# Heat Shock Protein Expression and Apoptosis in Myeloid Leukaemias.

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Biological Sciences

**Abbreviations used.**

| | |
|---|---|
| ALL | Acute lymphocytic leukaemia |
| AML | Acute myeloid leukaemia |
| ATP | Adenosine triphosphate |
| BCNU | Bischloronitrosourea |
| BSA | Bovine serum albumin |
| cpn | Chaperonin |
| CHX | Cycloheximide |
| CLL | Chronic lymphocytic leukaemia |
| CML | Chronic myeloid leukaemia |
| ConA | Concanavalin A |
| DAB | 3,3-diaminobenzidine |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyrobonucleic acid |
| EDTA | Ehtylenediamine tetra-acetic acid |
| *E.coli* | *Escherichia coli* |
| FCS | Foetal calf serum |
| FITC | Fluorescein isothicyanate |
| Hsp | Heat shock protein |
| kD | Kilodalton |
| MHC | Major Histocompatibility Complex |
| mM | Millimolar |
| mL | Millilitre |
| PBMC | Peripheral blood mononuclear cells |
| PHA | Phytohaemagglutinin |
| PI | Propidium iodide |
| RNA | Ribonucleic acid |
| TBS | Tris-buffered saline |
| TNF | Tumour necrosis factor |
| TPA | 12-O-tetradecanoyl-phorbol-13-acetate |

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## Acknowledgements.

I hereby declare that all parts of the research presented herewith were carried out solely by myself in the Department of Haematology at Warwick Hospital. This work was performed under the supervision of Dr. Alan Morris from the University of Warwick and Dr. Peter Rose, Consultant Haematologist at Warwick Hospital.

I would like to take this opportunity to extend my sincere gratitude to all those, both at Warwick Hospital and Warwick University, whose help and support has proved invaluable throughout the course of this project.

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Finally, I would like to mention all my friends and colleagues in the Haematology Department who have made my working days that much more enjoyable, especially Linda without whom none of this would have been possible.


## Declaration.

No material contained in this thesis has previously been presented for another degree, although parts of my research have previously been published and a copy of these papers is bound within this volume.

Ian Chant

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# Preface.

The heat shock response was originally described as a phenomenon of inducible gene expression in *Drosophila* in response to hyperthermia, but has rapidly become recognised as a ubiquitous response by virtually all cell types to a wide variety of environmental stresses. Much of the early work on heat shock protein (hsp) structure and function concentrated on the analysis of heat shock gene expression in *Drosophila*, but it soon became clear from studies involving higher eukaryotes and prokaryotes that the heat shock response is highly conserved and a high degree of homology in the nucleic acid sequence of related heat shock genes is evident in all species from bacteria to man. Over the past decade, the study of heat shock protein expression has diversified into broad areas of biological research. The importance of heat shock proteins as molecular chaperones which mediate the folding and assembly of polypeptide chains has led to a re-examination and broadening of our understanding of the principles of protein folding and transport. In immunology, heat shock proteins have been shown to act as major antigens involved in the immune response to pathogens, and mechanisms involving heat shock proteins have been implicated in the pathogenesis of a variety of autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus.

At the conception of this research, evidence existed that expression of heat shock proteins was related to the differentiation of cells, including haemopoietic cells, and abnormal expression in some tumour cells had been reported, although not in leukaemic cells. It was against this background that the ideas for this research project were conceived. Based in the Department of Haematology at Warwick Hospital, I had access to samples from leukaemic patients, providing an opportunity to examine heat shock protein expression in malignant cells from these patients. As the project evolved, the

significance of hsp expression was addressed by studying the relationship between heat shock protein expression and apoptosis. This mode of cell death has recently been shown to be crucial in carcinogenesis. A tumour is known to develop if the balance between cell division and cell death by apoptosis is disturbed, permitting a potentially malignant clone of cells to escape elimination. In addition, most, if not all, the cytotoxic drugs used to target malignant cells are known to exert their effects via the induction of apoptosis. The expression of genes which influence the susceptibility of cells to chemotherapy-induced apoptosis may therefore have a bearing upon the efficacy of chemotherapeutic regimens. Since heat shock proteins have been shown to protect cells against apoptosis induced by a variety of stresses, their expression in leukaemic cells is particularly worthy of investigation, both in terms of leukaemogenesis and the response of leukaemic cells to chemotherapy.

This research project has therefore evolved to question the role of heat shock proteins in the biology and treatment of leukaemia and to establish their role in the control of apoptosis, with particular reference to the stress response of cells exposed to the chemotherapeutic agents used in the treatment and clinical management of these malignancies.

# Chapter 1

# Introduction

# 1.1 Biology and function of heat shock proteins.

## 1.1.1. Heat shock proteins and the heat shock response.

The term heat shock protein (hsp) is used to describe several families of proteins whose expression is induced by hyperthermia. The first description of this phenomenon, now termed the heat shock response, arose from the observation that *Drosophila* larvae raised at 25°C exhibited a novel puffing pattern on their giant salivary gland chromosomes when they were exposed to higher temperatures of 30-32°C. (Ritossa, 1962). These chromosome puffs, indicative of DNA transcription, were subsequently shown by Ritossa to be also induced in the midgut and hindgut of the larvae, indicating that the heat shock response was not tissue-specific. Furthermore, the same puffing pattern could be induced in isolated salivary glands by treatment with 2,4-dinitrophenol, sodium salicylate, sodium azide and release from anoxia. These early observations already hinted at two important features of the heat shock response; namely, the universality of the response in all cell types, and the variety of cellular stresses which will induce this pattern of specific gene expression.

For many years, all research on heat shock gene expression was performed solely using *Drosophila* larvae and it was not until the late 1970's that the ubiquity of the heat shock response was demonstrated. The same typical pattern of gene expression in response to heat shock was then shown in chicken embryo fibroblasts (Kelley and Schlesinger, 1978) and in *Escherichia Coli* (Lemaux *et al,* 1978), and similar observations were soon reported in yeast (McAlister and Finkelstein, 1980) and plants (Barnett *et al,* 1980). The heat shock response has since been observed in all organisms examined, from archaebacteria to eubacteria, yeasts, plants, invertebrates and vertebrates, including

humans. Examination of the nucleic acid sequence of heat shock genes from distant species, and protein analysis using monoclonal antibodies, has since demonstrated that the heat shock proteins are among the most abundant and highly conserved proteins in nature (Hunt and Morimoto, 1985, Bardwell and Craig, 1987, Hartl, 1996).

Although the term 'heat shock protein' is still used widely, it has become clear that a variety of other cellular insults can induce the heat shock response. Ritossa's early experiments demonstrated that 2,4-dinitrophenol, sodium salicylate and sodium azide also generated the novel puffing pattern characteristic of heat shock gene transcription. Subsequently, a large number of both environmental and pathophysiological stresses (see figure 1.1) have been shown to induce the response, including heavy metals, ethanol, amino acid analogues, fever, and viral or bacterial infection (Lindquist and Craig, 1988). For this reason, the heat shock proteins are frequently referred to as 'stress proteins'. It should be noted that many of these inducers share a capacity to cause protein denaturation, a fact which hints at the major functional role of the heat shock proteins as molecular chaperones. This function, which is vital to the cell, both under normal circumstances and following cellular stress, will be discussed in greater detail later.

In the literature, terms used to describe heat shock proteins and molecular chaperones have frequently tended to be used interchangeably. A brief description of the terminology used to describe these groups of proteins therefore follows.

**Pathophysiological Stresses:**

Oxidative stress

Inflammation

Viral/chemical transformation

Oncogenes and protooncogenes

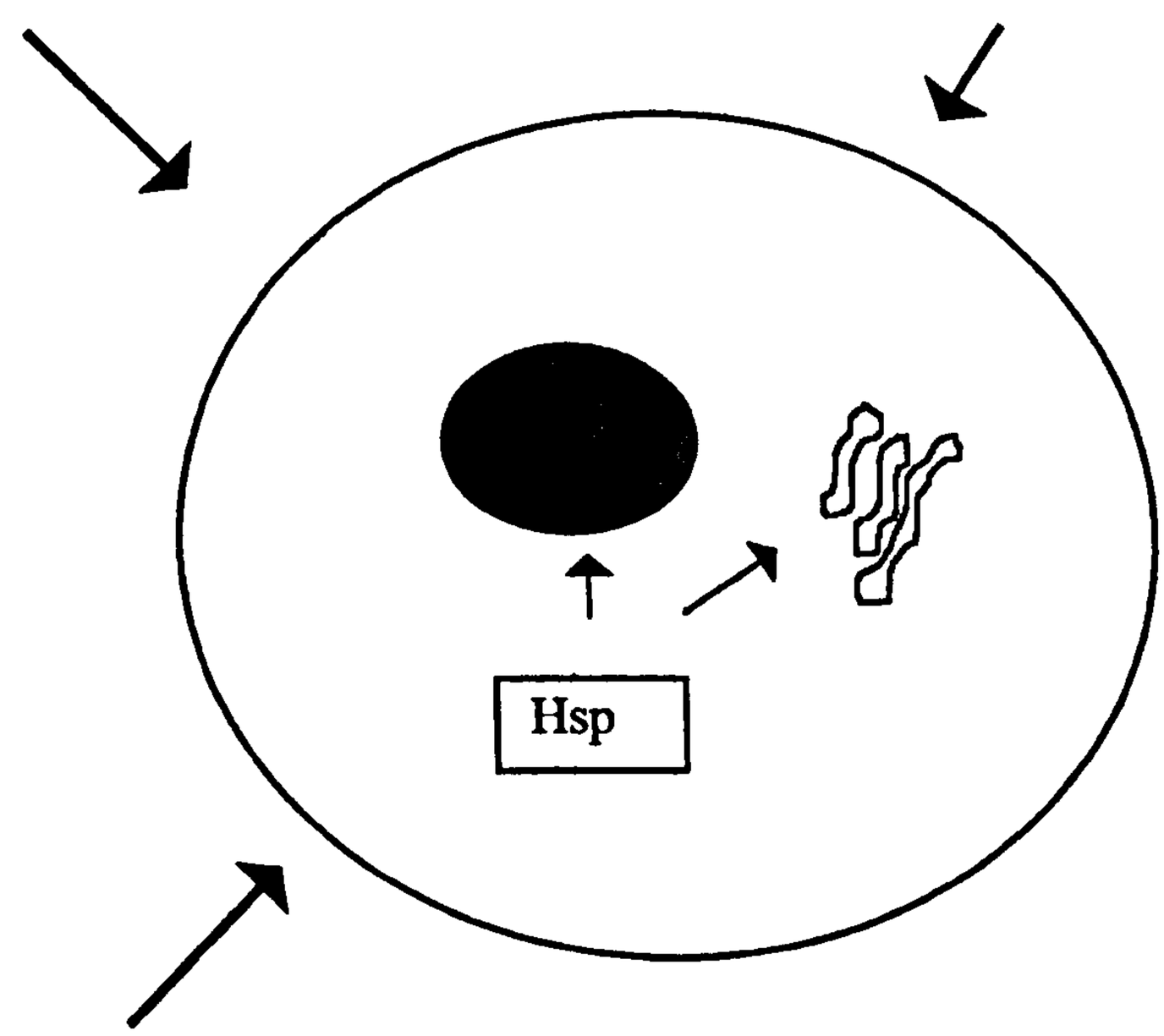
**Environmental Stresses:**

Heat shock

Amino acid analogues

Heavy metals

Ethanol




**Normal conditions:**

Cell cycle regulation

Proliferation and differentiation

Growth factors

**Figure 1.1.** The induction of heat shock proteins by a wide variety of environmental and pathophysiological stresses is a universal response common to all cell types and organisms.

## 1.1.2. Nomenclature.

Some confusion still arises in the literature regarding the nomenclature used to describe heat shock proteins, molecular chaperones and a sub-group of molecular chaperones known as chaperonins (cpn60). Heat shock proteins are also frequently referred to as 'stress proteins', a term which reflects the fact that hsp expression is induced by a wide variety of cellular insults other than hyperthermia, and is therefore perhaps a more appropriate nomenclature. Throughout this thesis, however, the prefix hsp will be used to describe these stress-inducible proteins.

The term *heat shock protein* covers an ever growing number of proteins which have in common the induction of their expression in response to a hyperthermic stress.

*Molecular chaperones* are defined as a family of unrelated classes of protein which mediate the assembly of other cellular polypeptides, but which are not components of the final functional polypeptide structure (Ellis and Hemmingson, 1989). As will be described, the ability of molecular chaperones to interact with other cellular proteins is vital to the cell, both during normal cell growth and when the cell is subjected to some form of environmental or pathophysiological insult. Many, but not all, molecular chaperones can be classified as heat shock proteins since their expression can be induced by hyperthermia and a variety of other cellular stresses. Similarly, most heat shock proteins are now known to perform a molecular chaperone function, and the importance of this function will be discussed. Finally, the term *chaperonin* is used to describe a specific sub-group of the molecular chaperones, comprising a class of sequence-related molecular chaperones which have been found in all bacteria, mitochondria and plastids so far examined (Ellis and van der Vies, 1991). One of the remarkable features of the chaperonins is the very high degree of structural and functional homology between members of this group indicating that these proteins are true evolutionary homologues. It

has been suggested that their distribution in the plastids and mitochondria of eukaryotes derives from a prokaryotic origin, providing further evidence for the evolution of eukaryotic cellular organelles via an endosymbiotic mechanism (Gupta *et al,* 1989, Gupta, 1998).

A growing number of cellular proteins whose expression is induced by hyperthermia and which have homology between species have been identified. The conservation of both structure and function between these groups has led to the classification of these hsp into four major families, and a discussion of the major features of each of these families follows.

## 1.1.3. Heat shock protein families.

Several distinct families of heat shock proteins have been identified in both eukaryotes and prokaryotes (see Table 1.1). The majority of these belong to four groups which are generally classified according to their approximate molecular weight, and although this varies slightly from organism to organism, a strong degree of homology between the amino acid sequences of proteins within these families allows easy identification of constituent members. Thus, the hsp90 family includes proteins with an apparent molecular weight of 90kD in mammals (83kD in yeast and bacteria), while the hsp70 family comprises several closely related members including the 73kD and 72kD proteins seen in mammals and the 70kD dnaK protein of *E.coli.*

The hsp60 (chaperonin 60) family comprises two subgroups: the GroEL family and the members of the TCP-1 ring complex (TRiC) family. The GroEL-type chaperonins found in eubacteria, mitochondria and chloroplasts are made up of two stacked heptameric rings of approximately 60kD, while the TRiC chaperonins in the eukaryotic cytosol are 8- or 9-membered double rings containing subunits of 55kD. Finally, the small heat shock proteins

comprise a number of related family members whose size generally ranges from 20 to 30kD in animals.

### 1.1.3.1. The hsp90 family.

This group includes cytosolic proteins with an apparent molecular weight of 90Kd in mammals and 83Kd in *Drosophila* and bacteria. Hsp90 is an abundant cytosolic protein, comprising 1-2% of total soluble protein under normal conditions and becoming even more abundant under stress. Hsp90 homologues have been found in the endoplasmic reticulum of higher eukaryotes, and have been present in all prokaryotic organisms so far examined (Parsell and Lindquist, 1993). It is only in recent years that evidence has emerged to indicate that hsp90 can act as an ATP-independent molecular chaperone, involved in protein folding and unfolding events (Jacob and Bukner, 1994, Bose *et al.,* 1996). Members of this family associate with a diverse range of cellular proteins including retroviral transforming proteins, steroid hormone receptors, cellular protein kinases, actin and tubulin (Hutchison *et al,* 1992; Dittmar,D.*et al.,* 1997), the common feature of most of these interactions being the stabilisation of the target protein in an inactive or unassembled state. For example, hsp90 is involved in both the initial folding of steroid hormone receptors and subsequent modulation of their DNA binding activities (Joab *et al.,* 1984, and reviewed by Pratt and Toft, 1997). Newly synthesised steroid hormone aporeceptors form a complex with hsp90 in which the receptor is partially unfolded and unable to bind DNA, and the subsequent binding of steroid hormones promotes dissociation of this complex allowing the receptor to bind to DNA. Only those receptors which are complexed with hsp90 are capable of binding to steroid hormones, which suggests that hsp90 maintains the receptor in a conformation which is competent for hormone binding, rather than simply inactivating the hormone-free receptors.

Another important group of proteins which interact with hsp90 are protein kinases (Parsell and Lindquist, 1993,) ). An example is the viral protein, v-Src, which forms stable complexes with hsp90 and is thus chaperoned until it becomes attached to the plasma membrane. It has been shown that the number of active, membrane-bound v-Src molecules is lower in cells which have reduced levels of hsp90, suggesting that the chaperoning activity of hsp90 is involved in the maturation of v-Src (Xu and Lindquist, 1993).

The finding that hsp90 was frequently isolated in complex with specific substrate proteins such as steroid receptors and kinases originally suggested that hsp90 is a highly specialised binding protein, but it has recently become clear that hsp90 has general molecular chaperone properties and is involved with protein folding within the cell. This property, which is common to all four major hsp families will be discussed in greater detail later.

### 1.1.3.2. The hsp70 family.

Members of this family are highly conserved ATPases, identified in both prokaryotes and most compartments of eukaryotic cells. In eukaryotes, three major members of the hsp70 system are found; a constitutively expressed protein with a molecular weight of 73Kd, and a highly heat-inducible protein of 72Kd, both of which are predominantly localised within the cytosol, and a third member, Grp78 (BiP), which is found in the lumen of the endoplasmic reticulum. Studies suggest that members of the hsp70 family function in a wide variety of processes, both under normal growth conditions and when the cell is stressed. Their role as molecular chaperones has long been appreciated and known to be important in the unfolding of cytoplasmic proteins and their translocation into mitochondria and endoplasmic reticulum (Pelham, 1986). Inside these compartments they then facilitate refolding of the transferred proteins to an active form, and also assist in the assembly of oligomeric complexes.

Mechanistic studies of hsp70 function have revealed just how adaptable the hsp70 chaperone machinery is, and an understanding of the sequence of events in the chaperone-mediated creation of a functional protein is beginning to become clear (Hartl, 1996). The adaptability of hsp70 in terms of substrate and function arises from the ability of the molecular chaperone system to function co-operatively, and hsp70 family members are known to act in concert with specific partner proteins which modulate the binding and release by hsp70 of other polypeptides (reviewed by Rassow *et al.,* 1995). For example, the hsp70 homologue in *E.coli,* DnaK, is regulated by a ~40kD chaperone (DnaJ) and a constitutively expressed 20kD protein (GrpE). These three proteins function in cohort to perform a number of specific cellular processes, which in general relate to *de novo* protein folding and maintenance of the structural integrity of existing proteins under stressful conditions. There is increasing evidence that in eukaryotic cells, similar cooperative pathways of chaperone function exist, providing a highly coordinated pathway which has evolved in order to assist nascent proteins along the folding and transport pathways.

When mammalian cells are heat shocked, levels of the inducible hsp72 protein increase and both hsp72 and hsp73 migrate from the cytosol to the nucleus. This response to stress is presumed to involve the association of hsp70s with unfolded or heat-denatured polypeptides which tend to aggregate and form insoluble complexes within the nucleus. Accordingly, most experimental studies which have examined the role of hsp in the stress response have focused on members of the hsp70 family, and in particular the heat- and stress-inducible hsp72 protein. For example, cells which are subjected to a mild heat shock acquire resistance to subsequent lethal heat stress, a phenomenon known as thermotolerance, and this state has been shown to correlate with hsp70 levels (Amin *et al,* 1995). Hsp70 has also been demonstrated to have a protective effect against a variety of other cellular stresses, both environmental and pathophysiological (see figure 1.1), and the protective role of the heat shock response will be discussed in further detail later (section 1.1.5.).

Hsp70 has been shown to interact with a variety of cellular proteins, including certain cell cycle-regulatory proteins . It appears that hsp70 may play a regulatory role in the cintrol of the cell cycle. For example, hsp70 expression varies throughout the cell cycle (Hang and Fox, 1996; He and Fox, 1997), but most interesting in terms of this project is the interaction with certain growth regulatory proteins, particularly the tumour suppressor protein, p53, and the retinoblastoma gene product, pRb (Nihei, *et al.*, 1994), The involvement of p53 and pRb in the development of tumours is well established, and it is therefore interesting that the interaction of these two tumour suppressor gene products with hsp70 has been suggested to modulate their function (Lane *et al.*, 1993; Hansen *et al.*, 1996). In addition, hsp70 can protect cells against the cytotoxic effects of tumour necrosis factor (Jaattela *et al.*, 1992) and from monocyte-mediated cytotoxicity (Jaattela and Wissing, 1993). These observations suggest that overexpression of this hsp could allow pre-cancerous cells to elude the normal mechanisms of immune surveillance, and the potential involvement of hsp70 in the development of malignancies will be discussed in greater detail later in this introduction.

### 1.1.3.3. The hsp60 (chaperonin) family.

The chaperonin family of hsp comprise two major subgroups: the GroEL (hsp60) family, and members of the TCP-1 ring complex (TRiC) family. Both have an essential function in promoting the ATP-dependent folding of proteins during normal growth and following cellular stress. (reviewed by Hartl, 1996). Members of the GroEL family are found in 1) eubacteria, where they have been shown to promote phage assembly, 2) mammalian mitochondria, where their major function is to promote the folding and assembly of imported proteins and the binding of heat-denatured mitochondrial proteins, and 3) in plant chloroplasts where they promote assembly and folding of imported proteins, e.g rubisco. The TRiC

chaperonins are found in archaebacteria and the eukaryotic cytosol, and although fewer studies on the function of this subgroup have been reported, the available data on the structure of these proteins suggests that they are also important in providing a folding compartment for polypeptide assembly.

### 1.1.3.4. The Small heat shock Proteins.

The family of small hsp encompasses a number of related species, with plant cells expressing about 20 different small hsp in the cytosol and chloroplasts, while yeast and mammalian cells have only one representative (Arrigo and Landry, 1994). Although the overall homology between different small hsp is rather low, they are grouped together according to the presence of conserved regions within the carboxy- terminus region of the protein, and by the similarities in their monomeric size. Despite their low molecular weights, the small hsp exist *in vivo* as higher ordered structures of 600-900 kDa (Waters *et al.*, 1996). The formation of these large oligomeric structures is a dynamic process, dictated by the physiology of the cells, whether they are subjected to stress, and by the phosphorylation status of these proteins (Arrigo, 1998).

In common with other hsp, small heat shock proteins perform a molecular chaperone function and can selectively recognise and bind non-native proteins, as well as promoting functional folding of polypeptides in an ATP-independent manner. Members of this family have also been implicated in a wide variety of different cellular processes, including RNA stabilisation (Nover *et al.*, 1989), growth and differentiation (Spector *et al.*, 1993), interaction with the cytoskeleton (Nicholl and Quinlan, 1994) and an association with non-native (unfolded or damaged) proteins following heat shock (Ehrnsperger *et al.*, 1997).

The functions listed above suggest an important role for the small heat shock proteins in the cellular response to stress. Indeed, during the last few years it has become clear that expression of the small hsp protects cells against various stresses, including heat shock and cytotoxic drugs (see Introduction, section 1.1.7), and the importance of the latter observation has been particularly highlighted by the finding that small hsp levels are a prognostic indicator in certain solid tumours, including breast cancer (Fuqua et al., 1994).

| Hsp family | Members | Function/localisation |
|---|---|---|
| **Hsp90** | Hsp90α<br>Hsp90ß | Most abundant constitutive hsp. Hsp90α more inducible than hsp90ß. Cytoplasmic anchor for target proteins. Maintains steroid receptors and kinases inactive until appropriate. Other functions in cell cycle regulation, development, immune function. Binds ATP, no known ATPase activity. |
| | GRP94 | Similar functions to hsp90, within Golgi and at plasma membranes. |
| | Hsp110 | Association with rRNA upon heat shock within nucleolus. |
| **Hsp70** | Hsp72<br>Hsp73 | Hsp72 largely inducible, hsp73 constitutive. Both located in cytoplasm and nucleus. Protein binding, transport, folding, assembly and turnover. Cell cycle regulation. Binds ATP, strong ATPase activity. |
| | grp78 (BiP) | Abundant protein, found in ER lumen. Complexes with unassembled secretory proteins. |
| **Hsp60**<br>**(cpn60)** | GroEL<br>homologue | Mitochondria. Present as large oligomers. Assembly of macromolecular protein structures. Cellular respiration. Binds ATP, weak ATPase activity. |
| | TRiC family | Eukaryotic cytosol. Promotes folding of actin and tubulin |
| **Small hsp** | Hsp28 | Large oligomer in peri-nuclear cytoplasm and Golgi. Embryogenesis, development, proliferation, cell transformation. |
| | Ubiquitin | Cytoplasm/nucleus. Facilitates protein degradation by ATP-dependent conjugation. Association with histone H2A - possible role in gene regulation. |

**Table 1.1**. Major mammalian heat shock protein families are classified primarily in terms of their approximate molecular weight in kilodaltons. Members of different families assume essential functions within individual compartments of the cell to provide a molecular chaperone machinery for other cellular proteins.

