a human peripheral blood leukocyte experimental system was that the vast majority of research into stress protein synthesis in humans had been conducted in *in vitro* settings and whilst the importance of such work cannot be denied, there was a need for more *in vivo* system research. The importance of investigations into expression of hsps in disease states is beginning to be appreciated, particularly in the fields of autoimmunity and inflammatory and cardiovascular diseases (reviewed in Pockley, 2002). The complexity of whole organism research is a challenge, given the vast myriad of interactions that occur between cells, receptors and protein molecules and the multiplicity of interactions no doubt, contribute to the modulation of stress protein expression. Therefore the work discussed in this Thesis examined a number of these interactions.

Taken together, the results of the experimental work presented in this Thesis highlight that leukocyte hsp70 expression is intricately associated with immune function, in response to viral infection, as a result of herbal supplementation with an immunomodulator and in response to immunological changes that occur during the menstrual cycle and with oral contraceptive use. The experimental work presented in this Thesis provided evidence of a number of different factors that modulate hsp expression in human leukocytes. These factors included infection with HIV-1 (Chapters 3 and 5), herbal supplementation (Chapter 6) and gender, menstrual cycle phase and oral contraceptive use (Chapter 7).

## 8.1 Expression of heat shock proteins is altered in HIV-infection

The current studies examined the expression of newly synthesized proteins in leukocytes by 1D-SDS-PAGE, the expression of relative amounts of constituitevely expressed and heat shock induced hsps in leukocytes by Western immunoblot and the level of hsps in the plasma of HIV-positive individuals and uninfected control subjects. Comparison of patterns of hsp expression in HIV-positive and HIV-negative individuals revealed that patients with HIV-disease displayed altered expression of several of the heat shock proteins. There were apparent differences in levels of expression of newly synthesized hsp110 and hsp90 between the HIV-positive and HIV-negative groups as measured by autoradiography. Significant differences were also apparent in absolute amounts of hsp70 as measured by Western immunoblot. Specifically, constitutive expression of hsp70 was down regulated in HIV-positive individuals with a resultant augmented fold-increase in heat shock induced hsp70 relative to constitutively expressed hsp70.

A limiting factor of the current studies was the high degree of inter-individual variability observed in the concentrations of plasma hsp70 measured by ELISA. This phenomenon was also observed by Njemini *et al.* (2003) indicating that the determinants of hsp70 release from leukocytes may be highly individual, and until the mechanisms of this release are elucidated, caution is urged in the use of this ELISA for clinical purposes. It is likely, however, that the results of the current study, indicating no difference in circulating hsp70 levels in HIV-positive individuals, were due to the recruitment of a cohort of clinically stable patients suggesting that hsp70 release from viable cells may be a result of active infection. Future studies of this nature, therefore, should compare hsp70 levels in the plasma of HIV-positive individuals at differing stages of disease progression.

In summary, the results of the studies presented in Chapter 3 of this Thesis suggested that the mechanisms of altered hsp expression in HIV-positive individuals were likely to be quite complex given the nature of chaperone-virus interactions. The significance of altered hsp expression in HIV-positive individuals remains to be determined, however, given the recent reports on the role of these proteins in cross-presentation of antigens (Millar *et al.,* 2003), α-defensin internalization (Nassar *et al.,* 2002; Zhang *et al.,* 2002) and pro-inflammatory cytokine production (Asea *et al.,* 2000), further investigation is merited. In this relatively small cohort no correlation

between fold increases in lymphocyte hsp70 expression and viral load, CD4 T cell count or antiretroviral treatment status could be determined. Studies involving much larger cohorts will be required to examine such correlations.