## 1.1.4. Heat shock proteins as molecular chaperones.

Although originally noted for the induction of their expression during conditions of cellular stress, the majority of heat shock proteins are also synthesised constitutively, and it is now realised that they have numerous cellular functions which are not confined to protection against stresses such as heat shock. Most heat shock proteins function as molecular chaperones, a term originally used to define a group of unrelated classes of proteins which mediate the correct assembly of other cellular proteins (Ellis and Hemmingson, 1989; Ellis, 1993). It is now clear that molecular chaperones play a varied and complex role in facilitating the fate of other proteins within the cell. The controlled binding and release of proteins by molecular chaperones is important, for example, during oligomeric assembly and in transport to and from subcellular compartments (reviewed by Hartl, 1996). Molecular chaperones also influence clathrin lattice dynamics, viral replication and transcriptional actvation, and they have been shown to have an important role in the maintenance and organisation of the cytoskeleton (Liang and McRae, 1997). Under conditions of stress, by extension of their role as mediators of protein folding/unfolding, some molecular chaperones prevent the denaturation of proteins while others act by dissociating protein aggregates and refolding the monomers thus derived. It has also been shown that molecular chaperones have a role in the disposal of denatured proteins by proteolytic degradation (Hayes and Dice, 1996).

The term molecular chaperone was first used to describe the function of nucleoplasmin, a nuclear protein which promotes the assembly of nucleosomes from individual histones and DNA (Laskey *et al,* 1978). The molecular chaperone concept was then further developed as a result of studies at Warwick University on the biogenesis of the plant enzyme, ribulose-bisphosphate carboxylase (rubisco), which is involved in the photosynthetic pathway of carbon dioxide fixation. The rubisco holoenzyme comprises

eight large subunits which are synthesised in the chloroplasts and eight smaller subunits which are imported into the chloroplasts following synthesis in the cytosol. Before assembly of the holoenzyme in the chloroplasts, rubisco subunits form a complex with a large binding protein from which they subsequently dissociate in an ATP-dependent reaction (Barraclough and Ellis, 1980). This binding protein was subsequently identified as the chloroplast homologue of bacterial GroEL and the mitochondrial hsp60 (Hemmingsen *et al.*, 1988), leading to the hypothesis that interaction between non-native rubisco and chloroplast cpn60 is required for assembly of the functional enzyme. As it became clear that molecular chaperones were involved in the folding of a variety of proteins, the term 'molecular chaperone' was introduced to describe how these proteins assist non-native polypeptide structures through the rigours of folding and oligomeric assembly, and in transport to and from subcellular compartments.

Under conditions of normal cellular growth, the many vital functions which are served by molecular chaperones derive from their ability to recognise and modulate the state of folding of other cellular proteins (reviewed by Ellis and van der Vies, 1991, Kelley and Georgopoulos, 1992, Hartl *et al*, 1994, Hartl, 1996). In the cell, nascent polypeptide chains emerge from ribosomes as unfolded linear chains and a comparable situation exists when polypeptide chains are transported across intracellular membranes in an extended state. In such an extended or partially folded state, hydrophobic surfaces become exposed, raising the possibility of aberrant inter- or intramolecular interactions and the formation of polypeptide aggregates. The major role of molecular chaperones appears to be the prevention of these incorrect intermolecular associations between unfolded polypeptide chains, thereby preventing their aggregation and assisting in the maintenance of polypeptide chains in a translocation-competent state during transport across intracellular membranes.

In terms of assisting protein folding, two distinct mechanisms of chaperone action have evolved to ensure the correct folding of polypeptide chains. Firstly, exposed hydrophobic surfaces are shielded by interaction with a molecular chaperone in order to prevent aggregation, particularly during synthesis or membrane translocation when productive folding is not yet possible. Secondly, molecular chaperones will sequester a completed but as yet unfolded protein, protecting it from the cellular environment whilst allowing folding to the native state to proceed. These two molecular chaperone functions are generally carried out by members of the hsp70 and hsp60 families respectively, in a sequential pathway of chaperoned protein folding (reviewed by Hartl *et al,* 1994). For example, in the synthesis and import of mitochondrial proteins, the first step involves cytosolic hsp70 interacting with nascent polypeptide chains as they are synthesised on ribosomes. In a translocation-competent state, the hsp70-polypeptide complex crosses the mitochondrial membranes where the still extended polypeptide chain interacts with mitochondrial hsp70 in the matrix compartment. Once in their correct cellular environment, chaperone-mediated folding and/or oligomeric assembly then occurs to give rise to the native protein structure.

A full discussion of the complex mechanisms whereby protein folding is achieved by molecular chaperones and their accessory proteins is beyond the scope of this introduction. However, the point to be made is that hsp, as molecular chaperones, are capable of interacting with a variety of proteins, including many growth regulatory proteins, and this has consequences which may impinge upon normal growth and cellular differentiation.

## 1.1.5. Heat shock protein expression during stress.

First noted for the induction of their expression following heat shock, the hsp were generally accepted as playing a role in the protection of cells against hyperthermia and those other stresses which were known to result in elevated hsp expression. Although the mechanisms by which hsp alleviate the cytotoxic effects of such a variety of stresses were not, and are still not, fully understood, it follows that elevated hsp expression during stress is a response to an increased requirement for molecular chaperone function in order to deal with the increased number of abnormally folded or damaged proteins which would arise following such cellular perturbations. The best studied aspect of the protective nature of hsp is, unsurprisingly, their role in the development of thermotolerance

### 1.1.5.1. Heat shock protein expression and thermotolerance.

Pre-exposure of cells to heat or other types of stress (e.g sodium arsenite, ethanol, $H_2O_2$) can lead to the acquisition of a transient resistance against the cytotoxic effects of a subsequent thermal stress (reviewed by Kampinga, 1993). This phenomenon, known as thermotolerance, involves a reduced susceptibility for protein denaturation and aggregation to occur. Although the mechanisms involved are not completely understood, it has been demonstrated that hsp clearly play a role in this thermal resistance. In a variety of systems, it has been shown that a positive correlation exists between levels of hsp70 and thermotolerance following heat shock (e.g Li *et al.*, 1982; Landry *et al*, 1989). Further evidence provided by studies using overexpression of specific hsp genes in leukaemic cell lines has revealed that hsp70 and hsp25 are particularly important in conferring resistance to thermal stress (Li *et al.*, 1991, Wissing and Jaattela, 1998). The reciprocal approach, using micro-injection of anti-hsp70 antibodies and antisense cDNA

to inhibit hsp70 expression has also demonstrated the role of this protein in thermal resistance (Johnston *et al.*, 1988; Riabowol *et al.*, 1988).

As mentioned, the exact mechanisms involved in hsp-mediated thermotolerance are unknown, although it can be speculated that the ability of hsp to interact with unfolded protein structures plays a part. Protein denaturation and the formation of protein aggregates are known to be the main processes involved in thermal cytotoxicity, implying that the protective effect of hsp derives from their ability as molecular chaperones to prevent this occurring. In support of this, *in vitro* experiments have shown that DnaK, the hsp70 homologue in *E.coli,* has the capacity to solubilise the aggregates of certain heat-denatured proteins. *In vivo*, it is therefore likely that stress-induced unfolding is associated with chaperone binding to proteins, preventing aggregation taking place. Other hsp-mediated mechanisms may also be important. For example, it has recently been suggested that a role for hsp in the recovery of cells from hyperthermia involves the improved ability of cells to re-establish the heat-induced shut-off of RNA and protein synthesis (Carper *et al.*, 1997), while other reports have hypothesised that protection of the actin microfilament network by hsp is a crucial factor in thermal resistance (Huot *et al.*, 1996).

The value of describing the thermotolerant cell in relation to aspects arising within this study is that the mechanism(s) involved in the development of a thermotolerant state probably share a common theme with those which are involved in the protective nature of hsp against a variety of environmental stresses. In particular, the potential role of hsp in protecting leukaemic cells against the cytotoxic effects of chemotherapeutic agents may occur in a similar fashion. It is relevant that the action of certain cytotoxic drugs (e.g the alkylating agents) results in the appearance of abnormal or damaged cellular proteins and thus one could envisage that hsp overexpression could be an adaptive

response of tumour cells to drug exposure thus leading to the acquisition of a drug resistant phenotype.

**1.1.5.2. Heat shock proteins protect cells from certain pathophysiological stresses.**

Expression of hsp, particularly members of the hsp70 family, has been shown to influence the susceptibility of cells to several stresses which may be of pathophysiological importance. For example, overexpression of hsp70 protects cells against monocyte-mediated cytotoxicity (Jaattela and Wissing, 1993), and the cytotoxic effects of tumour necrosis factor are inhibited by overexpression of hsp72 (Jaattela *et al.*, 1992). These two observations suggest a mechanism via which tumour cells could escape the normal immune surveillance mechanisms. This is particularly relevant since hsp expression has been reported as abnormal in a variety of tumour cells, and in recent years a number of studies have focussed, as this project has, on the relationship between hsp levels in tumour cells and clinical parameters such as drug sensitivity. A review of the published data relating to abnormal hsp expression in tumour cells appears later in this discussion (section 1.1.8).

**1.1.5.3. How do heat shock proteins prevent stress-induced cell damage?**

As has been mentioned, many of the cellular perturbations which lead to the induction of hsp expression have in common the effect of protein denaturation, causing the accumulation of abnormally folded proteins. For example, agents such as amino acid analogues will affect the folding of nascent proteins, while other stresses such as heat shock and alkylating agents will interfere with the folding of both nascent and mature protein structures. Such proteins will obviously become targets for molecular chaperone interaction via binding to exposed polypeptide surfaces (Beckmann *et al.*, 1992). The importance of this function under conditions of stress is illustrated by the fact that some

denatured or aggregated proteins can be repaired or restored back to their native conformation by the action of hsp60 or hsp70 (Martin *et al,* 1992).

On the other hand, certain pathophysiological stresses, such as hypoxia, the cytopathic effects of TNFα, and immune-mediated cytotoxicity, do not directly give rise to protein denaturation, but they may indirectly affect protein structure. All of these perturbants involve the intracellular generation of reactive oxygen species (ROS), with the potential result of protein oxidation. The finding that hsp overexpression enhances cellular resistance to oxidising agents such as hydrogen peroxide (Huot *et al.,* 1996) has led to the hypothesis that the hsp protect against pathophysiological stresses such as TNF by inhibiting the action of ROS (reviewed by Arrigo, 1998). Thus, the unifying theme of hsp protective action appears to derive from their molecular chaperone function permitting the repair of abnormal proteins and the accelerated recovery from the shutdown of normal RNA and protein synthesis. In addition, hsp are also involved in the elimination of proteins which are beyond repair, and a brief discussion of their role in protein degradation follows.

**1.1.5.4. The role of heat shock proteins in protein degradation.**

Molecular chaperones also stimulate the breakdown of proteins, and there is evidence to suggest that the function of chaperones in preventing or reversing protein aggregation is linked with their role in presenting misfolded polypeptides to the cellular mechanism for proteolytic degradation (reviewed by Hayes and Dice, 1996). This role is obviously important in cells following stress in order to prevent the formation of large aggregates of abnormal proteins which are beyond repair. However, it is equally important under normal conditions of growth that a mechanism for removing unwanted, short lived proteins exists. In mammalian cells, several pathways exist for the proteolytic degradation of proteins and these have been shown to be induced by cellular stress. One

of the best known examples is the ubiquitin pathway in which proteins are targeted for proteasomal degradation by ubiquitin 'tagging' (Ciechanover, 1994). Ubiquitin, itself a heat shock protein, requires several accessory proteins (these too are induced by heat shock) in order to effect the degradation of damaged proteins. The involvement of other hsp in this pathway is suggested by the observation that hsp70 promotes ubiquitin conjugation to abnormal proteins in reticulocyte extracts (Hayes and Dice, 1996). A second important pathway of protein degradation involves lysosomal proteolysis and it has been shown that hsp70 is required for the targetting of certain cytosolic protein to this site of destruction (Dice *et al.*, 1994).

Thus, as well as preventing protein aggregation, hsp play a more specific role in the breakdown of proteins via their ability to physically interact with damaged proteins and assist in their targeting towards the proteolytic machinery of the cell. A working model for hsp function during conditions of stress such as heat shock or exposure to cellular toxins therefore would be that when they fail in their functions of protein folding or disassembly, by acting as molecular chaperones they facilitate protein degradation.

## 1.1.6. Regulation of heat shock proteins during cell growth and differentiation.

One of the major reasons for the decision to examine hsp expression in leukaemic cells during the planning of this research project was the increasing volume of evidence demonstrating that hsp expression was linked to the growth and differentiation of cells. The differentiation of haemopoietic cells is a fundamental cellular programme which, in conjunction with cell growth, plays a critical role in the control of haemopoietic cell numbers. Although relatively little is known about the mechanisms regulating the differentiation process, its importance is highlighted by the fact that dysregulation of differentiation pathways is known to contribute to leukaemogenesis. It is therefore interesting that over recent years a relatively large volume of reports have been published which suggest that hsp expression is intimately linked to cell growth and differentiation, both in haemopoietic cell lines and a variety of other systems. This association cell growth was first suggested a decade ago by the observation that hsp70 and hsp90 are induced in lymphocytes by mitogenic stimuli (Ferris *et al.*,1988; Haire *et al.*, 1988), and a report from the same year found that heat shock, or agents known to induce the heat shock response, resulted in the differentiation of HL-60 cells (Richards *et al.*, 1988). Since that time, a variety of studies using myeloid leukaemia cell lines have demonstrated that the expression of all four major hsp families is differentially regulated during the process of differentiation.

Developmental studies have revealed that the expression of specific hsp genes is co-ordinately regulated during early embryogenesis and many differentiation processes (Walsh *et al.*, 1993; Shakoori *et al.*, 1992), and it has been shown that induction of hsp by heat shock, or chemical agents which induce hsp synthesis, results in the differentiation of cultured embryonal cells (Stoklosinski *et al.*, 1994). Furthermore the

importance of hsp expression in development has been demonstrated by the report that inhibition of hsp28 by anti-sense technology was found to abort the differentiation of murine embryonic stem cells via the induction of apoptosis (Mehlen et al., 1997).

In haemopoietic systems, studies of myeloid differentiation have shown that expression of hsp70 is induced by a variety of agents which cause differentiation of myeloid cell lines, including 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in both HL-60 (promyelocytic) and U-937 (monoblastoid) cells (Shakoori *et al.*, 1992; Twomey *et al.*, 1993), and levels of hsp70 and hsp28 showed transient increases following exposure of the same two cell lines to sodium butyrate, another agent which induces myeloid maturation (Garcia-Bermejo *et al.*, 1995). Retinoic acid-induced differentiation of HL-60 cells is accompanied by increasing levels of hsp28 protein, but a progressive reduction in mRNA levels, suggesting that the changes in protein levels are mediated post-transcriptionally (Spector *et al.*, 1995). The mechanism involved in the post-transcriptional modification of hsp28 probably involves phosporylation of this small hsp, as suggested by the observation that differentiation of HL-60 cells towards a macrophage-like phenotype is associated with increased phosphorylation and levels of hsp28 (Minowada and Welch, 1995).

Expression of hsp90 has also come into the picture with the finding that this stress protein plays a role in cell cycle control and differentiation of the monoblastoid cell line, U937 (Galea-Lauri *et al.*, 1996a).

It is not clear how hsp expression influences development and differentiation. Their expression may be a primary regulatory mechanism or it may reflect the phenotype of the cell in terms of its differentiation state. However, part of the explanation could be the ability of hsp to bind to growth factors such as p53 and pRb, as well as other cellular proto-oncogenes such as c-myc and $pp60^{src}$ which are involved in the control of cellular differentiation and proliferation.

## 1.1.7. Cell cycle-dependent expression of hsp,

A number of reports have been published describing changes in the levels of hsp and their localisation during progression through the cell cycle in a variety of cultured cell lines, but no clear picture of hsp expression in relation to the cell cycle is apparent. Patterns of hsp70 expression throughout the cell cycle have generally been examined using established mammalian cell lines and the results do not present a consistent picture, possibly due to the different cell lines used in these studies. Some authors have reported that in normal cells (such as mitogen-stimulated lymphocytes), hsp expression is cell cycle-regulated, wheras in tumour cells this is lost (e.g Ferrarini *et al.*, 1993), suggesting deregulation of hsp may be involved in the malignant phenotype. A previous study by Milarski *et al.*, (1989) found that hsp70 expression was cell cycle-regulated, with the subcellular localisation of hsp70 varying throughout the cell cycle. Co-precipitation experiments using nondenaturing immunoprecipitations demonstrated that these changes in the localisation of hsp70 were related to the formation of complexes between hsp70 and other unidentified proteins. It was noted that hsp70 associated with some proteins only at specific points in the cell cycle, whilst others could be co-precipitated throughout all phases of the cell cycle. These cell cycle-specific interactions were short lived, suggesting that hsp70 is involved in dynamic associations with specific cellular proteins throughout the growth cycle under normal circumstances. At the time of writng, the identity of these proteins which are transiently associated with hsp70 remains largely unknown, although it would seem likely that these interactions involve growth-related factors which are expressed at specific points in the cell cycle. The identification of the proteins whose expression and/or transport may be modulated by hsp70 remains an interesting enigma whose solution would shed light on the role of hsp in the control of cell growth.

By contrast, a study examining several different cell lines, including HeLa, CHO, and normal human skin cells, found that under normal conditions levels of hsp70 were not regulated through the cell cycle. However, following heat shock, substantial variations in both the inducible and constitutive forms of hsp70 became apparent, although this cell cycle-specific regulation varied for different cell lines (Hang and Fox, 1996; He and Fox, 1997). In a more recent report, using a sensitive PCR-based approach to analyse cDNA libraries prepared from cells in distinct phases of the cell cycle, hsp70 gene expression was shown to be linked with the S to $G_2$/M phase transition of the cell cycle, in this case under normal growth conditions in the absence of stress (Dittmar *et al.*, 1997). Developmental studies on embryonic cells have also shown that hsp gene expression is tightly regulated throughout the cell cycle. For example, Hsp90 expression is enhanced at the Go, and hsp70 increased at the G2/M phases of the cell cycle in neuroectodermal cells (Walsh *et al,* 1993). Furthermore, heat shock of these cells resulted in acquired thermo-tolerance whereby cell progression was delayed at the G1/S and S/G2 boundaries of the cell cycle, leading the authors of this report to suggest that hsp act as cell cycle regulators during neuroectoderm induction and differentiation.

## 1.1.8. Abnormal hsp expression in tumour cells.

Although there is no direct evidence linking hsp expression with tumourigenesis, the abnormal expression of hsp is likely to have detrimental effects on a variety of processes involved with the control of cell growth and differentiation. As outlined earlier (see section 1.1.7.), levels of most major hsp are intimately linked to these processes and it is therefore interesting to find that abnormal expression of hsp has been reported in some tumour cell types. For example, increased levels of hsp have been demonstrated in virus-transformed and chemically-induced tumour cells (La Thangue and Latchman, 1988;

Finlay *et al.*, 1988). It is difficult to be certain whether this represents a chronic stress response to abnormal proteins expressed in viral or chemically transformed cells, or simply that elevated hsp expression reflects an actively growing malignant pheotype. Ferrarini *et al.* (1992) demonstrated elevated expression of hsp90 in a range of tumour cell lines as well as high levels of the inducible hsp70 (hsp72) in some cell lines and some fresh tumour cells (lung and kidney carcinomas). Moreover, those cell lines which constitutively expressed hsp72 did not show increased synthesis of this heat-inducible protein following heat shock, suggesting that hsp72 expression was deregulated in these cells.

More recent reports have identified abnormal expression in primary tumour tissues, in particlar hsp28 which has been suggested to have prognostic relevance in some of the hormone-responsive tumours such as breast cancer and cervical cancer. In breast cancer, hsp90 and hsp70 mRNA is expressed at much higher levels in cancerous tissue than in non-cancerous tissue, and a close positive correlation between hsp expression and the proliferating cell nuclear antigen labeling index has been observed (Yano *et al.*, 1996). In endometrial carcinomas, hsp28 correlates with the degree of tumour differentiation, as well as the presence of oestrogen and progesterone receptors, whilst hsp28 is predominantly expressed in well-differentiated squamous cell carcinomas in patients with cervical cancer (reviewed by Ciocca et al., 1993).

Despite the connection between abnormal levels of hsp in tumour cells, the significance of elevated hsp expression is unclear. It is impossible to be certain whether deregulated or high levels of hsp expression contribute to tumourigenesis, or whether they reflect the malignant cell phenotype as a consequence of the differentiation state or abnormal growth of these cells.

## 1.1.9. Abnormal localisation of hsp to the cell surface in some tumour cells.

In addition to reports of abnormal levels of hsp expression in tumour cells, a growing volume of evidence suggests that many tumour cells differ from normal cells in that they express hsp on the cell surface. Under normal growth conditions, most hsp are located within the cytoplasm where their specific localisation is dictated by their individual function as molecular chaperones. For example, hsp70 family members are found in the cytosol, endoplasmic reticulum (ER) and mitochondria, where they perform biochemically similar functions in all three compartments but have access to distinct sets of protein substrates. Hsp70 members in the mitochondria and ER are involved with the folding of newly translocated proteins as well as the translocation process itself. Similarly, members of the hsp90, hsp60 and small hsp families are found within subcellular compartments where they perform individual functions as part of the larger whole molecular chaperone machinery.
At the outset of this work, cell surface localisation of hsp was controversial. Some authors had found no evidence of cell surface expression (Ferm *et al.*, 1989) whereas others had reported hsp expression on the surface of certain cell types. For example, human γ/δ T cells express a cell surface antigen which is recognised by a monoclonal antibody to hsp58 (P1), the human homologue of the *Escherichia coli* groEL protein (Jarjour *et al.*, 1990). Also, murine bone marrow-derived macrophages have been shown to express an epitope which is recognised by a monoclonal antibody, ML30, which has specificity for mycobacterial hsp60 (Wand-Württenberger *et al.*, 1991). Surface expression of hsp72, the inducible form of hsp70, was also reported on some tumour cell lines and fresh tumour cells (Ferrarini *et al.*, 1992), although the significance of this remained unclear. More recently, several reports have appeared which confirm the presence of hsp on the surface of certain cells, and a picture has emerged which suggests that they play an

important role as immunogenic determinants, particularly in stressed or transformed cells. Heat shock has been shown to induce cell surface expression of the inducible hsp72 on tumour cells, including Ewing's sarcoma and osteosarcoma cells, but not on normal peripheral blood lymphocytes or fibroblasts (Multhoff *et al.*, 1995). These authors suggested that the restricted stress-induced surface expression of hsp72 could be used as a means to target a tumour-specific immune response. Indeed, it has been recently reported by the same authors that the expression of hsp72 on the surface of tumour cells is a recognition structure for natural killer cells, and that the sensitivity of cells to lysis by natural killer cells correlates with the amount of hsp72 that is expressed on the cell surface (Multhoff *et al.*, 1997).

The ability of hsp to act as immunogenic targets probably derives from the involvement of hsp with intracellular antigen processing and the presentation of cell membrane-anchored antigens (Srivastava *et al.*, 1994; Li and Srivastava, 1994). By acting as molecular chaperones, hsp may mediate peptide transfer during presentation by MHC class I-restricted T cells. Heat shock proteins are associated with a broad spectrum of peptides, and it has been suggested that hsp-peptide complexes can be used as the basis for cancer immunotherapy (Srivastava and Udono, 1994). Recently, the potential clinical application of this observation has been demonstrated by the finding that, in an *in vivo* murine model, immunotherapy of mice by vaccination with hsp preparations derived from autologous tumour cells resulted in reduced tumour growth and prolonged survival (Tamura et al., 1997).

## 1.2. Tumour suppressor genes and their implications for leukaemogenesis.

### 1.2.1. The role of p53 in haematological malignancies.

Considerable attention has been focused on the tumour suppressor gene, p53, because mutations of this gene, located on chromosome 17, represent the most common genetic abnormality found in human cancer to date (Nigro *et al,* 1989, Hollstein *et al,* 1991). Although the precise molecular mechanisms of p53 function are unknown, it appears that p53 plays an important role in growth control, particularly during periods of cellular stress, and a model for p53 function has arisen whereby normal p53 acts as a monitor of genome integrity (Lane,1992). The exposure of cells to DNA-damaging agents induces a dramatic increase in the levels of p53 protein which results in cell cycle arrest and suppression of cell proliferation (Michalovitz *et al,* 1990, Kastan *et al,* 1991). Overexpression of wild-type p53 has also been shown to induce apoptosis in haemopoietic cells (Yonish-Rouach *et al,* 1991). Thus, p53 would appear to function as a cell cycle checkpoint control for DNA damage, halting the cell cycle to allow repair mechanisms to correct any genetic alterations. However, if repair fails, p53 may alternatively trigger apoptosis, thereby removing a potentially malignant cell. This model implies that loss of p53 function would permit escape of genetically damaged and potentially malignant cells from the normal mechanisms of surveillance and thereby be an important mechanism in carcinogenesis (Figure 1.2). Consistent with this hypothesis is the observation that loss of wild-type p53 expression is associated with a high frequency of gene amplification and genomic rearrangements (Livingstone *et al*, 1992, Yin *et al,* 1992).

Indeed, inactivation of the tumour suppressor function of normal p53 protein has been shown to be a critical event in the induction of malignant transformation. Inactivation at the DNA level most commonly occurs via point mutations of the p53 gene, but may occur by several other molecular mechanisms, including gene rearrangements, viral insertions and gene deletions, all of which lead to absence of expression or production of inactive mutant p53 protein. At the protein level, p53 function can be inactivated by binding to viral proteins such as the SV40 T antigen (Lane and Crawford,1979). In SV40-transformed cells, the p53 protein is normal but the tumour suppressor activity is lost because of complex formation with the viral large T antigen. Similarly, wild-type p53 has been shown to form complexes with other viral proteins such as the E1b of the adenovirus (Sarnow *et al,* 1982) and the EBNA antigen of the Epstein Barr virus (Szekely *et al,* 1993) leading to loss of function, whilst binding to E6 proteins from papillomaviruses has been shown to promote the degradation of p53 protein (Werness *et al,* 1990).

In haematological malignancies, alterations in p53 expression have been detected in a variety of disease states (reviewed by Imamura *et al,* 1994, Prokocimer and Rotter, 1994), and p53 mutations are often associated with disease progression. For example, in chronic myeloid leukaemia (CML), p53 alterations are rare during the chronic phase of the disease, but progression to CML with blast crisis is associated with the detection of p53 gene abnormalities in 20-30 % of cases ( Foti *et al,* 1991). Also, in the lymphoproliferative disorders, chronic lymphocytic leukaemia (CLL) and follicular lymphoma, p53 alterations are often acquired during the progression to a more malignant phenotype (Gaidano *et al,* 1991, Ichikawa *et al,* 1992). In cases of acute myeloid leukaemia (AML), p53 genotypic alterations are less common, the reported mutational frequency being about 6-15 % (Slingerland *et al,* 1991, Fenaux *et al,* 1992). However, despite the relative infrequency of p53 mutations, it has been shown that loss of tumour

suppressor function does occur in AML by mechanisms which act at the protein level. In a study of p53 expression in 49 AML patients, Zhang and Deisseroth (1992b) demonstrated that leukaemic cells from the majority (37/49) of these patients had elevated levels of p53 when measured by immunoprecipitation using monoclonal antibodies recognising both normal and mutant forms of p53. Furthermore, an antibody specifically recognising a mutant conformation of p53, normally associated with point mutations, immunoprecipitated the p53 protein from 32 of those 37 expressing high levels of p53. This was despite the finding that point mutations could be detected in only 3 patients by single-stranded conformation polymorphism (SSCP) analysis. It was later demonstrated by the same group that wild-type p53 protein in AML cells switches to a mutant conformation in response to growth factor stimulation, leading to the suggestion that conformational changes in normal p53 protein represent an important mechanism for the regulation of cell proliferation (Zhang and Deisseroth, 1994). Further evidence to support this theory comes from the observation that different conformations of p53 protein in AML cells relate to the *in vitro* growth characteristics of the cells, and this can be regulated by either exogenous or autocrine haemopoietic growth factors (Zhu *et al*, 1993).

In AML cells, as in many other tumour types, it is likely that the biochemical properties of either wild-type or mutant p53 are determined by the cellular environment and in particular by their interaction with other cellular proteins which influence the conformation of the p53 protein (Montenarh, 1992). It is clear that normal p53 protein can switch conformation from a normal wild-type which has tumour suppressor function, to an immunologically 'mutant' conformation which has tumour promoter properties. In light of the molecular chaperone function of certain heat shock proteins, it is therefore intriguing to find that p53 protein is frequently found complexed to members of the

hsp70 family, and there is evidence to suggest that this interaction with hsp70 has a role in the regulation of p53 conformation and therefore its activity.

### 1.2.2. Heat shock proteins interact with p53 protein and modulate its tumour suppressor function.

Evidence that members of the hsp70 family bind to p53 initially came from observations that various transformed cell lines expressing elevated levels of p53 co-immunoprecipitated a protein with an approximate molecular weight of 70kDa when probed with antibody to p53 (Pinashi-Kimhi, *et al,* 1986, Hinds *et al,* 1987). It was subsequently demonstrated that the formation of oligomeric complexes with the constitutively expressed hsp70 (hsc70) produced a mutant p53 protein which was more efficient at transforming a rat fibroblast cell line with the *ras* oncogene than the parent p53 (Finlay *et al,* 1988). The authors also showed that complex formation with hsc70 increased the half-life of p53, and therefore increased the concentration of p53, which they suggested may play a role in the enhanced ability of this complexed p53 to transform these cells.

As outlined earlier, p53 is capable of existing in a wild-type conformation and a 'mutant' as determined by immunochemical analysis using antibodies which react exclusively with the two different conformational forms of the protein (Bartek *et al,* 1990, Gannon *et al,* 1990). It has been demonstrated that hsp70 is involved in the switch between these two conformational phenotypes (Hainaut and Milner, 1992). These authors used a mutant murine p53 allele which is temperature sensitive in terms of p53 conformation; when expressed *in vitro* - at 30°C, the expressed p53 exists in the wild-type phenotype, while

at 37°C the mutant phenotype is adopted. Using a rabbit reticulocyte lysate *in vitro* translation system, they found that the formation of p53-hsp70 complexes took place after post-translational switching from the wild-type to mutant phenotype, a process which was ATP-independent. Conversely, the switch back from mutant to wild-type required hydrolysis of ATP and involved hsp70.

Further evidence to support the role of hsp in the regulation of p53 function comes from studies involving the expression of recombinant human p53 in bacterial systems and the study of DNA binding by p53 using a gel mobility shift assay (Hupp *et al,* 1992). The effect of several cellular factors including *E.coli* dnaK, a bacterial homologue of hsp70 which has previously been shown to bind p53 (Clarke *et al,* 1988), on the DNA-binding activity of p53 were studied. It was found that dnaK activated p53 DNA binding in an ATP-dependent manner. The ability of this hsp70 homologue to enhance the DNA-binding function of p53 is intriguing and may have important implications in terms of the control of cell growth and the response to cell damage. The current model of p53 function as part of a damage control pathway fits in very well with the idea that a stress-inducible molecular chaperone should be able to modulate the activity of p53.

The ability of a stress-inducible molecular chaperone to regulate the tumour suppressor function of p53 suggests that the examination of heat shock protein expression in AML cells may be useful both in terms of tumourigenesis and the stress response of leukaemic cells exposed to chemotherapeutic agents.

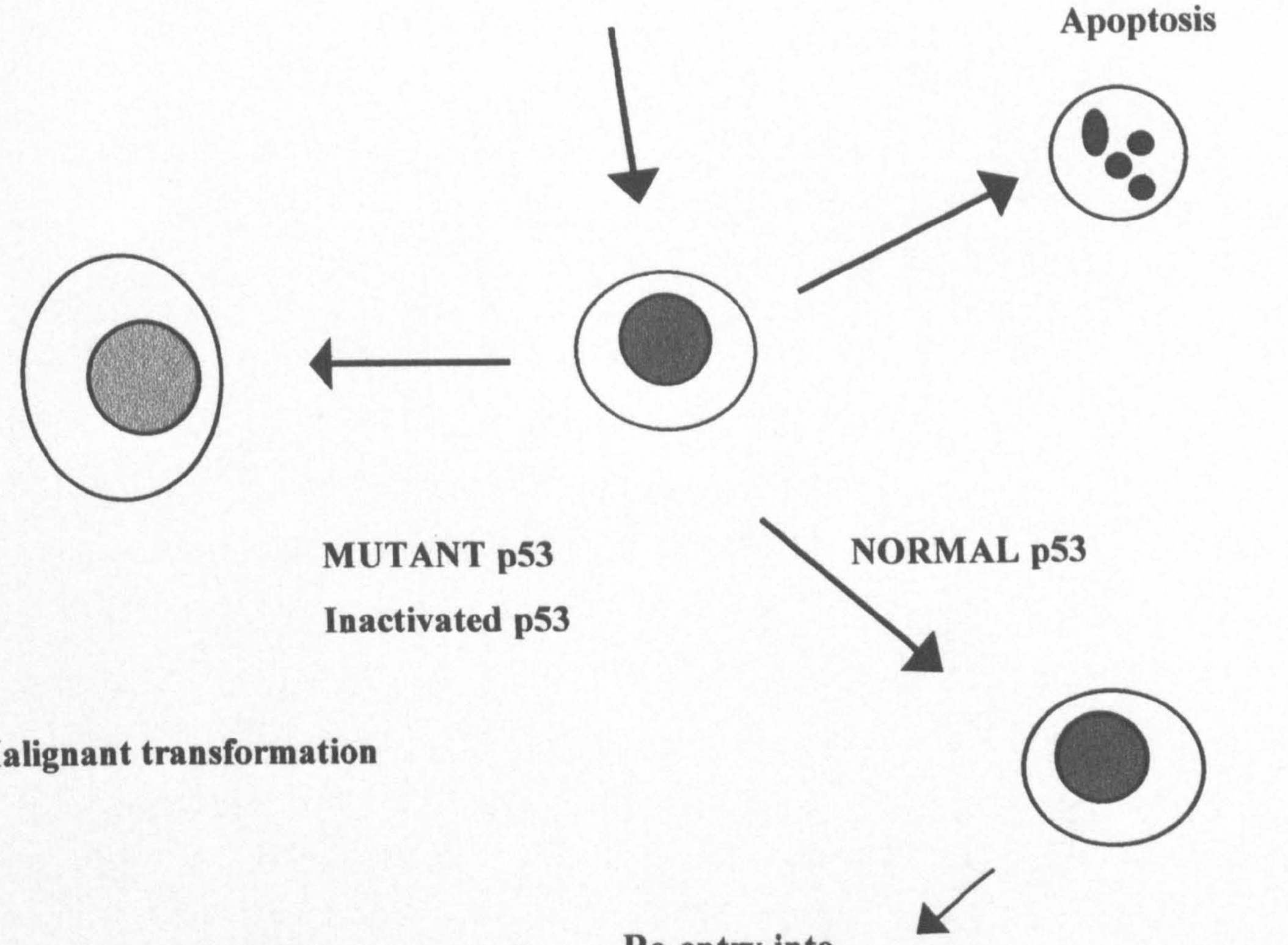



**Figure 1.2.** A proposed model for the role of p53 protein in the cellular response to DNA damage and the implicated role of inactivated p53 in carcinogenesis.

## 1.3. Apoptosis (Programmed cell death).

### 1.3.1 Introduction.

The term apoptosis is derived from a Greek word describing the process of leaves falling from trees. It was introduced to describe a type of cell death which exhibited a distinct set of morphological features (Kerr, *et al.*, 1972). One of the most characteristic of these features is the activation of endonucleases which cleave DNA at the internucleosomal sections. This reduces the DNA of apoptotic cells to a series of fragments, comprising multiples of 180-200 base pairs, and thus producing the characteristic DNA ladder seen on agarose gel electrophoresis. This event is closely followed by a cascade of other morphological changes including compaction and segregation of chromatin against the nuclear membrane, and condensation of the cytoplasm. Cell shrinkage occurs and individual cells become detached from their surrounding neighbours with preservation of the organelles, and nuclear and cytoplasmic budding takes place leading to the formation of membrane-bound fragments which are known as apoptotic bodies. These structures are shed from the cell, and *in vivo,* apoptotic bodies are phagocytosed by neighbouring cells including professional scavengers such as macrophages, but also cells of epithelial or fibroblast origin (reviewed by Majno and Joris, 1995). An important feature of this process is that apoptosis usually affects individual cells rather than all the cells in a particular area. The phagocytosis of apoptotic bodies and their degradation within the lysosomes of surrounding cells, avoids the induction of an inflammatory response due to the release of cellular contents. This is a crucial feature since it permits cell death without damage to adjacent cells. In addition, the process is rapid, with the onset of apoptosis

and formation of apoptotic bodies occurring within minutes of the apoptotic signal being interpreted by the cell.

## 1.3.2. The physiological role of apoptosis.

Apoptosis is a physiological mode of cell death which is now realised to play a major role in the maintenance of homeostasis in all systems of the body. It is particularly important during embryogenesis and metamorphosis, for example in the regression of the tadpole tail of frogs and for removal of interdigital webs during limb development in mammals (reviewed by Milligan and Schwartz, 1997). In adult mammals, the process plays a critical role in tissue turnover by maintaining the balance between cell number and cellular proliferation. For example, a balance between apoptosis and mitosis is required to maintain an equilibrium in the cell numbers of tissues which have slowly proliferating populations, such as the epithelium surrounding intestinal crypts and differentiating spermatozoa. The same principle applies to the haemopoietic system, where the demands of cell growth and differentiation are counterbalanced by the removal of unwanted cells by apoptosis (Lotem and Sachs, 1996). The triad of proliferation, differentiation and apoptosis are all controlled by a complex network of haemopoietic growth factors, and it is when dysregulation of these processes takes place that leukaemias may develop. This theme will be developed further in a later section.
The process is also especially important in the immune system. For example, it is responsible for the deletion of autoreactive T-cells in the thymus thereby permitting self tolerance, and for the selection of B-cells in germinal centres during humoral immune responses (reviewed by Ekert and Vaux, 1997). Cell-mediated immune responses are also effected by apoptosis; *in vitro* studies have demonstrated that T-cells and natural killer cells exert their cytotoxic effects on target cells by induction of apoptotic pathways (Shi, *et al,* 1992, Bishop & Whiting, 1983), and enhanced apoptosis is seen *in vivo*

during cellular immune rejection of allografts and in graft-versus-host disease (Kerr, *et al,* 1987).

## 1.3.3. Apoptosis is induced by a variety of stimuli.

To date, a myriad of stimuli have been reported to induce apoptosis, with a diverse array of cellular targets (reviewed by Wertz and Hanley, 1996). These include 1) reagents which act at the cell surface via receptor ligands, 2) disrupters of the cytosolic and organellar compartments, such as oxidants, 3) agents which disrupt the cytoskeleton, such as the anti-tumour drugs taxol and vincristine, and 4) reagents which act within the nucleus, such as certain chemotherapeutic drugs.

An example of the first category of molecular inducers is tumour necrosis factor (TNF), a cytokine which is responsible for cell death associated with septic shock, inflammatory disorders and anti-tumour activity (Smith *et al.,* 1994). The cellular response to TNF is mediated via cell surface receptors (TNFR), of which there are more than a dozen family members, including Fas. These receptors are responsible for regulating the survival and development of specific cells in response to environmental factors such as growth factors, and are particularly important in the haematopoietic system (Sachs, 1996). Although, the complete mechanism of the receptor-mediated death response is unknown, several components of the pathway have been identified, providing a link between the initial signal and the proteolytic activation of the caspases, enzymes which are the ultimate effectors of the biochemical changes which characterise apoptosis (see next section). Signaling through the TNFR or Fas receptor involves the recriutment and formation of complexes with adaptor proteins, such as FADD/MORT1 (from Fas-associated death domain protein) and FLICE (fas-activated protein-like ICE). The latter

is a protein which contains an ICE-related protease domain and can activate the caspase cascade (reviewed by Haimovitz-Friedman *et al.*, 1997).

Intracellular oxidants, such as menadione, cause damage to the cytosolic and organellar compartments and induce apoptosis via the generation of reactive oxygen species (Hockenberry, 1995). One of the consequences of oxidative damage is protein alteration, a factor known to induce heat shock protein expression. The induction of the heat shock response following oxidative damage may play an important role in the decision of the cell to commit itself to repair or cell death. This theory, in relation to other examples of apoptosis, will be elaborated upon elsewhere.

Of relevance to the treatment of leukaemia, the other two cellular targets mentioned above are both sites for chemotherapy-mediated cellular damage. For example, the anti-tumour drugs taxol and vincristine both exert their primary cytotoxic effect via the disruption of microtubule organisation, thereby inhibiting mitosis. This is particularly interesting in terms of the potential protective effect of hsp against cytotoxic agents, since members

Despite the diversity in targets and the variety of inducers of apoptotic cell death, the response of a cell to a particular inducer varies according to a range of factors including cell type and the pattern of characteristic gene expression in that cell, the differentiation state of the cell, and the cellular environment, in particular trophic factors such as hormones and growth factors. Thus different cell types appear to have the ability to sense and respond to similar insults in a fashion appropriate to their particular milieu.

## 1.3.4. Effector mechanisms in apoptosis.

The process of apoptosis can be divided into two phases. The first is the signalling phase whereupon the apoptotic machinery is activated, and the second is the effector phase of death. The key components of the second phase are a family of cysteine proteases (caspases) that are related to the interleukin-1ß-converting enzyme (ICE) (reviewed by Alnmeri, 1997). Members of this family of enzymes are involved in both the initial signalling events and the downstream proteolytic cleavages which result in the morphological events characterising apoptosis. They are all synthesised as proenzymes that are proteolytically processed to their active forms in response to an apoptosis-inducing stimulus.

ICE was first implicated in cell death when it was found to be homologous to the *Caenorhabditis elegans* death gene, *ced3*. Genetic studies have shown that *ced3* is involved in the apoptotic cell death that occurs during the development of this nematode worm. A total of ten caspases of human origin have now been identified, and these are known to act via a cascade mechanism to cleave a number of protein substrates within the cell (reviewed by Whyte, 1996; Nicholson, 1996). For example, caspase-3 is responsible for the cleavage of poly(ADP-ribose) polymerase and DNA-dependent protein kinases, proteins which are involved in DNA repair pathways. It has been suggested that degradation of these proteins cripples DNA repair and thereby permits the efficient action of endonucleases which leads to the degradation of DNA that is observed during apoptosis.

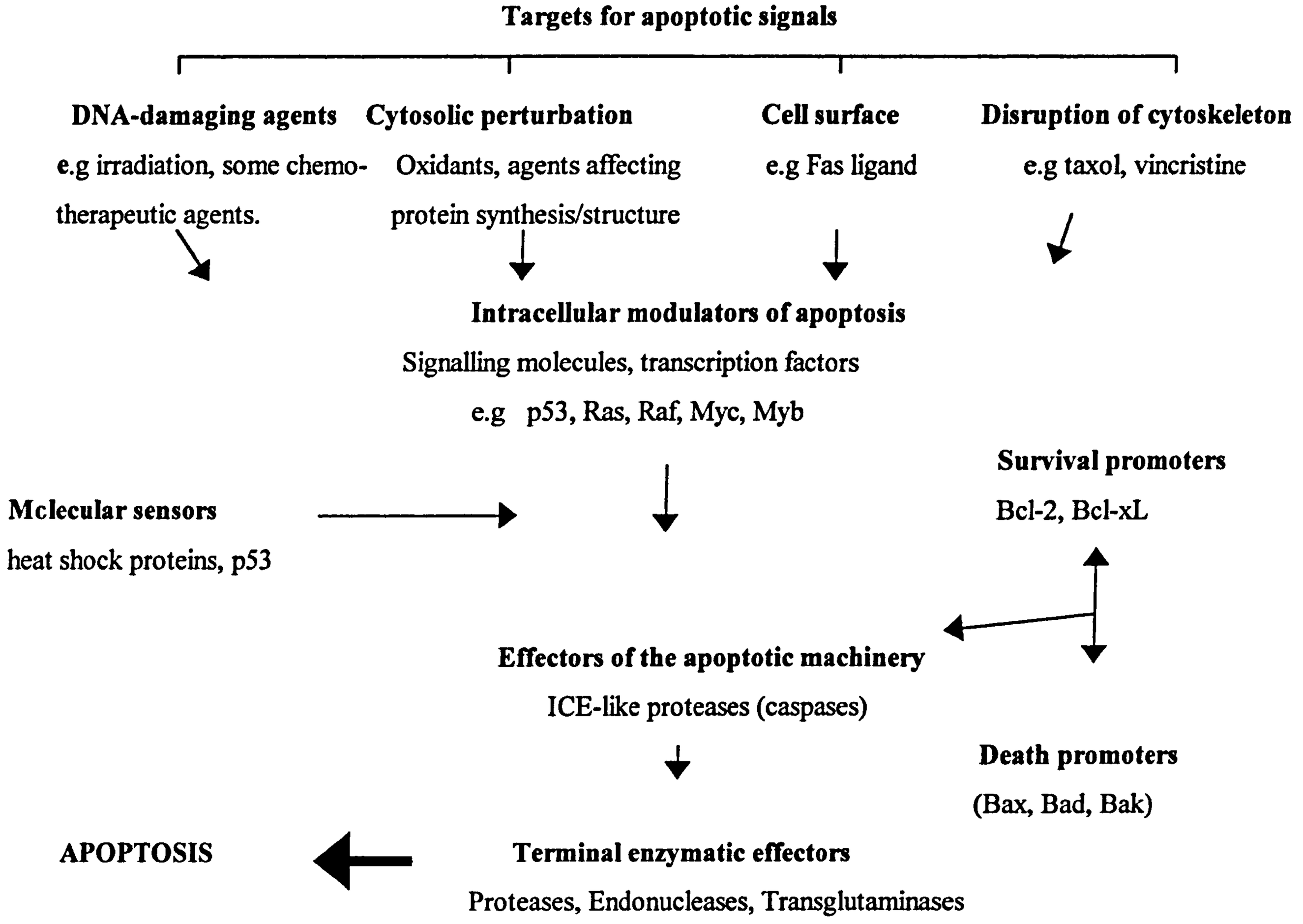



**Figure 1.3.** Generalised overview of apoptosis. A wide variety of inducing stimuli activate signalling mechanisms act to control activation of the effectors of apoptosis.

## 1.3.6. Apoptosis and the haematopoietic system.

### 1.3.6.1. The role of apoptosis in normal haematopoiesis.

Haematopoiesis is the term used to describe the normal production of the formed elements of the blood, including red blood cells (erythrocytes), white blood cells (monocytes, granulocytes, lymphocytes) and platelets. Each of these mature cell types has specific functions such as oxygen transport, response to infection, antibody production, etc., and the controlled production of these different haematopoietic cells is essential for the survival and development of a normal individual. It is when abnormalities in the normal developmental programme for blood cell formation occur, that haematological diseases such as leukaemia are seen.

Throughout life, the different lineages of blood cell are continuously produced to replace cells which have reached the end of their life span. In addition, the process must be flexible, allowing the increased production of one or more lineages in order to meet challenges such as infection or blood loss. Both *in vivo* and tissue culture studies of bone marrow cells have demonstrated that all blood cells of different lineages are derived from the same primary cell, referred to as the haematopoietic pluripotent stem cell. This stem cell is shown to have several important characteristics, including the ability to differentiate along several lineages, the capacity for extensive proliferation and self renewal, and the ability to respond to its environment via interaction with specific growth factors (reviewed by Minden, 1995). An overall view of haematopoiesis has arisen from these *in vivo* and *in vitro* studies whereby mature blood cells are derived from small numbers of pluripotent stem cells within the bone marrow, via the generation of progenitor cells which are committed to proceed along one of the maturation pathways (figure 1. 4).