## 8.2 Methodological developments in the measurement of stress protein synthesis in leukocytes

The experimental work discussed in Chapter 4 of this Thesis was conducted in order to develop scientifically robust methodologies to measure leukocyte hsp expression that would complement and extend the capabilities of the traditional methods of Western immunoblot, 1D-SDS-PAGE autoradiography and ELISA. The results of the current study revealed that not only was hsp70 expression measurable in cryporeserved lymphocytes, but also that these cells retained the ability to synthesise new proteins. This study also demonstrated that after cryopreservation, *de novo* lymphocyte expression of some hsps was altered. These differences were most notable after longer periods of storage (75/78 days) and included the up-regulation of proteins of approximately 56 kDa and 32 kDa in both control (37°C) and heat shock (42.5°C) samples. It is likely that these proteins are hsp56, also known as FK506 binding protein 59 or FK506 binding protein 52 (FKBP59/FKBP52), and hsp32 also known as heme-oxygenase (HO-1). The up-regulation of the latter proteins may be due to oxidative stress induction during cryopreservation. It is likely, therefore, that the concentration (10% v/v) of DMSO used during the cryopreservation process in the current studies was insufficient to counteract the oxidative burst that occurred, thus resulting in the upregulation of the stress proteins. Although lymphocytes are able to synthesize new proteins after cryopreservation, the finding that the process of cryopreservation itself induces hsp synthesis currently renders this technique unsuitable for the measurement of stress protein expression. Future studies could, however, determine the optimum concentration of DMSO to counteract the oxidative burst observed during cryopreservation of leukocytes, thus enabling the measurement of hsps in cryopreserved leukocytes.

Hsp70 was detectable in both serum and plasma and the current studies indicated that there was essentially no difference in these measurements, however, further studies with a larger cohort are recommended before a definitive statement can be

made. It was also demonstrated that hsp70 release from leukocytes into the plasma occurred in a time-dependant manner.

Using traditional methods, cells need to be separated from whole blood using Ficoll-Paque gradient centrifugation prior to a heat shock treatment. The results of the current study clearly demonstrated that heat shocking whole blood produced consistent and reliable up-regulation of hsp70. This finding is highly significant as it enables the measurement of the stress response in leukocytes by flow cytometric methods using whole blood instead of in separated leukocytes. Futhermore, the studies in Chapter 4 of this Thesis described the differential nature of hsp70 expression in leukocyte subpopulations, namely, helper and cytotoxic T lymphocytes, B lymphocytes, NK cells and monocytes. The results of the current study support previous findings that hsp70 expression is variable in leukocyte subtypes and is preferentially expressed by monocytes (Bachelet *et al.,* 1998). Furthermore, the benefit of using an isotype control antibody reduces the risk of false positive results, an issue of particular importance when examining cell subsets expressing small amounts of hsp70.

Significantly, Chapter 4 reported the process of development of a new, cutting-edge methodology for the measurement of leukocyte stress protein expression in whole blood samples. Importantly, this advancement included the ability to measure hsp expression in small volumes (1 – 2 ml) of whole blood, compared with traditional methods which required 20 – 30 ml samples. This technique has been further developed and applied to larger scale studies as detailed in the Chapters that follow.

### 8.3 Analysis of stress protein expression by flow cytometry: Differential hsp70 expression in leukocyte subpopulations

The results of the current studies revealed that hsp70 was expressed in a differential manner by human leukocyte sub-populations, confirming and extending previously reported findings (Oehler *et al.*, 2001*).* The current studies identified that under basal conditions (37°C), more monocytes expressed hsp70 than leukocytes or natural killer cells. Generally, the mean fluorescence intensity (MFI) was also greater in the monocyte sub-groups. It has been postulated that differences in hsp70 (and hsp27) expression in monocytes and lymphocytes may be a function of monocyte protection

against self-produced toxic metabolites upon activation (Jäättelä & Wissing, 1993). The present studies also demonstrated that induction of hsp70 by heat shock did not occur to the same extent in every cell despite exposure of the entire cell population to the same conditions. This finding suggested that hsp induction is differentially regulated and future studies, therefore, could be undertaken to examine the mechanisms of this regulation.