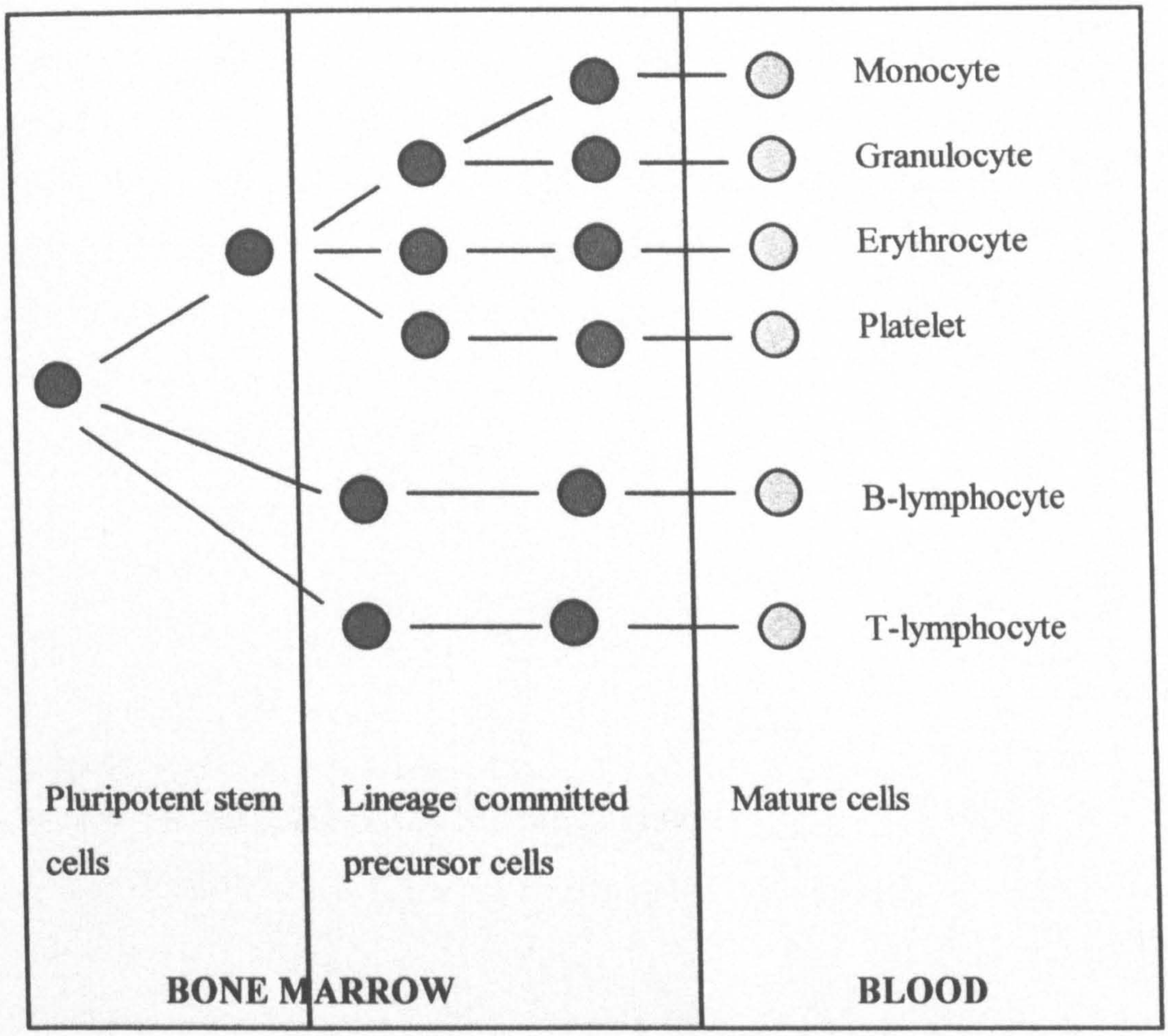



**Figure 1.4.** Normal haematopoiesis in the bone marrow gives rise to the formed cellular elements of the blood via a hierarchical organisation of differentiation and maturation from a pluripotent stem cell.

This ordered production of normal blood cells is the culmination of a series of interactions between a network of growth factors and receptors which control the maturation and differentiation of immature precursor cells. Over the past decade, the development of cell culture systems for the growth of haematopoietic cells has led to the discovery of an ever growing number of proteins which are responsible for regulating the proliferation and differentiation of immature cells along developmental lines of restricted lineage (reviewed by Sachs, 1996). These same growth factors also regulate cell viability, and protect both normal and leukaemic cells from apoptotic cell death. For example, normal myeloid progenitor cells, when cultured *in vitro,* require the availability of certain colony-stimulating factors (CSF) and interleukins (IL) in order to maintain viability, otherwise they will rapidly undergo apoptosis (Williams *et al.,* 1990). This programme for activation of apoptotic death in response to growth factor availability is retained throughout the developmental pathway, and cells at all stages of differentiation are subject to control by a complex network of signals, thus coupling the three processes of differentiation, multiplication and apoptosis.

Disorders in this normal developmental pathway give rise to leukaemias, and the haemopoietic system provides many examples of the significance of apoptosis, not only as a regulatory mechanism in normal haematopoiesis, but also as an element in the pathogenesis of the haematological malignancies. The implications of deregulated apoptosis in the development of leukaemia is therefore discussed in the following section.

**1.3.6.2. Leukaemogenesis and apoptosis.**

The homeostatic maintenance of cell numbers within a particular tissue or organ is delicately balanced between cellular proliferation and cell death. This balance is maintained by a complex regulatory network of growth factors which impinge upon both these processes, and under normal circumstances this permits considerable flexibility to

respond to changing requirements for cell numbers, as well as being important in the removal of unwanted or senescent cells. Over recent years it has become clear that in cancers, this balance between the rates of cell division and cell death is disturbed and that mechanisms which promote or suppress apoptosis can influence these rates. Thus, environmental circumstances or genetic lesions which affect the apoptotic pathway may lead to the expansion of a potentially malignant population of cells. The haematological malignancies provide many examples of the mechanisms which may be involved when deregulated apoptosis becomes a factor in the development of a malignant cell.

As outlined in the previous section, haematopoietic cells require the availability of various growth factors to maintain viability, and their absence results in rapid apoptotic cell death. Likewise, leukaemic cells are not immortal, and cells from most patients maintain the programme for apoptotic cell death which can be activated by the withdrawal of growth factors, as evidenced by the apoptotic cell death of leukaemic cells cultured *in vitro*. Interestingly, there are leukaemic cells from some patients which do not require exogenous growth factors in order to survive, and this reduced propensity to undergo apoptosis is associated with a worse prognosis (Lowenberg *et al.*, 1993; Hunter *et al.*, 1993). Haematopoietic growth factors also protect leukaemic cells against apoptosis induced by chemotherapeutic agents (Kaplinsky *et al.*, 1996), and this has implications for the treatment of leukaemia patients, not only because the production of autologous growth factors will influence the propensity of cells to undergo apoptosis, but also due to the fact that some chemotherapeutic regimens include the administration of certain haemopoietic growth factors.

In addition to the influence of growth factors, a number of genetic lesions have been identified, many of them already identified as oncogenes, whose expression is known to influence the apoptotic response of malignant haematopoietic cells.

### 1.3.6.3. Genes associated with apoptosis in the haematological malignancies.

Molecular analysis of apoptosis has revealed a number of genes whose expression is associated with this mode of cell death. In mammalian systems, many of these genes are also recognised as genes which modulate cell proliferation and/or growth, and have been implicated in leukaemogenesis. These include bcl-2, p53 and c-myc, genes whose expression has been shown to influence the susceptibility of cells to apoptosis induced by a variety of agents, including chemotherapeutic agents.

One of the most extensively studied of these regulators of apoptosis is the bcl-2 oncogene, encoding an integral membrane protein which occurs in nuclear membrane, endoplasmic reticulum, and mitochondrial membranes. Deregulated expression of bcl-2 can inhibit apoptosis in many cell types and in response to a variety of inducing treatments, including cytotoxic drugs (Korsemeyer, 1992; Chiou *et al,* 1994). In haemopoietic neoplasms, particularly the lymphoproliferative disorders, translocation of the bcl-2 gene to the heavy chain Ig locus (t14:18) is a common genetic abnormality which leads to deregulated bcl-2 expression and an associated resistance to apoptosis.

The mechanism(s) by which bcl-2 inhibits apoptosis is poorly understood, although studies on the nematode, *Caenorhabditis elegans,* demonstrate that ced-9 (a bcl-2 homologue) acts by inhibiting the activation of caspases (Alnmeri *et al,* 1997). Caspases are protein kinases which, as discussed earlier, are key effector molecules in the apoptotic process.

Many other genes resembling bcl-2 have now been identified (reviewed by Reed, 1995), some of which inhibit cell death (e.g bcl-xL, bcl-w), whereas others promote cell death (e.g bax, bak, bad). It is known that bcl-2 specifically interacts with other members of the family, forming homo- or heterodimers, and it is the resulting ratio of pro- and anti-apoptotic bcl-2 members which controls the activation of apoptotic pathways.

Another important genetic regulator of apoptosis is p53, a tumour-suppressor gene whose activity is abrogated by mutation or deletion in many human cancers, including leukaemias (Prokocimer and Rotter, 1994; Imamura *et al.*, 1994). The expression of p53 is known to be a critical response to cellular damage induced, for example, by ionising radiation and other DNA-damaging agents (Kastan *et al.*, 1991). Exposure of mammalian cells to ionising radiation can result in transient arrests of the cells at the $G_1$ and $G_2$ phases of the cell cycle (Kuerbitz *et al.*, 1992), and in apoptotic cell death (Cohen *et al.*, 1992). Early evidence that p53 may actually modulate apoptosis came from the observation that radiation-induced apoptosis of thymocytes requires p53 expression (Lowe *et al.*, 1993), although p53-independent mechanisms of apoptosis do exist as demonstrated by the fact that p53-null thymocytes undergo apoptosis in response to dexamethasone (Clarke *et al.*, 1993). A role for p53 as a $G_1$ cell cycle checkpoint is suggested by the observation that , whereas cells with wild-type p53 arrest in $G_1$ following DNA damage, cells with mutant p53 genes lack this checkpoint and go on to arrest in $G_2$. It has been suggested that this checkpoint provides the cell with a window within which DNA damage can be repaired, or else, if the damage is too great, the cell commits itself to apoptosis.

The decision of cells to undergo p53-mediated cell cycle arrest or apoptosis following cellular damage depends upon a variety of factors, including the extent of damage, growth factors, and the expression of other genes such as bcl-2 which influence the induction of apoptosis by p53 (Canman and Kastan, 1995). The inability of a cell to undergo apoptosis following DNA damage obviously has implications both for tumourigenesis and in the treatment of cancers. Mutations in the p53 gene may be one mechanism whereby potentially malignant cells could escape from apoptosis, while an inability to undergo p53-mediated apoptosis could be the cause of resistance to radiotherapy and chemotherapy.

Although mutations in the p53 gene are less common in haematological malignancies than in solid tumours, they have been associated with disease progression and poor prognosis (Foti *et al,* 1991; Gaidano *et al,* 1991; Ichikawa *et al,* 1992), an observation which may, in part, be due to impairment of p53-mediated apoptosis in response to chemotherapy.

The potential involvement of p53 in leukaemogenesis and the response of cell to chemotherapeutic agents was an important part of the rationale behind the ideas for this research project. As discussed in greater detail earlier (see Introduction, section 1.2.2), one of the interesting features of the heat shock proteins is their ability to modulate the conformation, and therefore the function, of p53 (Hainaut and Milner, 1992). Hypothetically, hsp expression and interaction with growth regulatory factors such as p53, may have implications for the control of growth versus apoptosis, as well as being an element in the response of tumour cells to drug-induced apoptosis mediated through p53-dependent pathways.

The expression of c-*myc* is an important regulator of the growth response of cells, influencing the decision of a cell to undergo proliferation or apoptosis in response to the availability of appropriate growth factors (Evan *et al.,* 1992). Enforced expression of c-*myc* promotes neoplastic transformation, and elevated expression of the protein is seen in a wide variety of tumour types (Packham and Cleveland, 1995). Paradoxically, in certain growth factor-dependent myeloid leukaemia cell lines which are maintained under growth restrictive conditions, deregulated expression of c-*myc* induces apoptosis rather than proliferation (Harrington *et al.,* 1994). It is thought that this apparent dual role is related to the relative abundance of appropriate growth factors, with cells in growth arrest being highly susceptible to apoptosis when c-*myc* is induced. In haemopoietic cells, the ability of deregulated c-*myc* to induce apoptosis or cell proliferation is also dependent upon p53 expression. For example, transfection with mutant p53 in a myeloid

cell line with deregulated c-myc expression suppresses the increased susceptibility to apoptosis induced by c-*myc* (Lotem and Sachs, 1996). These observations suggest that the suppression, by mutant p53, of this enhanced susceptibility to apoptosis is a cooperative mechanism in leukaemogenesis.

Finally, with regard to c-myc, it is interesting to note that heat shock of lymphoid cells has been shown to induce c-*myc* expression, as well as that of c-*fos* and c-*jun* (Bukh *et al.*, 1990), while studies using the regressing rat prostate gland as a model of apoptosis have demonstrated that the onset of apoptosis is accompanied by the expression of c-*fos* closely followed by the sequential induction c-*myc* and hsp70 (Buttyan *et al.*, 1988). It is difficult to be certain that these new transcripts are integral to the apoptotic process, and not simply part of an abortive stress response, but the co-ordinated expression of hsp70 with these oncogenes raises the possibility that it may be directly involved in the control of apoptosis.

Another gene whose expression has been linked with changes in apoptosis is c-abl, a cellular oncogene which is involved in a reciprocal chromosomal translocation t(9; 22) (q34; q11) with the BCR gene generating the so-called Philadelphia chromosome. This rearrangement is found in approximately 95% of chronic myeloid leukaemia (CML) patients, and 25% of adult acute lymphoblastic leukaemias cases. The resulting bcr-abl fusion protein has an oncogenic activity which is mediated via deregulation of abl tyrosine kinase function (Fernandes *et al.*, 1996). Expression of the bcr-abl protein confers resistance to apoptosis in CML cells following growth factor withdrawal (Bedi *et al.*, 1994), as well as death induced by a variety of chemotherapeutic agents (Bedi *et al.*, 1995; Dubrez *et al.*, 1998). In addition, anti-sense oligonucleotides for bcr-abl, when transfected into CML cells, increase the susceptibility of cells to apoptosis induced by certain cytotoxic agents (Rowley et al., 1996). This mode of resistance to apoptosis due to specific gene expression is a good example of how deregulated apoptotic mechanisms

are implicated in the leukaemogenic process, and also suggests why Philadelphia chromosome-positive leukaemias are particularly refractory to chemotherapy.

## 1.3. The Biology of Leukaemia.

Leukaemias are clonal disorders of haematopoietic tissue which arise due to a failure in the normal feedback control of clonal growth. This is usually caused by genetic changes in the regulatory gene sites of various growth factors or transcription factors, leading to the overproduction of one particular clone of cells representing a particular state of lineage maturation.

Leukaemias are broadly classified according to their myeloid or lymphoid lineage, and upon their maturation state. Acute myeloid or lymphoid leukaemias are characterised by clonal proliferation of their respective early precursor (blast) cells, whilst the chronic leukaemias are represented by myeloid or lymphoid cells at a later stage of differentiation. In each case the clonal proliferation leads to the replacement of normal haematopoietic cells in the bone marrow, invariably leading to anaemia, granulocytopaenia, and thrombocytopaenia, with the accompanying clinical manifestations of fatigue, infection and haemorrhages.

### 1.3.2. Acute myeloid leukaemia (AML)

#### 1.3.2.1. Targets of transformation in AML.

This disease results from the transformation of progenitor cells in the bone marrow exhibiting characteristics of one of the non-lymphoid lineages (granulocytes, monocytes, erythrocytes and platelets). AML is a therefore a heterogeneous disease, arising from the clonal proliferation of multipotent or lineage restricted precursors. In some cases of

AML, particularly in elderly patients, there is morphological evidence of trilineage involvement, the leukaemic cells exhibiting granulocytic, erythroid and megakaryocytic features, suggesting that leukaemic transformation has occurred at the level of a multipotent stem cell. This multiple lineage has been confirmed by both cytogenetic analysis and fluorescence in-situ hybridisation techniques (Van Lom *et al,* 1996, Keinanen *et al,* 1988). By contrast, AML in children and younger adults generally reveals that the disease is restricted to the granulocytic lineage without involvement of the erythroid and megakaryocytic populations. This implies that in these cases the leukaemia has arisen in a lineage restricted, committed granulocytic precursor cell (Figure 1.5).

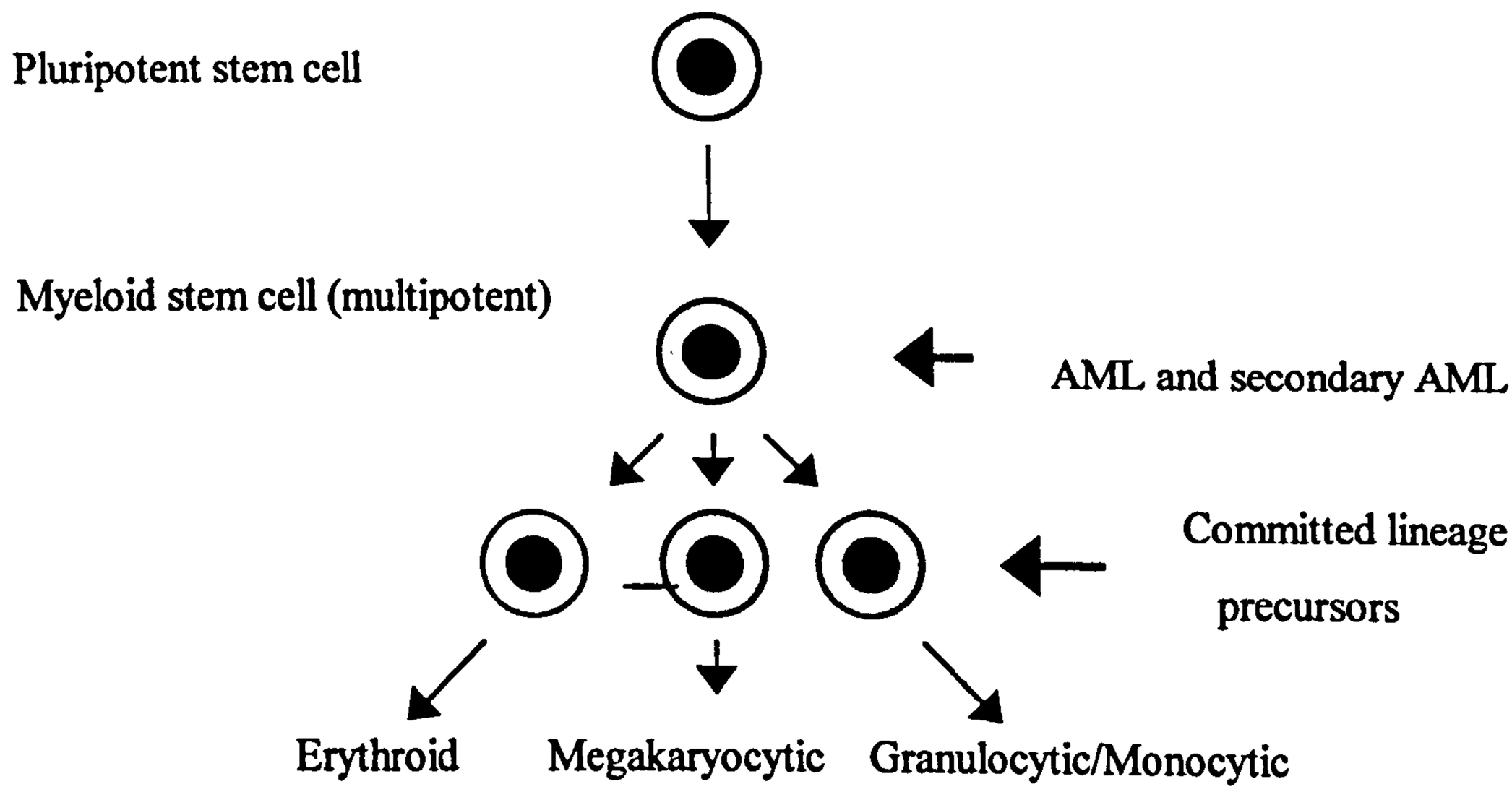



**Figure 1.5.** Target cells for transformation in AML. In elderly patients and patients with secondary AML there is evidence for transformation at the level of the multipotent myeloid stem cell. However, other AML types seem to arise in a lineage-committed progenitor cell.

### 1.3.2.2. Classification of AML.

Due to the heterogeneous nature of AML, sub-classification is important both in terms of disease prognosis and the standardisation of treatment strategies, and the most widely used system for the classification of AML is the French-American-British (FAB) system. This assigns a single lineage to each leukaemia based primarily upon morphological and cytochemical characteristics (Table 1.2). Eight AML subtypes have been distinguished, and are categorised according to the predominant differentiation pathway and the degree of maturation. For M1, M2 and M3 subtypes, granulocytic differentiation is predominant, for M4 the differentiation pathway is mixed granulocytic/monocytic, for M5 predominantly monocytic, for M6 erythroid and for M7 the platelet lineage.

| Leukaemia | Frequency | Lineage and differentiation characteristics |
|---|---|---|
| AML-M0 | <5% | Morphologically and cytochemically undifferentiated |
| AML-M1 | 20% | Myeloblastic |
| AML-M2 | 30% | Myeloblastic with maturation |
| AML-M3 | 10% | Acute promyelocytic leukaemia |
| AML-M4 | 20% | Acute myelomonocytic leukaemia |
| AML-M5 | 15% | Acute monoblastic leukaemia |
| AML-M6 | 5% | Erythroleukaemia |
| AML-M7 | <5% | Megakaryoblastic (platelet lineage) leukaemia |

**Table 1.2.** FAB classification of the acute myeloid leukaemias.

## 1.3.7 Apoptosis and the response of leukaemic cells to chemotherapy.

### 1.3.7.1 Apoptosis is induced by cytotoxic agents.

Most, if not all, of the cytotoxic drugs used in the treatment of leukaemias and other cancers exert their effects by inducing apoptosis in the target cells. (reviewed by Hickman and Boyle, 1996; Hickman, 1992). Among the growing list of drugs which have been reported to induce apoptosis are 1) DNA-reactive drugs such as cisplatin, chlorambucil, melphalan and bischloronitrosourea, 2) drugs which inhibit DNA topoisomerase I and II enzymes, such as camptothecin and etoposide, 3) inhibitors of mitotic spindle apparatus such as the Vinca alkaloids, and 4) antimetabolites such as methotrexate and phase specific drugs such as cytosine arabinoside and taxol (see table 1.3).

At present, the exact mechanisms whereby chemotherapeutic drugs induce apoptosis are unknown, but the fact that such a wide variety of drugs with differing modes of action can trigger apoptosis, suggests that either apoptosis can be activated by multiple mechanisms, or that it occurs via a common pathway which is activated by all these drugs. Although the primary action of most cytotoxic drugs is well understood, little is known concerning the mechanisms whereby the apoptotic machinery is switched on as a result of drug-induced damage.

In order to explain how such a wide variety of agents with disparate cellular targets all induce a common final mechanism of programmed cell death, it has to be assumed that cells possess the ability to decipher and assess the specific injury. The response of a cell to injury presumably relies on mechanisms whereby the individual cell reaches a decision, culminating in either the execution of apoptosis or successful repair of the damage and re-entry into the cell cycle. The existence of such a decision point would be an important

factor in the response of a cell to a particular injury, allowing the cell to decide whether the extent of the damage is such that either it can be successfully repaired, or else the damage is so great that the cell has no option but to proceed along an apoptotic pathway.

An understanding of this concept would provide us with a more complete understanding of why some cells die readily and others do not, and clearly this has important clinical implications. Apoptosis is a regulated phenomenon and the decision of a cell to initiate apoptosis may be determined by the relative expression of genes which promote or suppress apoptosis. Therefore, the expression of these genes which define the capability of a cell to undergo apoptosis following drug treatment may be a vital arbiter of the response to chemotherapy in leukaemia.

The possibility of enhancing the susceptibility of malignant cells to drug-induced apoptosis by modulation of the expression of these genes could lead to improved chemotherapeutic regimens.

**1.3.7.2. Inhibition of apoptosis and drug resistance.**

Inherent and acquired mechanisms of resistance to drugs are a major obstacle to the successful treatment of cancers. In the case of leukaemia, although the majority of patients achieve a complete remission with induction therapy, a large proportion will subsequently relapse with the development of resistance to a wide spectrum of chemotherapeutic agents. Many mechanisms of drug resistance have been described; these include increased metabolism of drugs, increased drug efflux, decreased drug uptake and amplification of the drug target (reviewed by Dalton, 1997; McKenna and Padua, 1997). However, it has become clear that mechanisms other than those affecting drug accumulation will determine the response of malignant cells to chemotherapy. As

suggested by Dive and Hickman (1991), the response of a tumour cell to a drug may not depend exclusively on the initial perturbation or biochemical lesions imposed by the drug, but also on how the cell responds to these lesions. In other words, whether or not apoptosis is induced in that cell.

As discussed earlier, a number of genes have been identified as being important in the control of apoptosis, and it has indeed been shown that these factors will influence the susceptibility of malignant cells to drug-induced apoptosis. For example, bcl-2 overexpression in a myeloid leukaemia cell line has been associated with a high level of resistance to a range of chemotherapeutic agents (Lotem and Sachs, 1993a). In addition, transfection of a human lymphoma cell line with the bcl-2 gene resulted in a dramatic increase in resistance to apoptosis, despite the fact that similar levels of drug-induced damage were evident in both parent and transfected cells (Fisher *et al,* 1993), whilst downregulation of bcl-2 with antisense oligonucleotides has been shown to induce apoptosis and increase the sensitivity of AML blast cells to the drug, cytosine arabinoside (Keith *et al,* 1995). The clinical significance of these *in vitro* observations has been emphasised by the finding that patients with acute myeloid leukaemia (AML) whose cells express high bcl-2 have a worse response to chemotherapy (Campos *et al,* 1993, Maung *et al,* 1994).

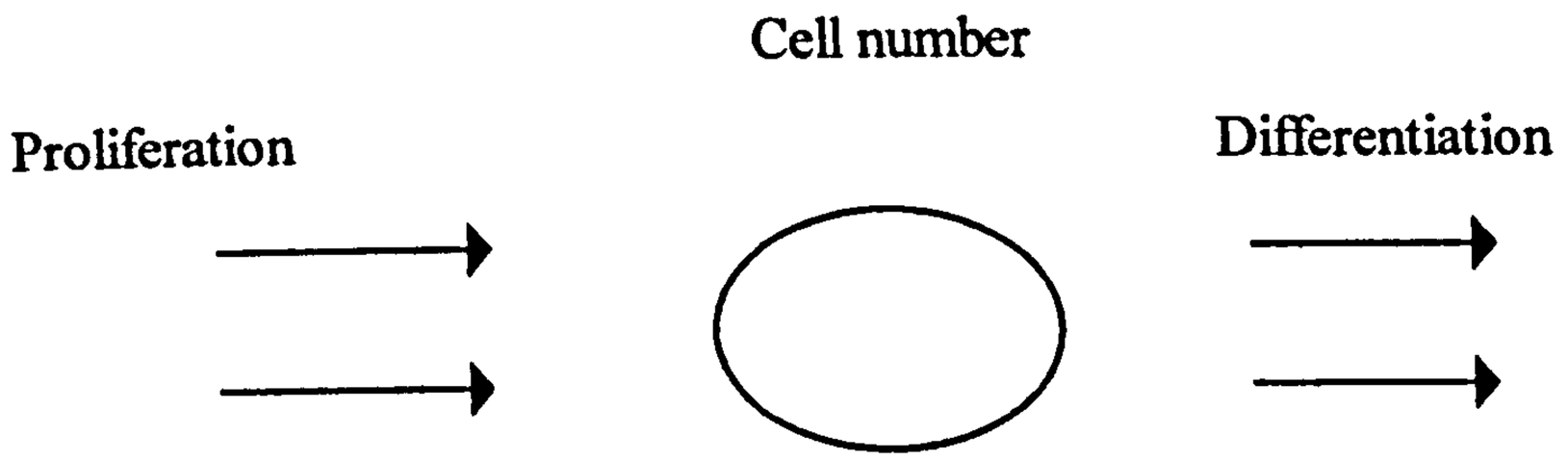
The expression of the tumour suppressor gene, p53, is another factor closely involved in the control of apoptosis which has been shown to influence the ability of cytotoxic agents to induce apoptosis in haematopoietic cells. Haematopoietic stem cells from mice deficient in wild-type p53 are more resistant to the induction of apoptosis by a variety of agents (Lotem and Sachs, 1993b). Conversely, acute overexpression of wild-type p53 using an adenovirus vector enhances the sensitivity of normal human fibroblasts to .

Again, the clinical relevance of these *in vitro* studies has been demonstrated by the

finding that mutation of the p53 gene in AML patients is a strong prognostic indicator of response to chemotherapy and survival (Wattel *et al,* 1994; Kurosawa *et al.,* 1995). The relevance of these observations is to demonstrate that mechanisms exist which can rescue leukaemic cells from drug-induced apoptosis. Thus, the suppression of apoptosis by mechanisms which act downstream of the initial site of cellular perturbation may represent an important mode of drug resistance. The identification of those factors which modulate this downstream response to cellular damage is therefore critical to an understanding of why some tumour cells fail to undergo apoptosis. Presumably, individual cells possess a threshold which defines whether that cell survives or initiates apoptosis. It seems probable that cellular factors are involved which act as sentinels of damage, and one obvious example would be the product of the tumour suppressor gene, p53. Exposure of cells to DNA-damaging agents results in an increase in p53 levels by a post-transcriptional mechanism (Kastan *et al,* 1991) which probably involves an increase in protein translation and an increase in p53 protein half-life. One identified signal for p53 induction is the presence of DNA strand breaks. It has been demonstrated that p53 protein will bind to the ends of single stranded DNA and to sequences of DNA containing mismatches (Jayaraman and Prives, 1995; Lee *et al,* 1995), and it has been suggested that binding of p53 to these sites might be a signal for p53 activation. However, it does not seem likely that DNA-binding would lead to an increase in either the transcriptional rate or the half-life of p53 and there must be alternative signals, or some protein which binds to p53 after DNA damage; the binding of hsp70 to p53 springs to mind. Interestingly, it has recently been shown that the exposure of cells to hypoxia results in p53 induction and leads to apoptosis in a p53-dependent manner demonstrating that signals other than DNA damage induce p53-dependent apoptosis. This is interesting because hypoxia is a recognised inducer of hsp expression which leads one to speculate whether hsp could be involved in the apoptotic response of the cell to hypoxia. In this

manner hsp induction may play a dual role; as a signal to the cell that potentially lethal stresses are being exerted, and possibly a direct role in the apoptotic pathway via the modulation of p53 function.

**Normal cell growth:**




**Leukaemogenesis:**

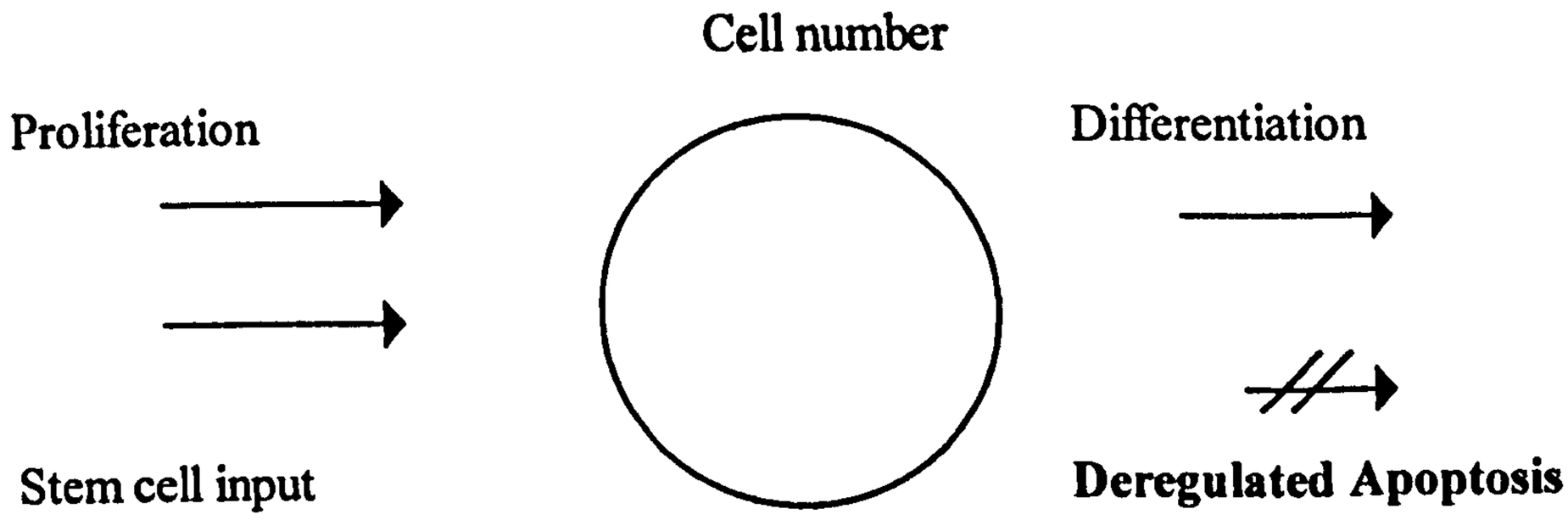



**Figure 1.5.** Normal haemopoiesis relies on a balance between the demands of proliferation and differentiation and the removal of unwanted cells by apoptosis. Dysregulation of the apoptotic process by genetic lesions involving regulatory proteins such as bcl-2 and p53 are implicated in loss of this balance and leukaemogenesis.

## 1.3.8. Heat shock protein 70 (hsp70) and apoptosis.

Studies focusing on the genetics of apoptosis have identified hsp70 as being one of the prominent genes whose expression is induced during the early phases of the process. For example, studies using the regressing rat prostate gland as a model of apoptosis have demonstrated that the early stages of the process are accompanied by an increase in hsp70 expression (Buttyan *et al.,* 1988), whilst sodium butyrate-induced apoptosis in lymphoid cells is preceded by a transient over-expression of hsp70 (Filippovich *et al.*, 1994). It is impossible to say whether this indicates that hsp70 expression is integral to the onset of apoptosis, or simply that these increased hsp70 levels are part of the normal (perhaps abortive) stress response. What is clear, however, is that over-expression of certain hsp, and in particular hsp70, confers resistance to apoptosis induced by a number of different agents and in a variety of cell types.

### 1.3.8.1. Heat shock proteins and thermotolerance.

As described earlier, hsp overexpression is directly related, both quantitatively and temporally to the development of thermoresistance. Heat shock is one agent which has been shown to induce apoptosis in a variety of cell types, and although early studies did not identify the cytotoxic effect of heat as acting via this mode of cell death, the observation that a mild hyperthermic exposure induces a thermotolerant state which is quantitatively related to the expression of hsp, in particular hsp70, was the first suggestion of a relationship between hsp and apoptosis (Li and Werb, 1982; Landry *et al.,* 1982). Cell lines which have been stably transfected with hsp-encoding genes, including both hsp70 and hsp25, are protected from hyperthermia, as assessed by colony forming assays (Landry *et al.,* 1989) and more recently by measuring indicators of apoptosis (Li *et al.*, 1996).

### 1.3.8.2. Hsp protect against apoptosis induced by a variety of stimuli.

Hsp70 overexpression also protects cells against apoptosis induced by agents other than heat shock. For example, a mild heat shock of neuronal cells protects these cells against apoptosis induced by transfer to a serum-free medium (Mailhos *et al.*, 1993) whilst transfection with the hsp70 protects susceptible cells from apoptosis induced by TNF (Jaatella *et al.*, 1992). The involvement of hsp in apoptosis is also suggested by experiments examining apoptosis of haemopoietic cells induced by quercetin, a bioflavanoid which specifically inhibits hsp70 translation (Wei *et al.*, 1994), while hsp70 antisense mRNA has been shown to induce apoptosis in haemopoietic cells (Wei *et al.*, 1995).

The potential involvement of hsp in protecting tumour cells against apoptosis induced by chemotherapeutic agents is suggested by data which shows that elevated hsp expression is associated with chemoresistance both in human tumours (Ciocca, 1993). Similar observations have been made in experimental models; in heat-shocked rat tumour cells the induction of hsp70 is associated with vincristine resistance (Lee *et al*, 1992) while Chinese hamster cells over-expressing the human hsp70 gene are more resistant to a variety of cytotoxic agents (Huot *et al*,1991).

## Experimental aims.

As has been outlined in the introduction, hsp expression displays several important characteristics which would appear to be extremely relevant to the study of leukaemogenesis. Hsp expression is intimately linked with differentiation and cell cycle control, possibly via the ability of hsp to interact with and modulate the function of growth factors such as p53. In addition, hsp overexpression protects cells against a variety of stresses, including cytotoxic drugs which mediate their effects via the induction of apoptosis. Hsp expression in leukaemia is therefore interesting in terms of both the development of the malignant cell and in the problems associated with the treatment of these diseases, ie. the development of chemoresistance.

The primary aims of this research project are therefore to address this potential link between hsp expression and haematological malignancy by answering the following questions:

1. Is hsp expression abnormal in leukaemic cells isolated from patients with myeloid leukaemia, both in terms of the subcellular localisation and the levels of hsp?

2. Is there a correlation between hsp expression and disease status, either in terms of disease progression or the potential prognostic relevance of hsp expression?

3. Relating to the previous point, do levels of hsp expresion influence the susceptibility of leukaemic cells to apoptosis?

4. In particular, does hsp over-expression protect leukaemic cells against apoptosis induced by chemotherapeutic agents?

# Chapter 2

# Materials and Methods

## 2.1. Collection and storage of leukaemic cells.

### 2.1.1. Leukaemic patients.

Leukaemic cells were harvested from patients attending Warwick Hospital, either as in-patients or as out-patients presenting at the Haematology clinics. These patients included those with *de novo* disease and those whose disease had relapsed. The initial diagnosis of leukaemia was made in the Haematology Department and the disease classified according to morphological and cytochemical criteria. Samples were also collected, when possible, from patients during the course of treatment. However, this was not possible in every case due to the very low numbers of mononuclear white blood cells which were present in the peripheral blood of patients undergoing ablative chemotherapy.

### 2.1.2. Harvesting of mononuclear cells from whole blood.

Peripheral blood mononuclear cells were separated from whole blood samples, or on occasions from bone marrow aspirates, collected from inpatients of Warwick Hospital or those attending the Haematology Clinic at Warwick Hospital. These samples were in all cases collected into tubes containing EDTA as an anticoagulant.

Blood or bone marrow samples were diluted with an equal volume of sterile physiological saline, and, using a pasteur pipette, carefully overlayed on top of an equal volume of lymphoprep (Nycomed) in a 15 ml. centrifugation tube or sterilin universal container.

Samples were centrifuged at 800 xg for 15 minutes at room temperature, taking care to leave the centrifuge brake off in order to avoid excessive swirling of the separated layers. Following

centrifugation most of the upper serum layer was removed with a sterile pasteur pipette, and the mononuclear cells, which form a distinct band on top of the separation medium, were aspirated and resuspended in 15 mls of RPMI 1640 (Gibco, U.K).
Samples were centrifuged at 400 xg for 10 minutes to wash the cells, and resuspended in a suitable volume of RPMI 1640. At this stage, the cell concentration cells was determined using an automated haematology analyser (Abbott Cell-dyn 3500), and the cell suspension was adjusted to $10^6$ cells/mL using RPMI 1640.

### 2.1.3. Freezing of mononuclear cells.

Cell suspensions were adjusted as above to a concentration of approximately $10^6$ cells/mL in RPMI 1640, and foetal calf serum (Gibco) was added to a final concentration of 10% (v/v/). Mononnuclear cells (0.9 mL) were then aliquoted into biofreeze vials (Costar) and 0.1ml of dimethyl sulphoxide (DMSO) was added to act as a cryo-preservant. The vials were wrapped in tissue and placed at -70°C in an insulated box to allow a slow rate of freezing. Vials were then transferred to liquid nitrogen within 3 days.

### 2.1.4. Thawing of cells and measurement of cell viability.

Vials of cryopreserved cells were rapidly thawed by incubation of the vial in a 37°C waterbath and the thawed cell suspensions were transferred to a sterile universal container. Cells were washed by the addition of 10 mL of RPMI 1640 culture medium followed by centrifugation at 400 xg for 5 minutes, and then resuspended in 1mL of RPMI 1640 culture medium.
Cell viability was assessed in each case by flow cytometric analysis of propidium iodide-stained cells. To 100µl of the mononuclear cell suspension, 10µl of DNA staining buffer

comprising 50μg/mL propidium iodide (Sigma) and 0.5 mg/mL Rnase A (in PBS), was added, and this was incubated for 15 minutes at room temperature. Cells were then analysed for uptake of propidium iodide by flow cytometric analysis.

As in other cell viability assays which are based on dye exclusion, non-viable cells take up the dye, and the advantage of using propidium iodide is that uptake of the dye can be measured by flow cytometry permitting the analysis of a large number of cells.

### 2.1.5. Leukaemic cell lines.

Four myeloid leukaemia cell lines were used during the course of this study to investigate the kinetics of the heat shock response following hyperthermia, and to study the relationship between hsp levels and apoptosis in myeloid cells. All four cell lines were obtained from the ECAAC culture collection of cells and tissues. KU812 and KG1a are lines derived from patients with myeloid leukaemia, HL-60 is a myelomonocytic cell line and THP-1 is derived from a patient with pro-monocytic leukaemia.

Cell lines were maintained as described in the specification data from the ECAAC.

Briefly, KU812, HL-60 and THP-1 were maintained in suspension in RPMI 1640 containing Glutamax ™, plus 10% foetal calf serum (FCS) and 50μg/mL gentamicin (all obtained from Gibco). The KG1a cell line was maintained in Dulbecco's Modified Essential Medium (Gibco), supplemented with 10% FCS and 50μg/mL gentamicin. All cell lines were cultured at 37°C in a humidified incubator, and maintained in continuous logarithmic growth with sub-culturing by 1:4 dilution in fresh complete medium at 48-72 hour intervals.

## 2.2. Analysis of hsp expression.

Immunochemical analysis of hsp expression throughout the course of this project was performed using a range of monoclonal antibodies to the major hsp families (see Table 2.1). Anti-hsp70 (72/73) and anti-hsp60 (clone LK1) were both obtained from Sigma, and used at a dilution of 1:50 for immunocytochemical analysis and 1:100 for immunofluorescent staining. ML30 was provided by Dr. N.Mann from the University of Warwick, and was used at a final concentration of 5µg/mL for both techniques. A monoclonal antibody to hsp90 was obtained from Stressgen biotechnologies Ltd, and this was diluted as for the anti-hsp70 and hsp60 antibodies.

| Monoclonal Antibody | Specificity |
|---|---|
| Anti-hsp60 Clone LK1 | Produced using recombinant human hsp60 as immunogen, recognising an epitope located between residues 383-447 of human hsp60. |
| ML30 | Recognises amino acids 275-295 of the 65kDa hsp from *Mycobacterium leprae* and *M. tuberculosis*. Will cross-react with human hsp65. |
| Anti-hsp70 (72/73) | Produced using purified hsp70 from bovine brain as immunogen. The antibody localises both the constitutive (hsp73) and the inducible (hsp72) forms of hsp70. |
| Anti-hsp90 | Clone AC-16, recognises both the constitutive and inducible forms of hsp90. Does not bind to the native (unbound) form of hsp90. |

**Table 2.1.** Monoclonal antibodies to the major hsp families used in the analysis of hsp expression in normal and leukaemic mononuclear cells.

## 2.2.1. Cytospin preparations of mononuclear cells.

Hsp expression in normal and leukaemic mononuclear cells was investigated by immunocytochemical staining of cytospin cell preparations. For all leukaemic patients involved in this study, cytospins were prepared using freshly harvested mononuclear cells as it was found that cryopreservation and subsequent thawing of cells affected the morphological appearance of the cells. Cytospins were prepared with a Shandon cytocentrifuge, using 50-100 µl of cells suspended at $10^5$-$10^6$ cells/mL in RPMI 1640 culture medium containing 5% bovine serum albumin (BSA). The addition of BSA to the cell suspension resulted in improved preservation of cell morphology, presumably by buffering the cells against the deleterious forces of centrifugation onto the glass slide. Cells were centrifuged at a setting of 900 xg for 3 minutes, using disposable plastic cuvettes, and glass microscope slides used were cleaned before use in 70% ethanol. Prepared cytospins were air-dried for at least 60 minutes prior to staining. Those not stained immediately were stored at -30°C in a slide tray wrapped in aluminium foil until use, and these were allowed to reach room temperature for a minimum of 60 minutes prior to removing the foil wrapping.

## 2.2.2. Localisation of hsp expression by immunocytochemistry.

Visualisation of antigen distribution following incubation with specific hsp monoclonal antibodies was achieved using an avidin and streptavidin/biotin system labelled with horseradish peroxidase. The principle of this procedure is based upon the affinity between avidin or streptavidin and biotin to detect a secondary biotin-labelled anti-mouse monoclonal which binds to the primary mouse antibody.

Cytospins preparations of mononuclear cells were fixed in acetone:methanol (1:1) for 10 minutes at room temperature, prior to rinsing with distilled water and incubation in tris buffered saline (TBS) for 5 minutes. In order to block non-specific binding, cytospins were incubated for 20 minutes with normal rabbit serum (Dako), diluted 1:20 in TBS. Following blocking, the serum was tapped off and the excess blotted away.

The primary hsp antibody (see above), diluted optimally in TBS, was added and the slides incubated in a humid staining tray for 1 hour. Unbound antibody was removed by rinsing twice with TBS and slides were placed in a TBS bath for 5 minutes. This was followed by incubation with a biotinylated rabbit anti-mouse monoclonal (diluted 1:50 in TBS), for 30 minutes. During this incubation the ABComplex was prepared by combining the avidin and streptavidin/biotin reagents according to the manufacturers instructions. Slides were rinsed with TBS as before to remove unbound secondary antibody, prior to incubation with the ABComplex/HRP for 30 minutes. After rinsing with TBS, stained slides were incubated with DAB substrate for 5-10 minutes and then rinsed with distilled water. Cells were counterstained with Meyer's haematoxylin and air-dried before mounting with a coverslip. Intracellular distribution of hsp antigens was examined by light microscopy.

## 2.3. Immunofluorescent analysis of heat shock proteins.

The method of choice for demonstration and quantitation of heat shock protein antigen expression was flow cytometric analysis of immunofluorescence-stained cells. Indirect immunofluorescence techniques using a secondary fluorophore-conjugated antibody to label cells incubated with a primary hsp monoclonal antibody are highly sensitive. The ability to stain either 'live' or fixed and permeabilised cells permits the analysis of cell surface antigens as well as intracellular hsp expression. Hence, this technique was utilised in order to investigate both cell surface and intracellular hsp expression using unfixed and fixed haemopoeitic cells respectively.

Immunofluorescent staining followed by flow cytometric analysis was the method of choice for this study, both for examining the possibility of surface hsp expression, and for quantifying specific hsp levels (Chapters 3 and 4). Flow cytometric analysis offers several advantages over other techniques for protein analysis in this case. Leukaemic cells are particularly suited to flow cytometry, naturally existing as single cells in suspension, thereby eliminating the need for extensive sample preparation. The technique permits the rapid analysis of large numbers of cells (>10,000 cells /min) with high reproducibility. Particularly important, and a notable advantage compared with Western blotting, during flow analysis cells are examined at the single-cell level. This is particularly advantageous when a population of cells displays heterogeneous patterns of expression for a particular parameter. In addition, flow cytometry permits the simultaneous analysis of multiple parameters. Distinct populations of cells can be identified by specific light scatter characteristics, and the ability of the modern flow cytometer to detect fluorescent probes with different emission spectra means that double or triple-labelling techniques can be utilised for multiparametric analysis.

## 2.3.1. Analysis of surface hsp antigen expression.

Washed mononuclear cells were suspended in phosphate-buffered saline, pH 7.4 (PBS) containing 1% bovine serum albumin (BSA) to a final concentration of $10^6$ cells/mL. Cell suspensions (100μL) were added to round-bottomed microtitre plate wells (Costar), to which 10μL of optimally diluted primary hsp monoclonal antibody was added. In every case, an isotype-matched negative control antibody was added to a second aliquot of cells. The microtitre plate was covered with an adhesive plate sealer, and the plate incubated at room temperature for 60 minutes. The microtitre plate was then centrifuged (up to 2000 rpm prior to braking) and the supernatent removed. Cells were then washed twice in PBS in order to remove unbound antibody, and the cells were resuspended in 100μL PBS. Finally, 5μL of a secondary goat anti-mouse/FITC monoclonal antibody was then added to each microtitre plate well, followed by incubation at room temperature for 45 minutes. Cells were washed once in PBS prior to resuspension in 200 μL of PBS. Flow cytometric analysis of immunofluorescence-stained cells was performed using a Coulter Profile II, and the potential presence of surface hsp expression was determined by comparison of fluorescence intensity between samples stained with the hsp- and isotype-matched monoclonal antibodies.

As will be described in Chapter 3, initial experiments revealed the absence of specific staining with any of the hsp antibodies used. In order to increase the level of sensitivity, a three-stage immunofluorescence technique was utilised. Cells were stained with the primary hsp monoclonal as above, followed by the addition of a biotinylated rabbit anti-mouse monoclonal (Dako), diluted 1:50 in PBS. Cells were incubated for 30 minutes and washed twice as above, prior to the addition of a streptavidin/FITC conjugate (also from Dako). After another 30

minute incubation, cells were washed once and resuspended in PBS prior to flow cytometric analysis as before.

## 2.3.2. Analysis of intracellular hsp expression.

Mononuclear cells were fixed by the addition, slowly with gentle mixing, of 1mL of 1% paraformaldehyde (in PBS). After 15 minutes, cells were centrifuged at 400 xg for 5 minutes and following removal of the supernatent were washed twice in PBS. Cells were re-suspended in PBS to a final concentration of $10^6$ cells/mL, and for each hsp monoclonal plus isotype-matched antibody, 90μL of cell suspension was added to a microtitre plate well. 10μL of primary antibody plus 10μL of 0.25% saponin (to permeabilise the cells) was added, and the microtitre plate covered with an adhesive seal prior to incubation at room temperature for 45-60 minutes. The microtitre plate was then centrifuged up to 2000 rpm in order to pellet the cells and the supernatent removed, followed by resuspension of the cells in 300μL of PBS and centrigugation as before in order to wash the cells. The washed cell pellet was resuspended in 100μL of PBS, and 10μL of goat anti-mouse/FITC (Dako) added. The plate was incubated in the dark at room temperature for 30 minutes prior to washing once in PBS as before and resuspension of the cells in 200μL of PBS. Stained cells were analysed by flow cytometry, gating on the cells using side scatter and forward scatter characteristics to eliminate debris, and green (FL1) fluoresence of the cells was determined. Specific staining with hsp monoclonal antibodies was compared with the isotype-matched antibody.