The role of hsps in viral infection has, at the time of writing, not been fully elucidated, and conflicting theories have emerged based upon the close association hsps have with a range of viral proteins which may, in fact, assist viral pathogenesis. On the other hand, an anti-viral role for hsps could be perceived. It may be that hsps avert cleavage and processing of precursors into mature virions by binding newly synthesized viral capsid proteins thus preventing their participation in viral assembly (Macejak & Sarnow, 1992). During the course of the present studies, several samples from seroconverters, long-term non-progressors and hepatitis C co-infected subjects were examined for leukocyte hsp70 expression. Interestingly, these sub-groups displayed quite unique patterns of hsp70 expression, unlike either the HIV-negative or clinically stable HIV-positive cohorts examined in these studies.

There is currently no published data describing hsp expression in HIV-positive individuals at seroconversion, those who are long term non-progressors, or those who are co-infected with hepatitis C. In the present studies, the numbers of individuals in each of the sub-groups were limited, therefore, further studies involving much larger numbers of individuals will be required before a definitive statement can be made regarding the differential nature of hsp expression in the various phases of HIV-infection.

The observation in the current studies of the differential nature of hsp70 up-regulation in patients with HIV-disease suggested that the up-regulation of hsp70 following viral infection did not occur in a similar fashion to the up-regulation that is observed following heat shock. This observation may be due to the differences that occur in cellular localization of hsps following heat shock and viral infection.

Taken together, the results of this study reveal that infection with HIV-1 results in alterations in leukocyte hsp70 expression and that the expression of hsp70 varies considerably with disease status, suggesting that hsp70 expression may be a

reflection of host-virus interactions resulting in alterations to the cellular stress response. The mechanisms of altered hsp70 expression in HIV-positive individuals are yet to be elucidated and appear to be quite complex. These mechanisms may include, but not be limited to, host cellular activation status, induction of the innate and/or adaptive immune response, an increased requirement for effective presentation of antigen, or hsps ability to act as an innate anti-viral factor. Furthermore, preliminary findings of the current studies 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. 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.

### 8.4 Modulation of stress protein synthesis by *Echinacea* spp.

A variety of herbs are commonly used in presumptive immune modulating formulations and Echinacea, in particular, is perhaps the most widely used of all medicinal herbs. Echinacea is used prophylactically as an immunomodulator and antioxidant and for the treatment of urinary tract infections, eczema and psoriasis as well as to aid wound healing. Conflicting reports exist, however, as to whether Echinacea is suitable for use by patients who are HIV-positive (Echinacea Monograph, 2001). Some patients with HIV/AIDS take Echinacea to treat the symptoms of colds and/or flu, however, many use Echinacea to stimulate the immune system. Potentially, stimulating the immune system by increasing the number of T lymphocytes could provide more 'target cells' for the virus to infect. It is also plausible that if the cells of the immune system are already activated in response to the HIV-infection, further activation by Echinacea could result in cell and tissue damage (AIDS Infonet, 2002).

The importance of the research presented in this Chapter and future research into the effects of Echinacea and other herbal products should be measured by the large and increasing proportion of the Australian adult population using such products. The importance of this type of research to industry cannot be underestimated given that the complementary medicines industry in Australia is estimated to be worth $800 million. Moreover, the Therapeutic Goods Administration (TGA, 2005) has

announced a requirement for the neutraceutical and complementary medicine industries to provide evidence-based support to substantiate claims of efficacy for their products. The mechanisms of actions of most complementary therapies are poorly investigated and a scientific understanding of these mechanisms are thus critical both with respect to current efficacy and quality control issues as much as for the future development and marketing of these products.