In order to compare levels of hsp expression in cells from different patients and in different cell samples, the intensity of green fluoresence was recorded as a numerical figure given by he

mean peak channel of fluoresence, which although not an absolute measure of antigen expression, is directly proportional to the level of antigen expression.

## 2.3.2. Flow cytometric analysis of p53 and bcl-2 expression.

Direct immunofluorescent staining of haemopoietic cells with FITC-conjugated antibodies to bcl-2 and p53 (Dako) was performed according to suppliers specifications. Briefly, washed cells ($10^6$ cells) were fixed for a minimum of 15 minutes in 1 mL of ice cold 70 % ethanol. These were then washed twice in PBS and either 10µl of anti-bcl-2/FITC or 10µL anti-p53/FITC antibody were added. Appropriate isotype-matched negative control antibodies were added in each case to a second aliquot of cells. Cells were incubated in the dark for 45 minutes at room temperature, and washed in PBS prior to flow cytometric analysis.

## 2.3.2. Dual staining for analysis of cytoplasmic antigens and cell cycle.

Fixed and permeabilised haemopoietic cells were analysed for hsp expression and cell cycle analysis by a dual staining technique utilising indirect hsp staining with a FITC-conjugated antibody plus staining of nuclear DNA with propidium iodide (PI). Simultaneous measurement of green (FITC) and red (PI) fluorescence by flow cytometry permits analysis of hsp expression during different stages of the cell cycle.

Washed mononuclear cells were suspended in PBS containing 1% BSA to a final concentration of $10^6$ cells/mL. For each analysis, 100ml of mononuclear cell suspension (1 x $10^6$ cells) was added to each of 2 plastic tubes (or microtitre plate wells) and 1 mL of 0.25% para-formaldehyde (in PBS) was slowly added to each tube whilst vortexing. The cells were

incubated for a minimum of 15 minutes at room temperature in order to fix the cells, prior to washing twice in PBS, centrifuging at 300 x g for 5 minutes. In order to permeabilise the cells, 1 mL of cold (4°C) 70% methanol was added to each tube, followed by incubation for 60 mins. at 4°C. Tubes were then centrifuged at 300 x g for 5 minutes, and the supernatent aspirated.Following a single wash with PBS, cells were resuspended in 100ml of PBS.
10μL of appropriately diluted anti-hsp monoclonal was added and the cells stained for 45 minutes at room temperature. In order to remove unbound antibody cells were washed once in PBS and resuspended in 100μL of PBS. To each tube, 10μL of secondary goat anti-mouse/FITC (GAM/FITC) and 200μL DNA staining medium containing 50μg/mL propidium iodide and 0.5 mg/mL Rnase A (both from Sigma) in PBS.
and these were incubated in the dark for 30 minutes. Finally, cells were washed by the addition of 2 mL PBS containing 2% FCS and centrifuged at 300 x g for 5 minutes. The supernatent was aspirated and the cells resupended in 200μL of PBS prior to flow cytometric analysis.
Stained cells were gated using red fluoresence (PI stain), and green fluorescence of these cells was measured. A dual histogram plot of red *versus* green fluoresence demonstrated the cell-cycle specific expression of hsp.

## 2.4. Analysis of apoptosis in haematopoietic cells.

### 2.4.1. Morphological assessment of apoptosis.

During apoptosis, cells demonstrate a number of distinct morphological changes which can be visualised by light microscopy. The apoptotic process is characterised in most cell types by compaction and segregation of chromatin against the nuclear membrane with condensation of the cytoplasm. The cells generally shrink, and the latter stages are

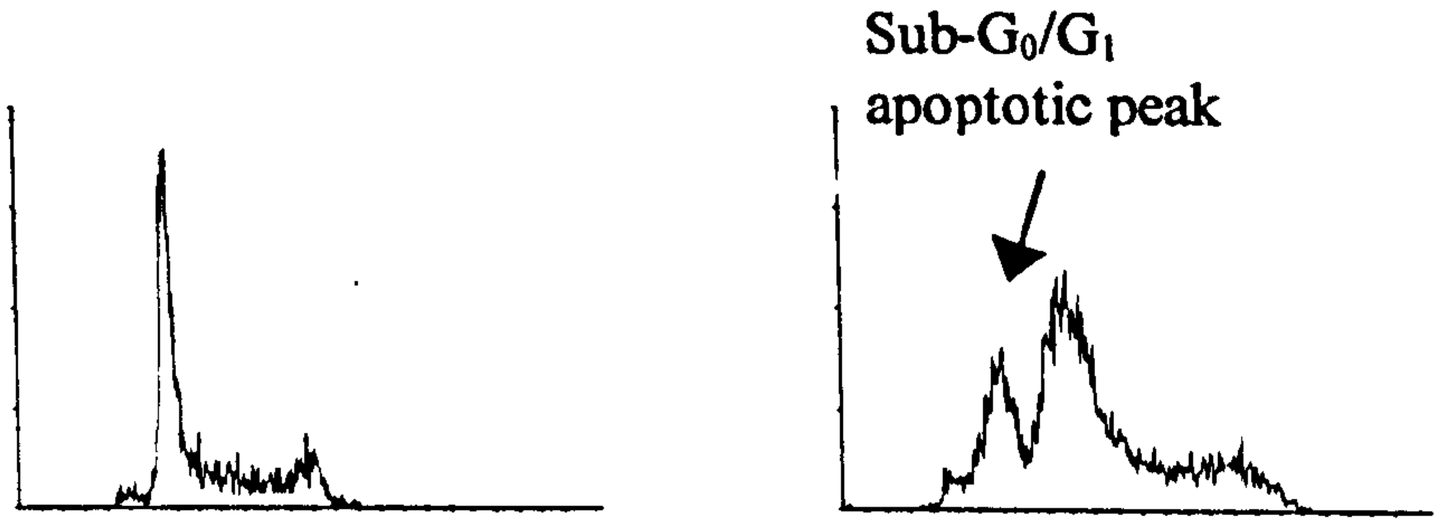
characterised by nuclear and cytoplasmic budding which leads to the formation of membrane-bound fragments which are known as apoptotic bodies. These visible morphological changes were used in order to identify apoptotic cells in cytospin preparations of haemopoietic cells following staining with Romanovsky dye. Quantitation of the proportion of apoptotic cells within a population was performed by counting a minimum of 500 cells in Romanovsky-stained cytospin preparations, identifying apoptotic cells by cell shrinkage associated with nuclear compaction, cytoplasmic condensation and the presence of apoptotic bodies.

## 2.4.2. Quantitation of apoptotic cell populations: propidium iodide staining of ethanol-fixed cells.

Staining of ethanol-fixed cells with propidium iodide, a (fluorescent) dye which intercalates with nucleic acids, has been used routinely for cell cycle analysis and quantitation of cellular DNA. It has been observed that apoptotic cells have a reduced DNA content. This may be due to loss of smaller DNA fragments following endonuclease activation and diffusion of these low molecular weight DNA products from the cell. A second explanation is that the marked nuclear condensation which is characteristic of apoptosis, renders some nucleic acids inaccessible to propidum iodide binding (Darzynkiewicz *et al.*, 1997).

Flow cytometric analysis of ethanol-fixed and propidium iodide stained cells demonstrates that apoptotic cells appear as a 'sub-$G_0/G_1$' peak, due to the lower DNA content and therefore reduced fluorescence. This permits the percentage of apoptotic cells within a population to be quantified using the software incorporated into the flow cytometer to set markers around the sub-$G_0/G_1$ peak of fluorescence. This apoptotic population can be clearly demonstrated in leukaemic cell lines following incubation with

cytotoxic drugs, e.g HL60 (a promyelocytic leukaemia line) cells treated with 10μg/mL bischloronitrosourea (BCNU) produce a characteristic apoptotic peak, and the appearance of this peak correlates well with the definitive identification of apoptotic cells in a cytospin preparation by morphological criteria.




*Method for PI staining.* Mononuclear cells were washed twice in PBS by centrifugation at 400 xg for 5 minutes. After removal of supernatent, the cells were then fixed by the addition, slowly and with gentle mixing, of 1 mL ice cold 70 % ethanol (EtOH). Cells are left in EtOH to fix for a minimum of 15 minutes. Once fixed, the cells are stable when left at 4°C in EtOH for up to 2 weeks in my experience and possibly longer.
Fixed cells were washed twice in PBS as before and resuspended in 200μl of DNA staining medium, comprising 50μg/mL propidium iodide and 0.5 mg/mL Rnase A in PBS. Cells were stained for a minimum of 30 minutes prior to flow cytometric analysis.

## 2.4.3. Quantitation of apoptotic cell populations: annexin V-FITC staining of apoptotic cells.

One of the features associated with apoptosis is a change in membrane symmetry which results in the exposure on the cell surface of phosphatidylserine residues. These demonstrate a high affinity for binding to annexin V, and this property has been utilised in order to recognise apoptotic cells. This technique was utilised using a commercially available kit to verify the integrity of results obtained using the simpler propidium iodide staining technique describd above.

An apoptosis detection kit (R&D Systems) was used according to the manufactures insructions to analyse apoptosis in cell lines induced to undergo apoptosis following treatment with cytotoxic drugs or hyperthermia. Briefly, cells are incubated with an annexin V-FITC conjugate and propidium iodide (PI). Early apoptotic cells will be distinguished by their ability to bind annexin V-FITC, but the technique can also distinguish between apoptotis and necrosis by the fact that the former exclude PI because they still have an intact membrane. Flow cytometric analysis of annexin V-FITC and PI-stained cells can therefore be used to distinguish between live, apoptotic and necrotic cells. This technique was used in some experiments to verify the integrity of the results obtained by the propidium staining of fixed cells. In a comparison of the number of apoptotic cells within a population of HL-60 cells taken at different time intervals following treatment with 20μg/mL BCNU, there was no significant difference ($p=0.021$) between the number of apoptotic cells detected by these two techniques.

# Chapter 3.

# Analysis of the cellular localisation of heat shock proteins in myeloid leukaemia cells.

## 3.1 Introduction.

In mammalian cells the expression of heat shock proteins (hsp) is generally considered to be intracellular, restricted to the cytoplasm and including the endoplasmic reticulum and mitochondrial compartments (Mizzen *et al.*, 1989). The pattern of hsp expression under normal circumstances would obviously reflect the role of hsp as molecular chaperones, with specific hsp family members functioning within intracellular locations in the folding and unfolding of cellular proteins (see Introduction, section 1.1.5). However, under conditions of stress it has been demonstrated that members of the hsp70 family are found in the nuclear compartment. For example, both the constitutive and heat-inducible hsp70 rapidly accumulate within the nucleus of heat-stressed (42°C, 1 hour) human fibroblasts. Members of the hsp70 family interact with nuclear oncogenes such as *c-myc* and p53; nuclear co-localisation of hsp70 and viral *myc* proteins is observed in *myc*-overexpressing cells (Koskinen *et al.*, 1991), whilst the constitutive hsp70 (hsp73) forms complexes with mutant p53 in *ras*-transformed cells and human tumour cells (Nihei *et al.*, 1994, Fourie *et al.*, 1997 ). In addition, the formation of hsp: p53 complexes containing not only members of the hsp70 family, but also hsp90, is thought to represent the components of a molecular chaperone programme which affects the subcellular distribution of p53 protein (Merrick *et al.*, 1996).

As outlined in the Introduction, such interactions may play an important role in terms of cell growth and oncogenesis (see Section 1.2.2.). An examination of the cytoplasmic/nuclear distribution of hsp in leukaemia cells is therefore essential in order to demonstrate whether abnormal localisation of hsp is associated with leukaemogenesis. Another important aspect concerning the localisation of hsp is the possibility that they may also be expressed on the cell surface, and if so, then what is the significance of this observation? The potential expression of hsp on the surface membrane of cells was still a

controversial topic at the time of starting this investigation. Although some authors had found no evidence of cell surface expression (e.g Ferm *et al.*, 1989), others had reported hsp expression on the surface of certain cell types. For example, human γ/δ T cells express a cell surface antigen which is recognised by a monoclonal antibody to hsp58 (P1), the human homologue of the *Escherichia coli* groEL protein (Jarjour *et al.*, 1990). Also, murine bone marrow-derived macrophages have been shown to express an epitope which is recognised by a monoclonal antibody, ML30, which has specificity for mycobacterial hsp60 (Wand-Württenberger *et al.*, 1991). The significance of hsp expression on the surface of such cells is not clear, although it has been suggested that hsp recognition may play a role in autoimmunity and microbial infections and recent reports have implied that hsp expression on the surface of malignant cells may be important in terms of cancer immunity (see Introduction, section 1.1.9).

No reports exist in the literature concerning the specific localisation of hsp in primary leukaemic cells. It was therefore particularly important to analyse the cellular distribution of hsp in these cells, since cytoplasmic/nuclear redistribution clearly has biological implications. In addition, evidence of abnormal expression of hsp on the surface of cells may be relevant both in terms of cancer immunity, and as a mechanism for specifically targeting tumour cells with hsp-immunotoxin complexes.

Working in the Haematology Deparment at Warwick Hospital, I had access to blood samples from patients with a variety of haematological malignancies. In particular, I was interested in patients with acute myeloid leukaemia (AML) since loss of p53 function in AML appears to occur by mechanisms which occur at the protein level rather than by specific genotypic alterations. As described in the Introduction (section 1.2.1), p53 protein in the majority of AML cells is recognised by monoclonal antibodies specific to the mutant conformation of p53, despite the fact that genotypic alterations are relatively rare (Zhang and Deisseroth, 1992b). In addition, wild type p53 protein in AML cells has

been shown to switch to a mutant conformation in response to growth factor stimulation (Zhang and Deisseroth, 1994). In light of the report by Hainaut and Milner (1992) that members of the hsp70 family are involved in the switch between a wild-type and 'mutant' p53 conformation, it is tempting to suggest that in AML cells the loss of p53 function which occurs at the protein level is the result of a switch in conformation mediated by hsp70. For this reason, the analysis of hsp expression in leukaemic cells focussed primarily on cells from patients with AML.

## 3.2 Design of experimental protocol.

### 3.2.1. Normal and leukaemic haemopoietic cells.

Cells from patients with a variety of haematological malignancies presenting to the Haematology Department at Warwick Hospital were harvested by density gradient centrifugation as described in Chapter 2. As previously described, we were particularly interested in patients with AML, although availability of clinical material was dictated by the number of patients presenting with this disease. At Warwick Hospital, approximately 10 new patients with AML would present within a 12 month period, and a smaller number of patients with the disease in remission would relapse. In initial studies, I therefore also examined cells from patients with the other major haematological malignancies, namely chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL) and acute lymphocytic leukaemia (ALL).

### 3.2.2. Immunochemical analysis of hsp in haemopopietic cells.

*Flow cytometry.* Analysis of the expression and localisation of hsp was performed using immunochemical techniques (see Chapter 2, section 2.2). Immunofluorescent staining followed by flow cytometric analysis was the method of choice, both for examining the possibility of surface hsp expression, and for quantifying specific hsp levels (Chapter 4).

*Immunohistochemistry.* Subcellular localisation of hsp expression was in turn analysed by immunocytochemical techniques using specific hsp monoclonal antibodies to stain cytospin preparations (see Chapter 2, Methods, section 2.2). Examination of stained preparations by light microscopy provides details of cell morphology as well as visually demonstrating the nuclear/cytoplasmic distribution of hsp in leukaemic cells.

*Monoclonal antibodies.* Immunofluorescence and immunohistochemistry were both performed using monoclonal antibodies to several of the major heat shock protein families. At the outset of this section of experimental investigation, antibodies were available which recognised members of the hsp60, hsp70 and hsp90 families (see table 3.1, below). At the time of performing these analyses, the only anti-hsp70 monoclonal antibody available was one which recognised both the constitutive (hsp73) and inducible (hsp72) members of this family, and both qualitatative and quantitative analysis of hsp70 was performed initially using this antibody. Recently, an antibody has become commercially available which specifically recognises just the inducible (hsp72) protein. The initial studies of hsp70 localisation have therefore been recently repeated using the new anti-hsp72 antibody, although not in every case possible on the same patient samples due to availability of clinical material. Further details of the monoclonal antibodies used can be found in Chapter 2, section 2.2.

| Monoclonal Antibody | Specificity |
|---|---|
| Anti-hsp60 Clone LK1 | Produced using recombinant human hsp60 as immunogen, recognising an epitope located between residues 383-447 of human hsp60. |
| ML30 | Recognises amino acids 275-295 of the 65kDa hsp from *Mycobacterium leprae* and *M. tuberculosis*. Will cross-react with human hsp65. |
| Anti-hsp70 (72/73) | Produced using purified hsp70 from bovine brain as immunogen. The antibody localises both the constitutive (hsp73) and the inducible (hsp72) forms of hsp70. |
| Anti-hsp90 | Clone AC-16, recognises both the constitutive and inducible forms of hsp90. Does not bind to the native (unbound) form of hsp90. |

**Table 3.1.** Monoclonal antibodies used to determine the localisation of the major hsp in haemopoietic cells. These unconjugated antibodies were used as primary monoclonals to examine surface expression by indirect immunofluorescence techniques, and subcellular localisation by immunohistochemical staining of cytospin preparations.

## 3.3. Results.

### 3.3.1. Heat shock proteins are not expressed on the surface of acute myeloid leukaemia cells.

Mononuclear cells from 16 patients with AML were harvested by density gradient centrifugation (Lymphoprep) from whole blood samples collected into EDTA anticoagulant. Cytospin preparations of harvested cells were made immediately following separation, and, where possible, immunofluorescent staining of unfixed cells followed by flow cytometric analysis was performed to examine whether hsp expression could be

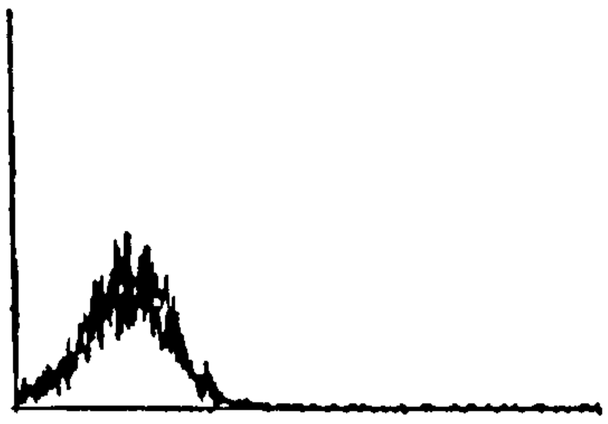
detected on the cell surface. Aliquots of leukaemic cells were also cryopreserved and, in certain cases, immunofluorescent staining was performed retrospectively on thawed samples. In these instances, cell viability was checked by the ability of cells to exclude propidium iodide, and in all cases this exceeded 89%.

Immunofluorescent staining of unfixed cells using the four monoclonal antibodies listed in table 3.2 was performed initially by an indirect two-step protocol, using the primary unlabelled hsp monoclonal antibody, followed by a FITC-labelled goat-anti mouse antibody. An isotype-matched negative control adjusted to the same immunoglobulin concentration was used for each individual aliquot of patient cells to demonstrate non-specific binding to the cell surface. In subsequent experiments, a three-step technique was employed, using an avidin-biotin conjugate followed by FITC-labelled streptavidin in order to enhance the sensitivity of the procedure (see Chapter 2, Methods). Following immunofluorescent staining by both techniques, cells were washed and fixed in 2% paraformaldehyde prior to flow cytometric analysis (Coulter Epics II).

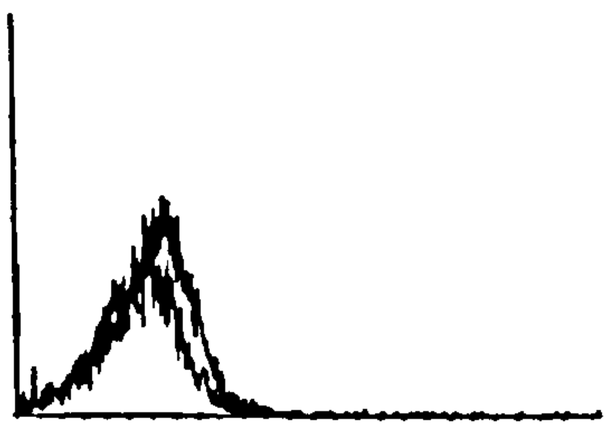
Analysis of leukaemic cells from all sixteen AML patients revealed no increase in specific staining with any of the four anti-hsp monoclonal antibodies when compared with the negative control antibody (Figure 3.1). This was apparent when using both the two-step immunofluorescent staining procedure and the more sensitive avidin-biotin technique. Final immunoglobulin concentrations of 0.2-20μg/mL of each of the four antibodies were employed in order to verify that the lack of hsp antigen was not due to inappropriate antigen/antibody ratios. Whilst increasing immunoglobulin concentrations led to a slight increase in fluorescence, this was not specific as demonstrated by a similar increase, up to saturating concentrations, of the isotype-matched control antibody.

Analysis of leukaemic cells was performed on both freshly isolated cells, and cells which

had been cryopreserved in liquid nitrogen. This is an important point to emphasise since it is recognised that cryopreservation can lead to the deterioration of some antigenic epitopes.



a)



b)

**Figure 3.1.** Flow cytometric analysis of AML cells reveals an absence of surface expression for any of the major hsp. Patient cells were probed with antibodies to ML30, hsp60, hsp70 (72/73) and hsp90 followed by a goat anti-mouse/FITC conjugate. Histograms of cell number versus LFL1 demonstrate no increase in specific staining with the a) hsp72/73 and b) hsp90 monoclonal antibodies compared with the isotype-matched negative control (red line).
Similar results (not shown) were obtained in further experiments using a more sensitive three-stage avidin-biotin immunofluorescent technique, as described in Chapter 2, Methods, section 2.2).

As described earlier, at the time of these experiments, the only commercially available hsp70 monoclonal antibody available recognised both the constitutive and inducible isoforms, although recently an antibody specifically recognising the inducible (hsp72) antigen has become available. Accordingly, I have recently re-analysed cells from a group of 10 AML samples for the surface expression of this antigen. Again, using both double- and triple immunofluorescence techniques, I have been unable to demonstrate the presence of hsp72 on the surface of AML cells (results not shown).

### 3.3.2. Hsp are not expressed on the surface of other malignant or normal haemopoietic cells.

Immunofluorescent staining of cells from 6 patients with acute lymphoblastic leukaemia (ALL), 4 patients with B-cell chronic lymphocytic leukaemia (B-CLL) and 2 patients with chronic myeloid leukaemia revealed no increase in specific staining using any of the four antibodies listed in table 3.1. In addition, normal peripheral blood mononuclear cells were harvested from 10 healthy volunteers, and again in these cells there was no detectable expression of any of the major hsp on the surface membrane of these cells (results not shown).

### 3.3.3. Hyperthermic treatment of myeloid leukaemia cells does not induce surface hsp expression.

Hsp expression was also analysed on the mononuclear cells from 6 AML patients following exposure to a hyperthermic stress (42°C for 30 minutes). Subsequent to heat shock, cells were placed in an incubator at 37°C for six hours prior to immunofluorescent staining. In addition to the four antibodies listed in table 3.1, the antibody specific to the highly heat-inducible hsp72 protein was used. Again, no detectable expression on the surface of these cells was apparent (results not shown).

### 3.3.4. Hsp expression is also undetectable on myeloid cell lines.

Four haemopoietic cell lines, KU812 (myeloid), KG1a (myeloid), THP-1 (myelomonocytic), and HL-60 (promyelocytic) were analysed for surface expression of

hsp. Exponentially growing cells were stained with primary monoclonal antibodies to the five anti-hsp antibodies available and analysed as before by flow cytometry. In repeated experiments, none of the four cell lines was found to express hsp on the cell surface (results not shown). In addition, cell lines were exposed to a hyperthermic stress by incubation of the cells at 42°C for 30 minutes. Analysis of cells following recovery at 37°C revealed that surface hsp expression was undetectable on any of the four cell lines using samples taken at time intervals of 4, 8, 16, 24 and 48 hours post heat shock.