The results of this pilot study demonstrated the immunomodulatory properties of Echinacea and represented one of the very few studies involving a widely used, commercial preparation of Echinacea in tablet form. Importantly this Echinacea preparation consisted of chemically defined concentrations of alkylamides from the roots of two distinct species of Echinacea. We suggest that supplementation with Echinacea may induce an immune response through altered expression of leukocyte hsp70 and increased white cell counts. There is also some support for the protective effects of Echinacea against free radical induced damage to erythrocytes. These immunomodulatory effects may be attributable to the alkylamide constituents of Echinacea and, as such, further research investigating the effects of alkylamide extracts on hsp expression is warranted. This research should also seek to investigate the mechanisms of altered hsp70 expression by examining the response to Echinacea and its constituents by individual leukocyte subsets. A greater understanding of the mechanisms of action of Echinacea and other herbal medicines is essential given the large and increasing percentage of the population using these products as passive immunomodulators.

## 8.5 Modulation of stress protein expression by gender, menstrual cycle phase and oral contraceptives

The studies presented Chapter 7 arose as a result of an observation of possible gender differences in hsp70 expression during the studies that were presented in Chapter 4 of this Thesis.

Taken together, the results of the current studies revealed that leukocyte hsp70 expression, serum leptin, serum cortisol and serum prolactin were differentially modulated by gender, menstrual cycle phase and oral contraceptive use indicating alterations in immune response and stress response pathways. Given the intricate

links between the endocrine system, in particular the HPA axis, the cardiovascular system, immune function and stress activated pathways, the results of the current studies indicated that further studies should be conducted to investigate the connections among these systems and the mechanisms by which leukocyte hsp70 expression is modulated.

This is the first report of changes in leukocyte hsp70 expression in response to gender, menstrual cycle phase and oral contraceptive use. These results have important implications for the design and interpretation of human clinical studies investigating leukocyte hsp70 expression in females or mixed gender cohorts. The results presented in Chapter 7 of this Thesis highlighted the gender-based differences in hsp expression, however, individual differences should not be discounted when planning clinical trials. The use of each participant as their own 'control' is, therefore, strongly recommended.

Whether estrogen has direct effects on leukocyte hsp70 expression remains to be determined. The results of the current small-scale, pilot study, investigating the effects of gender, menstrual cycle phase and oral contraceptive use on hsp expression did not find a direct association between serum estradiol and leukocyte hsp70 expression. As discussed in Chapter 7, a number of researchers have reported effects of estrogen on hsp70 expression, however, these effects appear to be localized to particular tissues and cell types, suggesting that estrogen modulates hsp70 expression via differential mechanisms.

A universal problem encountered when conducting research using human cohorts is the difficulty in recruiting sufficient numbers of participants to allow for a valid interpretation of the data. In the current studies, this difficulty was compounded as the author's candidature was conducted at a small regional University without a medical faculty. The recruitment of all of the HIV-positive individuals and many of the HIV-negative control subjects, therefore, was conducted through large, metropolitan clinical settings, namely The Albion Street Centre and St Vincent's Hospital, Sydney.

In conclusion, the research discussed in this Thesis represented the first report of altered hsp70 expression in HIV-disease. As such, the publication Agnew *et al.* (2003) was a seminal work in this field. The development of a small volume (1 – 2

ml), whole blood flow cytometric assay to measure hsp expression in leukocyte sub-populations was also a key outcome of the current work. This assay is a scientifically robust technique that can be applied to investigate the mechanisms of alterations in hsp expression by examining expression in specific cell types, whereas previous methods were restricted to total leukocyte hsp expression and required large sample volumes (20 – 30 ml whole blood). This assay has the potential to lead to the identification of clinical markers for disease progression. The finding that a commercially available Echinacea preparation induced an immune response through altered expression of leukocyte hsp70 and increased white cell counts lends support to and provides a definable mechanism for the premise that Echinacea is unsuitable for use by HIV-positive individuals. The identification that leukocyte hsp70 expression, serum leptin, serum cortisol and serum prolactin were differentially modulated by gender, menstrual cycle phase and oral contraceptive use is indicative of alterations in immune response and stress response pathways. The encapsulation of the current studies, therefore, provides a platform for future studies to investigate the precise mechanisms of interactions between the immune and endocrine systems and stress activated pathways.