### 3.3.5. Myeloma cell lines undergoing apoptosis express hsp72 and hsp90 on the cell surface.

Recently, as part of a novel research project examining the potential use of a bacterial toxin (verocytotoxin) to induce apoptosis in myeloma cells, I have been examining the influence of hsp expression on the induction of apoptosis by verocytotoxin, as well as various cytotoxic agents. In order to examine hsp expression in these cells undergoing apoptosis, I developed a double-labelling protocol using annexin V-FITC in combination with probing with the hsp antibodies using an RPE-labelled secondary antibody. One of the earliest gross morphological events during apoptosis is the loss of cell membrane phosholipid asymmetry, resulting in the exposure of phosphotidylserine on the outer cell membrane. Annexin V has a selective affinity for negatively charged phospholipids, which are normally only found on the inner side of the cell membrane, and the differential staining of cells with annexin V has been shown to be specific to cells during the early stages of apoptosis.

These experiments demonstrated that the induction of apoptosis, as indicated by increased annexin V binding, is associated with the expression of both hsp72 and hsp90

on the cell surface (Figure 3.2). Exposure of myeloma cell lines to recombinant verocytotoxin (2-20pg/mL) induces apoptosis, the early stages of which are evident by a time interval of 6 hours following exposure to verocytotoxin.

## 3.4 Intracellular localisation of hsp expression in haematopoietic cells.

The intracellular expression of hsp was next analysed in haematopoietic cells using an immunoperoxidase technique to stain cytospin preparations. Fixation of the cells in acetone:methanol (1:1) permeabilises cell membranes and therefore provides information on the intracellular location of the antigen of interest.

### 3.4.1. Normal mononuclear cells.

Cytospin preparations of mononuclear cells from 6 normal healthy individuals were prepared. Romanowsky staining of these revealed that in each case the cells present were >96% lymphocytes and monocytes as assessed by morphological criteria. Immuno-peroxidase staining of normal lymphocytes and monocytes reveals that members of the hsp60, hsp70 (72/73) and hsp90 families are all expressed within the cytoplasm of these cells, even in the absence of stress. Cytospin preparations of mononuclear cells from six normal individuals were probed with the monoclonal antibodies, ML30, anti-hsp70 (72/73) and anti-hsp90. The expression of all three hsp families was evident in lymphocytes and monocytes as a diffuse cytoplasmic staining. No evidence was found of

nuclear localisation with any of the three antibodies employed. Plate 3.1 is a Romanowsky-stained cytospin of normal mononuclear cells following density gradient centrifugation and washing. The mononuclear cell fraction recovered can be seen to be comprised of lymphocytes and monocytes. Plate 3.2 demonstrates the typical diffuse cytoplasmic staining seen in these normal mononuclear cells with the monc clonal antibody to hsp72/73. The same distribution of antigen was seen in these cells when probed with ML30 and anti-hsp90 (data not shown).

Cytospin preparations of cells harvested from normal individuals were also stained with an anti-p53 monoclonal antibody, recognising both mutant and wild-type conformations of p53. Light microscopic examination of immunostained cells revealed no detectable expression of the p53 tumour suppressor gene product in normal mononuclear cells from any of the six individuals used in this study.

### 3.4.2. Mitogen-stimulated lymphocytes.

Normal mononuclear cells exposed to concanavalin A (5μg/mL) and pokeweed mitogen (1μg/mL) transformed and proliferated during *in vitro* culture as evidenced by the appearance of mitotic figures and large cells of immature appearance, identifiable by light microscopy analysis of Romanowsky-stained cytospin preparations (Plate 3.3). Immunoperoxidase staining of cytospins prepared from lymphocytes after 48 hours exposure to concanavalin A and pokeweed mitogen revealed that in both cases intense staining of the larger transformed lymphocytes was evident with antibodies to hsp72/73 and hsp90. Plate 3.4 illustrates the intense staining pattern seen for hsp72/73 in the larger mitogen-transformed lymphocytes compared with the smaller normal lymphocytes. The ability to distinguish morphologically between smaller untransformed lymphocytes

and the larger transformed cells within the same field of view acts as a useful internal control. Again, the expression of hsp72/73 and hsp90 is largely confined to the cytoplasm, although in some transformed lymphocytes there is clearly some nuclear staining with the antibody for hsp72/73.

Intracellular distribution of hsp60 in mitogen-transformed lymphocytes, as determined by immunohistochemical staining with ML30 and the anti-hsp60 monoclonal antibodies, was confined to the cytoplasmic compartment. Furthermore, unlike hsp72/73 and hsp90, there did not appear to be any difference in the pattern or intensity of staining compared with normal lymphocytes (results not shown).

Interestingly, probing with an antibody to p53 (recognising both wild-type and mutant conformations) reveals that mitogen-stimulated lymphocytes express p53 located in both the both cytoplasm and the nucleus (Plate 3.5).

### 3.3.3. Leukaemic cells from patients with AML.

Cytospin preparations of leukaemic cells from 16 AML patients were stained with antibodies to hsp60, hsp72/73 and hsp90. In the absence of stress, significant levels of all three heat shock protein antigens were evident in all 16 cases, and again the heat shock proteins were generally localised within the cytoplasm (plate 3.6). Immunoperoxidase staining using an isotype-matched negative control antibody revealed no non-specific staining. However, myeloblasts from one patient clearly demonstrated significant nuclear localisation of hsp72/73 (plate 3.7). Immunohistochemical staining using the p53 antibody revealed that no p53 expression was evident in 15/16 samples, but cells from that patient which demonstrated nuclear localisation of hsp72/73, also exhibited significant nuclear expression of p53 (plate 3.8). This is an interesting observation,

although isolated, since this particular patient had developed resistance to standard cytotoxic therapy.

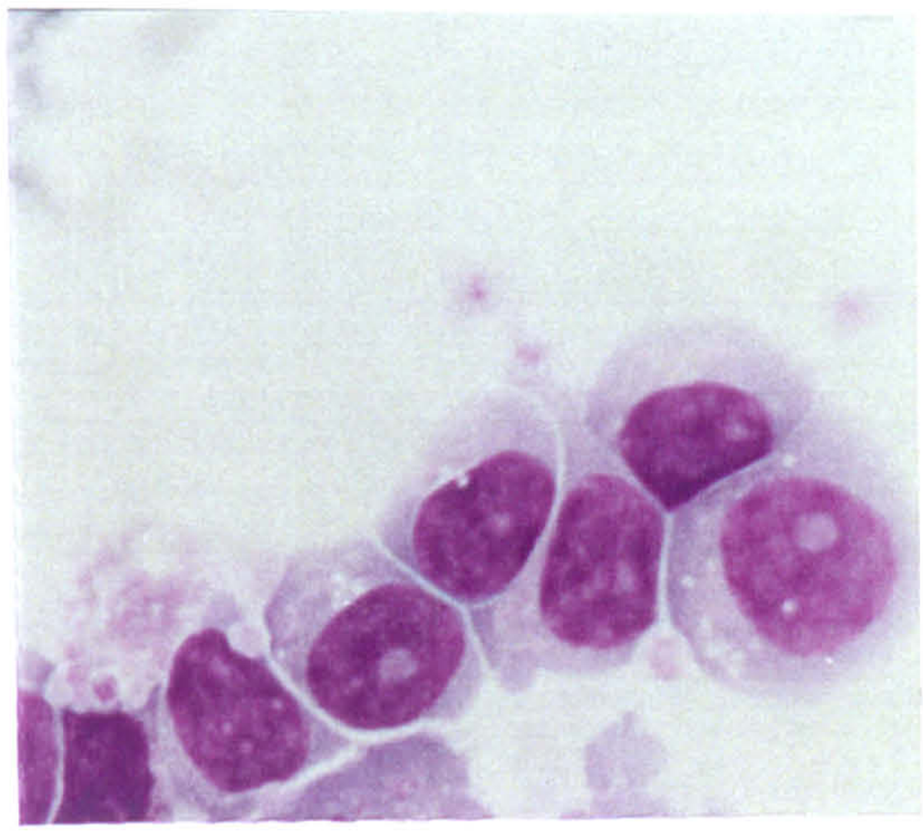


**Plate 3.1.** Romanowsky-stained cytospin preparation of normal peripheral blood mononuclear cells.

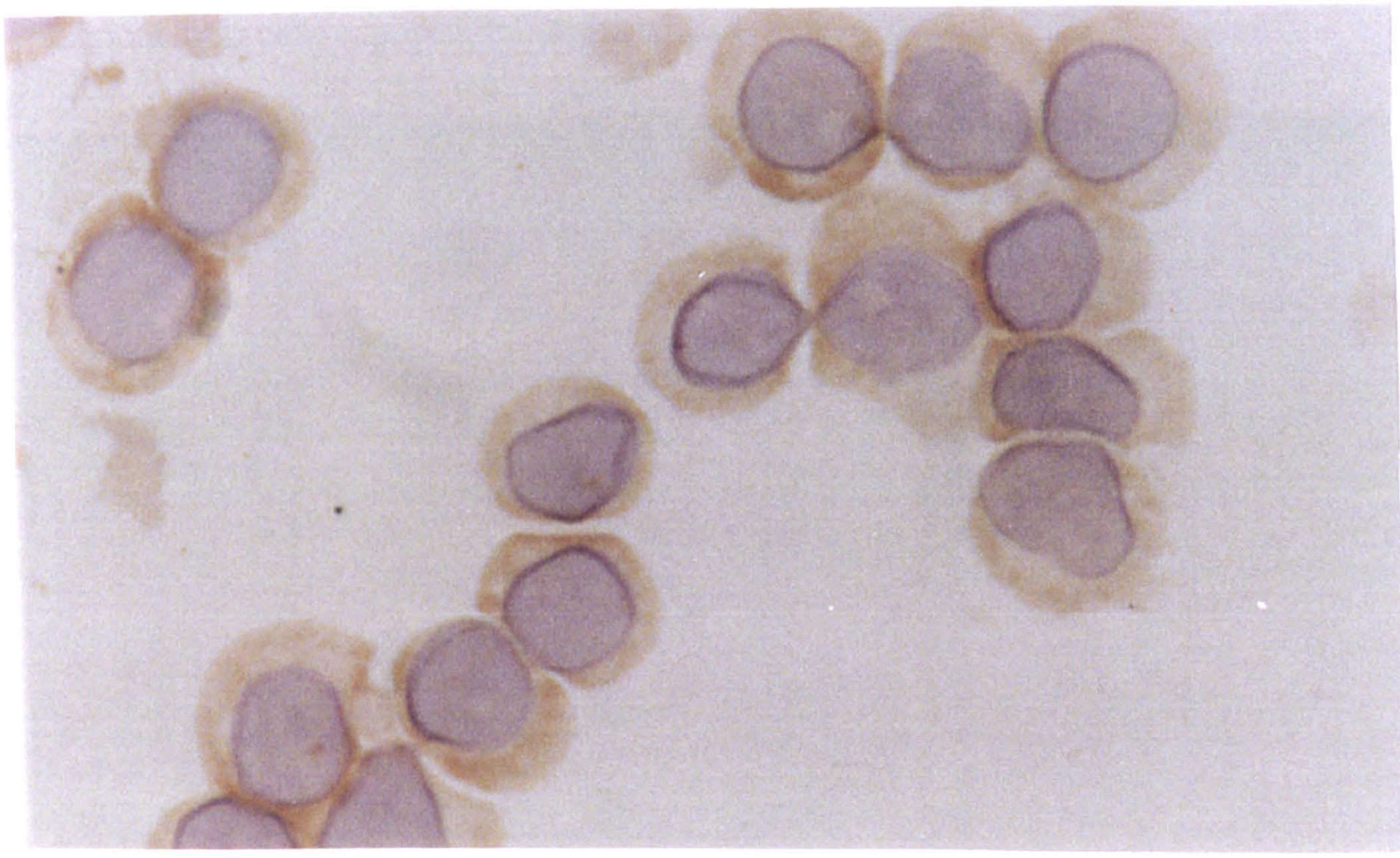


**Plate 3.2.** Immunoperoxidase staining of peripheral blood mononuclear cells with monoclonal antibody to hsp72/73, demonstrating a diffuse pattern of distribution throughout the cytoplasm.

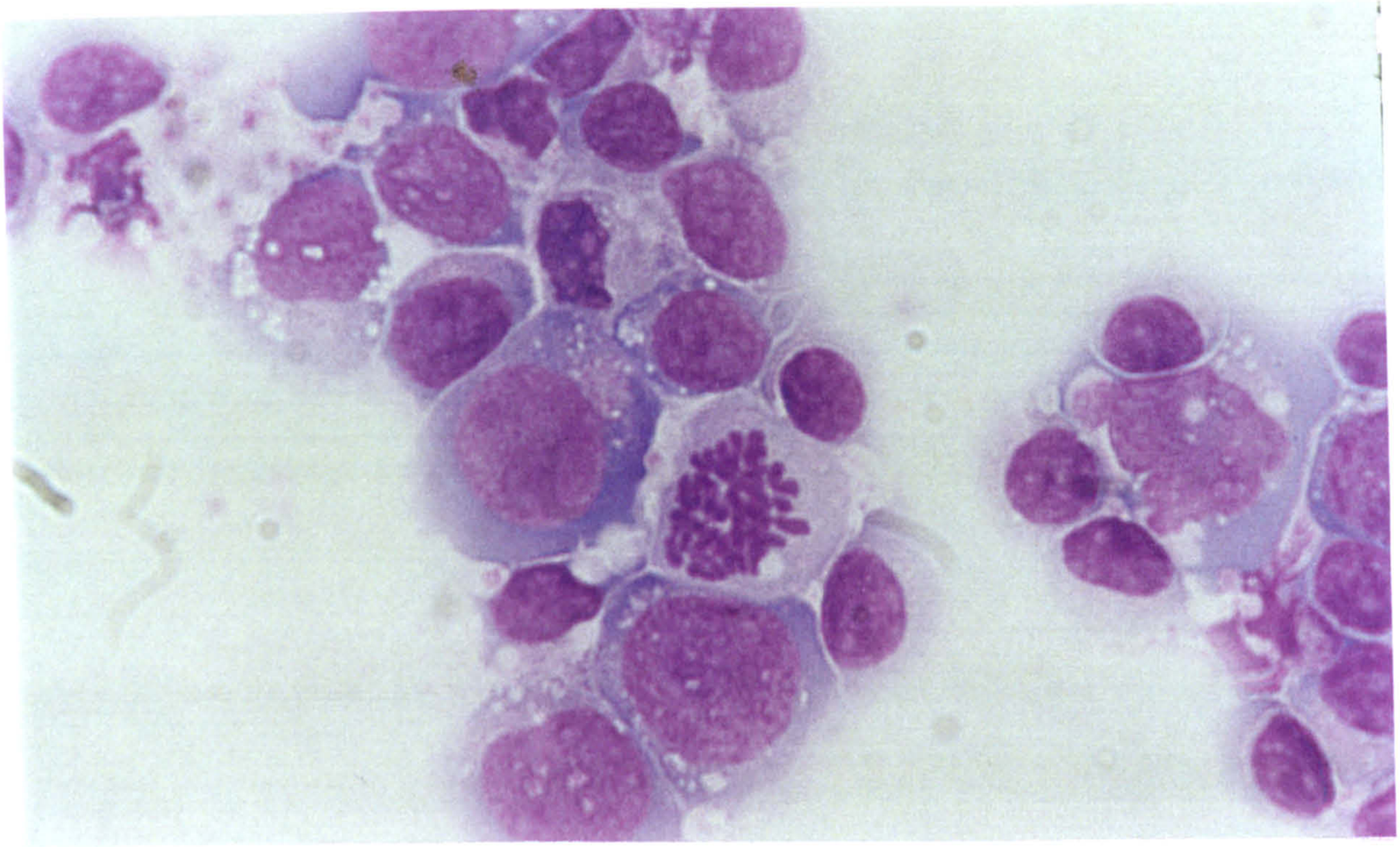


**Plate 3.3.** Romanowsky-stained cytospin preparation of normal peripheral blood mononuclear cells exposed for 48 hrs to the mitogen, concanavalin A (5μg/mL).

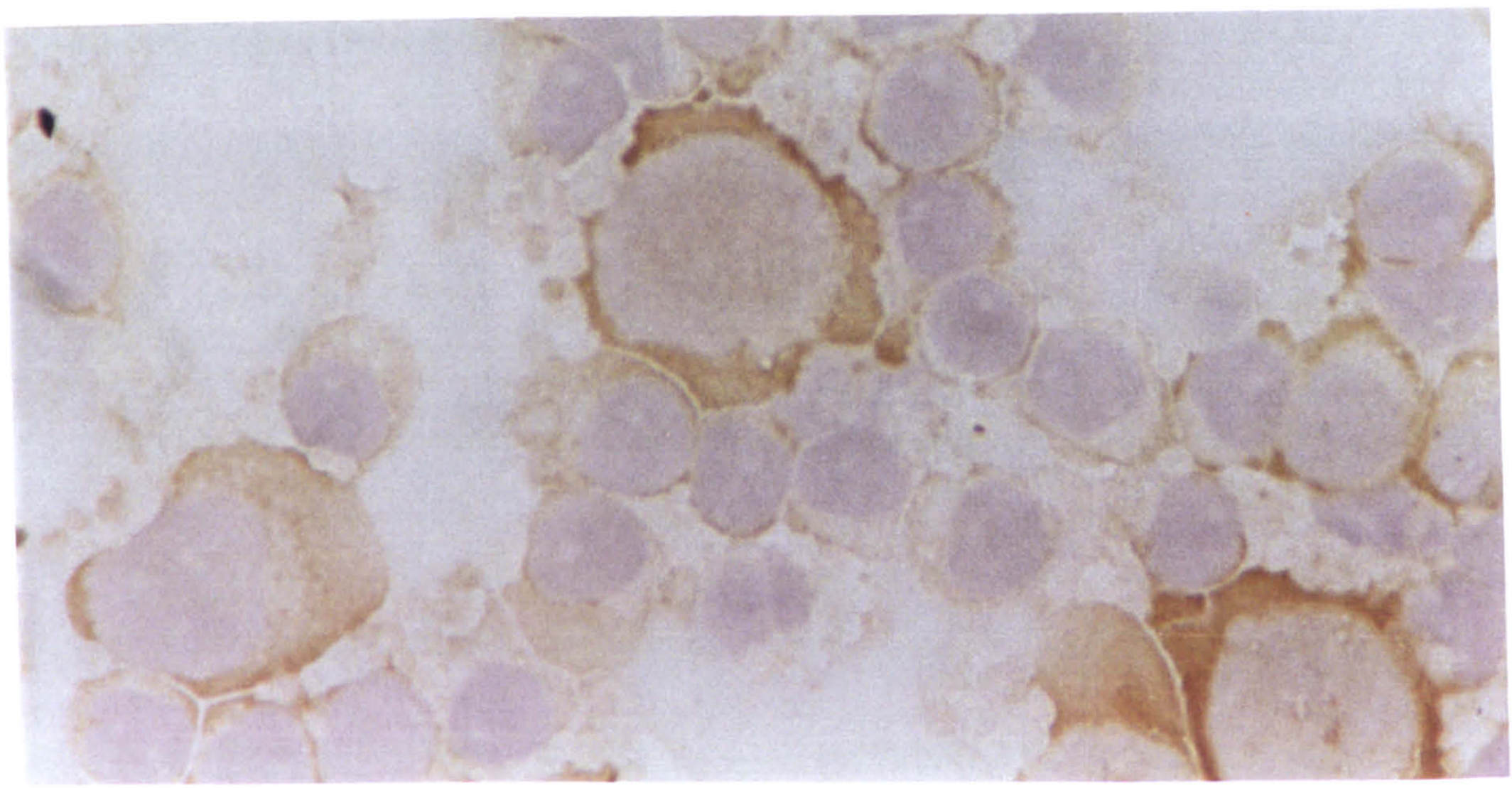


**Plate 3.4.** Immunoperoxidase staining of mitogen (concanavalin A, 5μg/mL)-transformed normal lymphocytes with anti-hsp72/73. The larger transformed lymphocytes demonstrate more intense cytoplasmic staining.

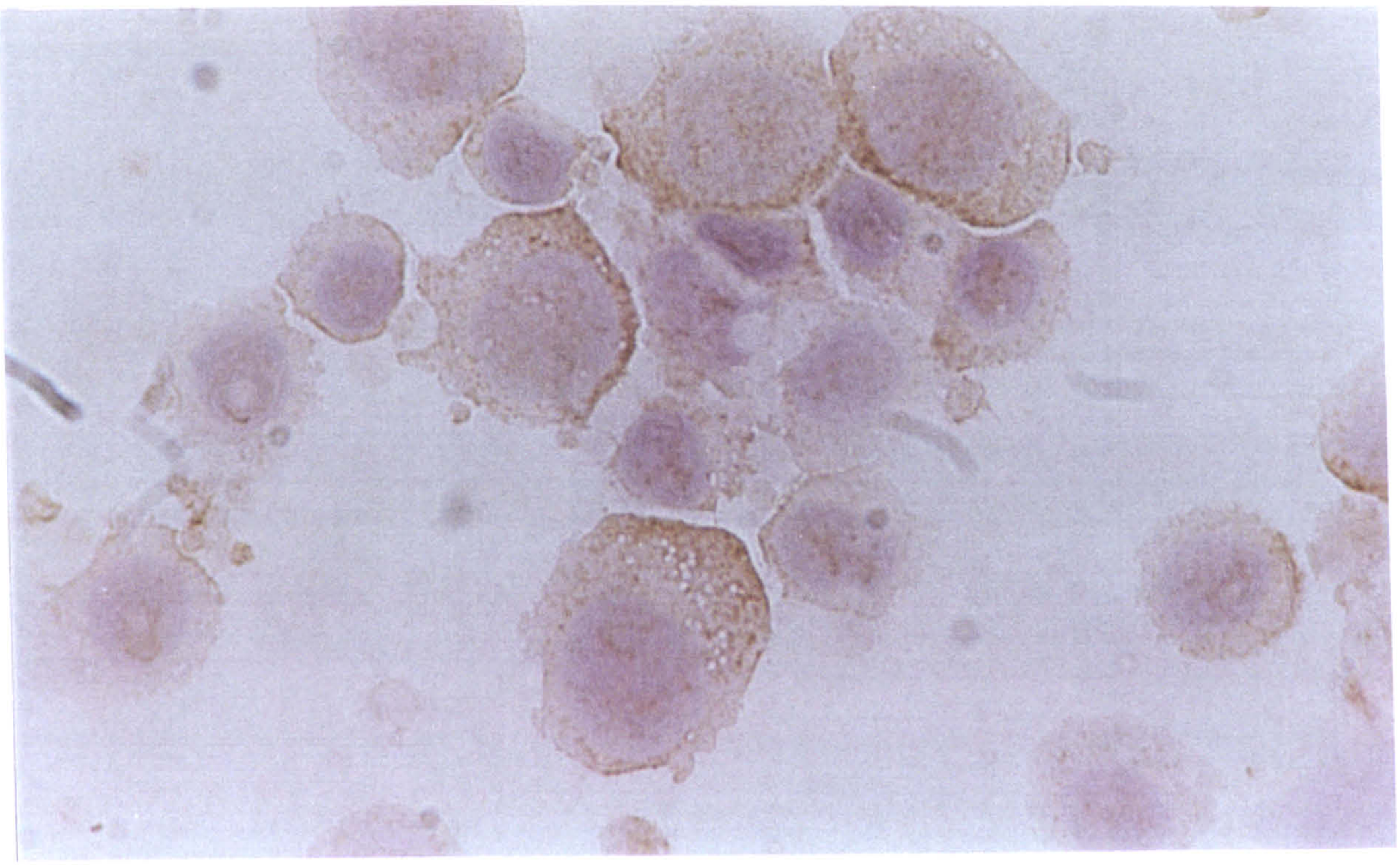


**Plate 3.5.** Immunoperoxidase staining of mitogen-transformed lymphocytes demonstrates accumulation of p53 in both the cytoplasmic and nuclear compartments. Normal PBMC were exposed to the mitogen, concanavalin A, for 48 hours and cytospin preparations stained using an antibody recognising both the wild-type and mutant conformations of p53 protein.

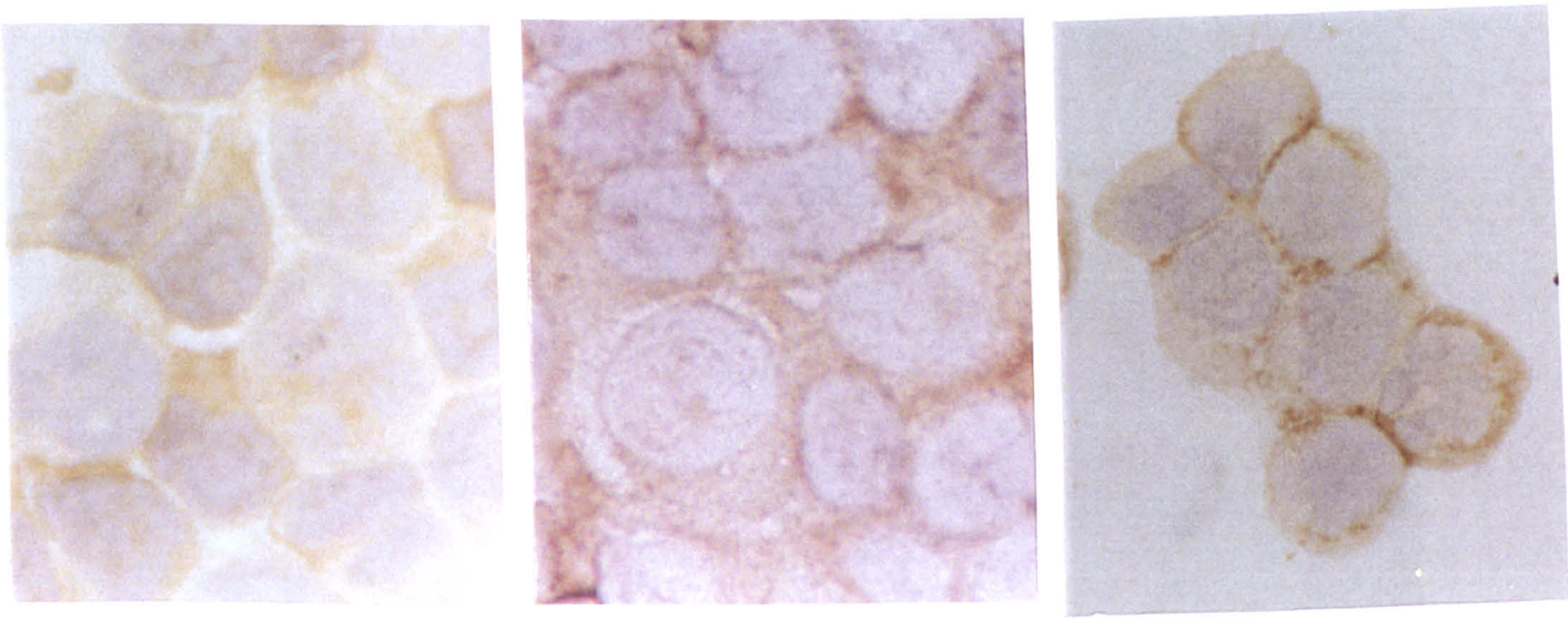


**Plate 3.6.** Immunoperoxidase staining of myeloblasts from a patient with acute myeloid leukaemia (AML). Cytospin preparations were stained with a) anti-hsp60, b) anti-hsp72/73 and c) anti-hsp90. Typically, AML cells demonstrate diffuse cytoplasmic staining with all three antibodies.

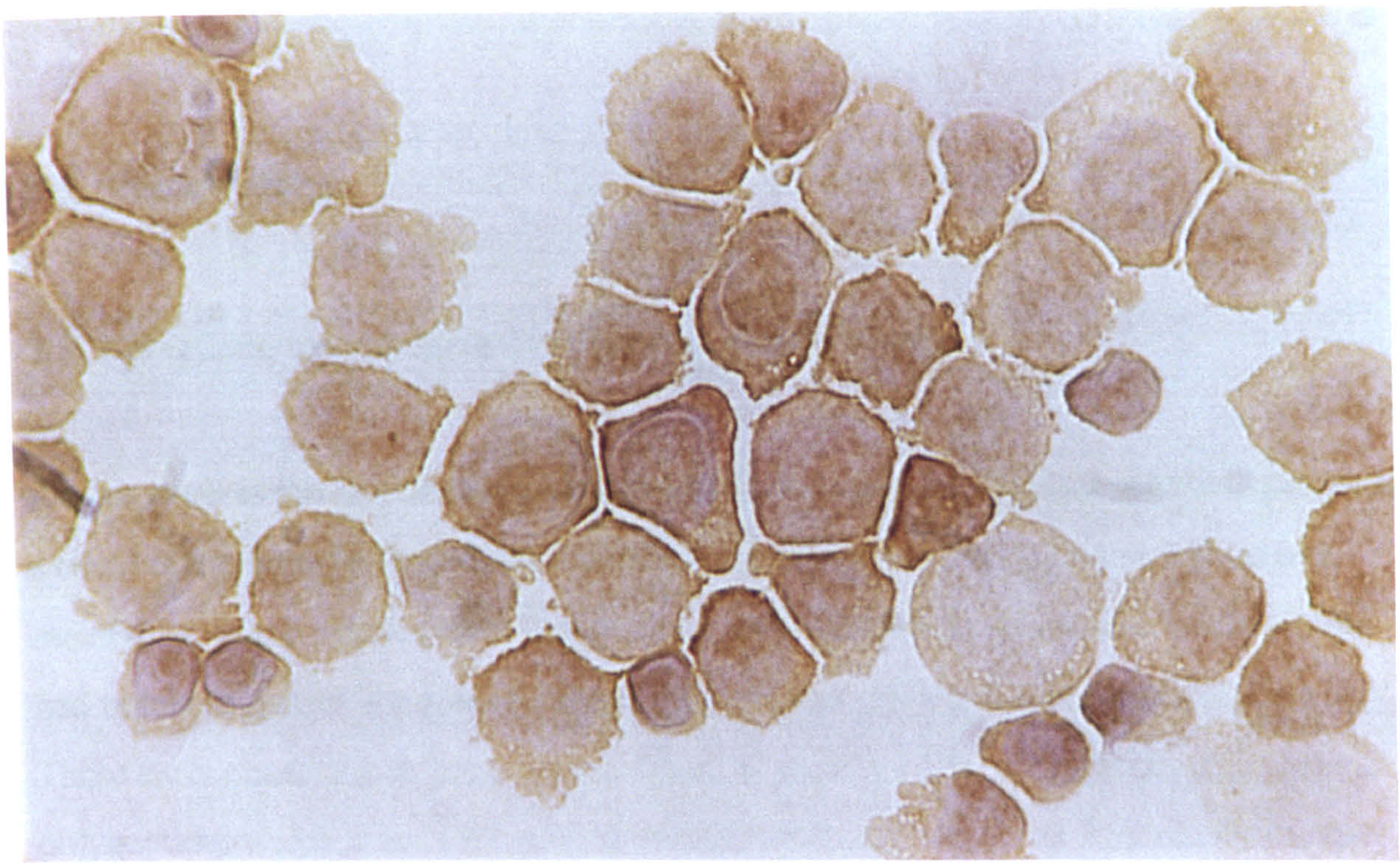


**Plate 3.7.** Myeloblasts from one patient (1/16) with AML demonstrate nuclear staining with an antibody to hsp72/73. These cells were harvested from a patient who remained refractory to standard cytotoxic therapy.

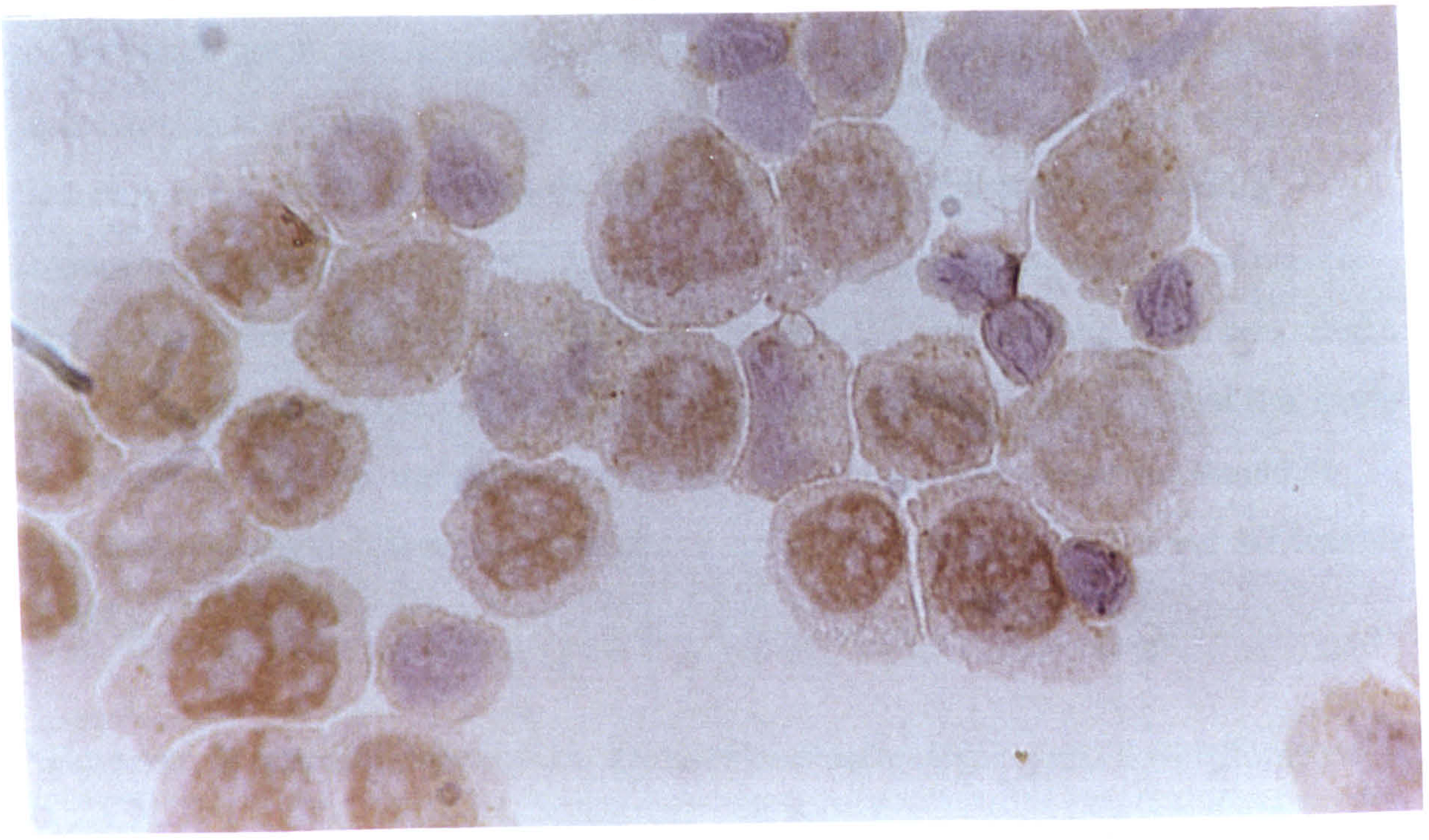


**Plate 3.8.** Cells from the same patient also demonstrate nuclear expression of p53 protein.

## 3.5. Discussion.

### 3.5.1.Surface expression of heat shock proteins.

As described in the Introduction, at the outset of these investigations conflicting reports existed in the literature regarding the existence of hsp expression on the cell surface membrane. Some authors have found no evidence of surface expression, whilst others had reported surface expression of epitopes reactive with antibodies raised against hsp. These early observations demonstrated that, at least in a few very specific cell types, polypeptides which react with anti-hsp monoclonals and which are in some cases heat-inducible, are expressed on the cell surface membrane. The possible relevance of these observations has been suggested by several subsequent reports which propose a role for hsp molecules in antigen processing and presentation. It has been reported that members of the hsp70 family are expressed on the surface of resting antigen presenting cells as demonstrated by immunogold labelling with an anti-hsp72/73 monoclonal antibody (Manara *et al.*, 1993). Furthermore, these authors found that incubation with a monoclonal antibody to hsp72/73 inhibited the antigen presenting function of human monocytes. It appears most likely that the appearance of hsp on the cell surface occurs as a result of the chaperoning function of hsp, and it has been suggested that this mode of peptide transfer through direct interaction with a molecular chaperone would be preferable to direct diffusion of peptides to cell surface destinations (Li and Srivastava, 1994).

However, in repeated experiments, immunofluorescent staining and flow cytometric analysis of a variety of primary leukaemia cells revealed no detectable expression of any of the major hsp families on the surface of these cells. Cells from 16 patients with AML

were examined, as well as 6 patients with ALL and 4 patients with B-CLL, a total of 28 individuals. The design of the experiments was such that a range of primary hsp antibody concentrations was employed in order to ensure that the correct antibody/antigen ratio would be achieved. In addition, both an indirect two-step technique, and then subsequently a much more sensitive protocol using a three-step avidin-biotin staining procedure were used. However, in no analysis of any sample was there any evidence of specific cell surface staining for hsp90, hsp72/73 or ML30-reactive epitopes.

In addition, the effect of a hyperthermic shock on the possible surface expression of AML cells was analysed. Leukaemic cells, suspended in RPMI 1640 culture medium, were heat shocked (42°C, 30 min.) and then placed in an incubator at 37°C for six hours. After this recovery period, cells were stained as before using monoclonal antibodies to ML30, hsp90 and hsp72/73, but in addition with an antibody specific to the highly heat-inducible hsp72 protein. Again, repeated experiments failed to demonstrate the induction of hsp expression at the cell surface. The data indicate that hsps are expressed infrequently, if ever, on the surface of leukaemic cells.

Indirect immunofluorescence is a sensitive technique which is capable of detecting low levels of antigen, and has been one of the techniques which has been utilised by other authors to report surface hsp expression in certain sub-sets of haemopoietic cells. Having found that surface expression of hsp was not detectable using a two-step indirect approach, I subsequently performed a series of experiments using avidin-biotin amplification, again finding that none of the major hsp families were expressed on the cell surface. Repeated experiments and titration of both primary and secondary antibody concentrations were used to ensure that the correct antigen:antibody ration was achieved and therefore not the reason for the inability to demonstrate any surface expression ni the cells examined.

Although I have been unable to demonstrate any evidence of surface hsp expression in normal or leukaemic haemopoietic cells, I have recently examined the role of hsp expression in myeloma cell lines which have been induced to undergo apoptosis in response to the cytotoxic drug, bischloronitrosourea (BCNU). As part of this investigation, I have looked for surface expression of hsp, and preliminary experiments have indicated that apoptotic cells do in fact express both hsp70 (the specific heat-inducible hsp72) and hsp90 on the cell surface. In these experiments, the induction of apoptosis by BCNU was demonstrated by increased annexin V binding, which was evident on myeloma cells at approximately 6 hours after the addition of BCNU. Double labelling of these cells, revealed that early apoptotic cells which were identified by annexin V binding expressed hsp on the cell surface. At this stage, the significance of these observations remain unclear, although it is feasible that hsp may have a functional role in the apoptotic process. It is possible that these hsp function as direct antigenic determinants which act as an apoptotic 'flag' and a signal to neighbouring phagocytic cells. There is a precedent for this hypothesis in the expression of ubiquitin, which itself can be classified as an hsp since it is induced by hyperthermia, and is well known to act as a flag to phagocytic cells. Alternatively, hsp may be functioning as molecular chaperones by interacting with other cellular protein(s) which are transported to the cell surface during the apoptotic process. This interesting observation is currently being investigated further.

### 3.5.2. Localisation of hsp by immunocytochemical staining.

Examination of immunostained cytospins of normal mononuclear cells demonstrates that these cells express detectable levels of hsp60, hsp70 and hsp90 in the absence of stress. No nuclear localisation was evident in any of the preparations from ten normal individuals. In each case, hsp60, hsp70 and hsp90 were immunolocalised within the cytoplasm, as evidenced by a diffuse cytoplasmic staining pattern. The expression of the tumour suppressor gene product, p53, was also analysed in these cells and no evidence of cytoplasmic or nuclear p53 accumulation was evident by immunoperoxidase techniques.

The finding that normal mononuclear cells constitutively express detectable levels of hsp, even in the absence of any stress, reflects the importance of these molecules as molecular chaperones.

Stimulation of normal peripheral blood lymphocytes with the mitogens concanavalin A or pokeweed mitogen induced transformation and proliferation with an associated upregulation of hsp70 and hsp90. As outlined in the Introduction (Section 1.1.6), a link between hsp expression and cell growth and differentiation is suggested by a number of reports which describe changes in hsp expression during the growth and differentiation of haemopoietic cell lines (e.g Galea-Lauri *et al.*, 1996, Mivechi *et al.*, 1994). In my analysis of mitogen-stimulated lymphocytes, the expression of hsp in these cells was predominantly cytoplasmic, although hsp72/73 was also localised within the nucleus. It is therefore interesting to note that p53 staining of mitogen-stimulated lymphocytes revealed nuclear accumulation of this tumour suppressor gene product. This finding may be significant in terms of the relationship between hsp70 and p53, and may have relevance to the proliferation of haemopoietic cells. For example, it has been reported that wild-type p53 protein in AML cells switches to a mutant conformation in response

to growth factor stimulation, leading to the suggestion that conformational changes in normal p53 protein represent an important mechanism for the regulation of cell proliferation (Zhang and Deisseroth, 1994). Further evidence to support this theory comes from the observation that different conformations of p53 protein in AML cells relate to the *in vitro* growth characteristics of the cells, and this can be regulated by either exogenous or autocrine haemopoietic growth factors (Zhu *et al,* 1993).

Leukaemic cells from AML patients express significant levels of hsp60, hsp70 and hsp90 in the absence of stress. Light microscopy of immunperoxidase-stained cytospins revealed that the expression of all three hsp was predominantly cytoplasmic. In fifteen of sixteen cases, no nuclear staining was evident with any of the three hsp monoclonal antibodies, but a strong pattern of nuclear staining for hsp72/73 was evident in the myeloblasts of one patient. Furthermore, immunoperoxidase staining with an antibody to p53 of cytospins from the same patient revealed significant nuclear expression of this tumour suppressor gene product, whilst immunostaining of the cells from all fifteen other AML patients revealed no significant staining for p53, either cytoplasmic or nuclear. This nuclear co-localisation of hsp72/73 and p53 is interesting since these cells were from a patient whose disease had undergone a second relapse and had become particularly resistant to chemotherapy. However, the relevance of this observation is uncertain, and since it was only apparent in this one patient, it is impossible to definitively ascribe a clear association between nuclear co-localisation of hsp72/73 and p53 and any clinical parameters such as drug resistance or tumour aggressiveness. Clearly, it would be desirable to investigate this further, and a much larger sample of AML patients including a larger number with drug resistant malignant cells would be required. In order to address this, cells from AML patients continue to be harvested at Warwick Hospital so that a larger study may shed light on any potential association between hsp:p53 localisation and drug resistance.

# Chapter 4

# Quantitation of Heat Shock Protein expresson in myeloid leukaemia cells.

# 4.1. Introduction.

In Chapter 3, it was demonstrated that both normal and malignant haemopoietic cells, and mitogen-stimulated lymphocytes, express detectable intracellular levels of all three major hsp families, predominantly localised in the cytoplasm. As described in the main Introduction, the significance of hsp expression in tumour cells has important implications, both in terms of cell growth and proliferation, and by virtue of their protective role which may be important in terms of the response of tumour cells to chemotherapy. Indeed, it has been reported that hsp expression in breast cancer cells has prognostic implications in terms of drug resistance (Fuqua *et al.*, 1994). At the outset of this investigation, no reports existed in the literature describing hsp levels in primary leukaemia cells, and an analysis of hsp expression in a variety of patients with myeloid leukaemias was therefore undertaken in order to examine whether abnormal levels of hsp expression could be detected in these cells.

# 4.2 Patients and methods.

## 4.2.1. Collection of leukaemic cells.

Mononuclear cells were harvested by density gradient centrifugation from peripheral blood samples of 12 patients with acute myeloid leukaemia (AML), and 9 patients with chronic myeloid leukaemia (CML). The AML patients were diagnosed according to the French-American-British (FAB) classification of acute myeloid leukaemia (table I) which

sub-classifies AML according to morphological criteria. These included 6 *de novo* cases of AML, 3 patients who transformed to frank AML (2 myelodysplastic and 1 chronic myeloid leukaemia) and 3 patients whose disease had relapsed (2 patients at first relapse, 1 patient second relapse). Of the 9 patients presenting with CML, retrospective cytogenetic analysis was available for only 3 of these patients, and this demonstrated that all three possesses the Philadelphia chromosome translocation.

All leukaemic cells used in this study were harvested from blood samples collected on the day of initial presentation, prior to the administration of any chemotherapeutic drugs. In addition, none of the patients exhibited a temperature above 37.5°C. Normal mononuclear cells from ten healthy volunteers were also harvested.

| **AML FAB type** | **Patient** |
|---|---|
| M2 Myeloblastic with maturation | K.W, S.B, M.K, C.J, M.B |
| M3 Acute promyelocytic | K.H |
| M4 Acute myelomonocytic | L.R, B.T, A.M |
| M5 Acute monoblastic | M.S, M.M |
| M7 Megakaryoblastic | D.G |

**Table 4.1.** Classification of AML patients according to the French-American- British (FAB) system

### 4.2.2. Quantitation of intracellular heat shock proteins in leukaemic cells.

Measurement of intracellular hsp levels was performed by immunofluorescent analysis of fixed and permeabilised patient cells using the same antibodies described in chapter 3. The development of the optimal staining procedure is described in Chapter 2, Materials and Methods. Flow cytometric analysis was used to quantify hsp levels, again because of the same advantages described in the previous chapter.

## 4.3 Results.

### 4.3.1. Expression of hsps in normal mononuclear cells.

Normal peripheral blood mononuclear cells (PBMC) exhibited positive immuno-fluorescence staining with antibodies to the three major heat shock protein families, hsp60, hsp72/73 and hsp90. Figure 4.1 illustrates the typical flow cytometric histograms of fluorescence staining with the three anti-hsp antibodies for one individual.

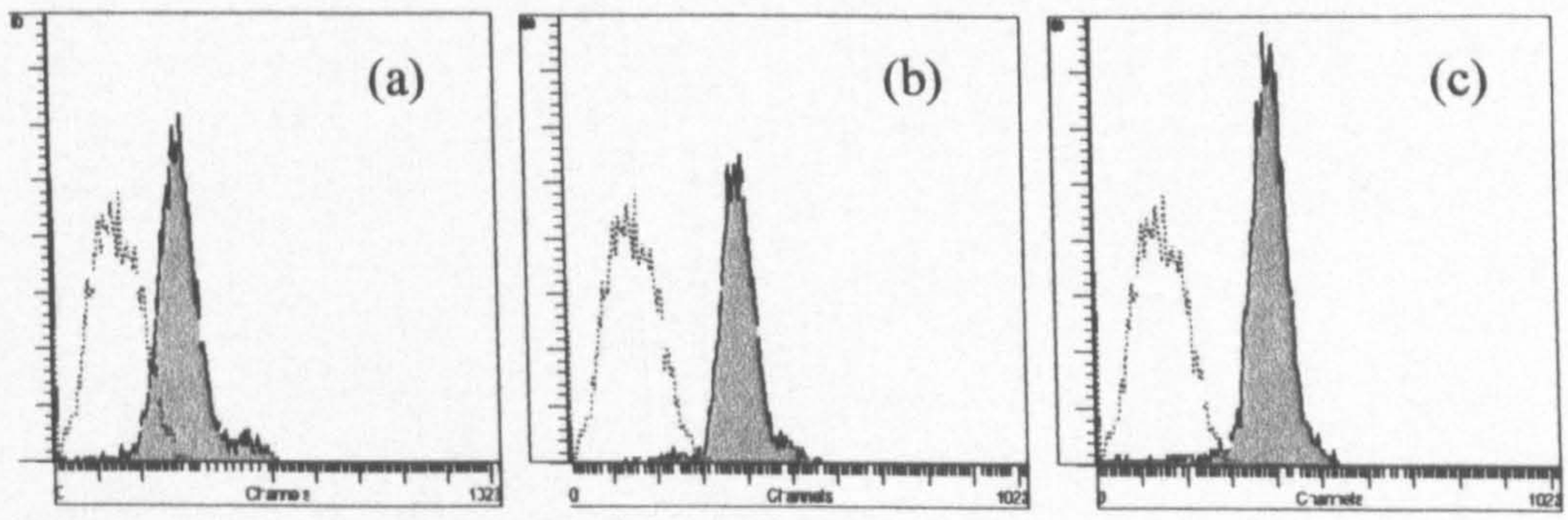



**Figure 4.1.** Representative profiles of flow cytometric analysis of a) hsp60, b) hsp72/73 and c) hsp90 protein levels in normal mononuclear mononuclear cells. Immunofluorescent staining is presented as cell numbers versus log fluorescence intensity. Comparison with an isotype-matched negative control is indicated by the dotted line.

Typically, the cells fluoresce as a single narrow peak, indicating a relatively homogeneous pattern of hsp expression in these mature blood cells.

The values for the mean peak channel (MPC) of fluorescence obtained by flow cytometric analysis in the ten samples of normal PBMC were as follows; Hsp60; mean = 6.3, range 2-12.5, hsp72/73; mean = 16.3, range 4-30, hsp90; mean = 32.3, range 13-52.

## 4.3.2. Expression of hsps in CML mononuclear cells.

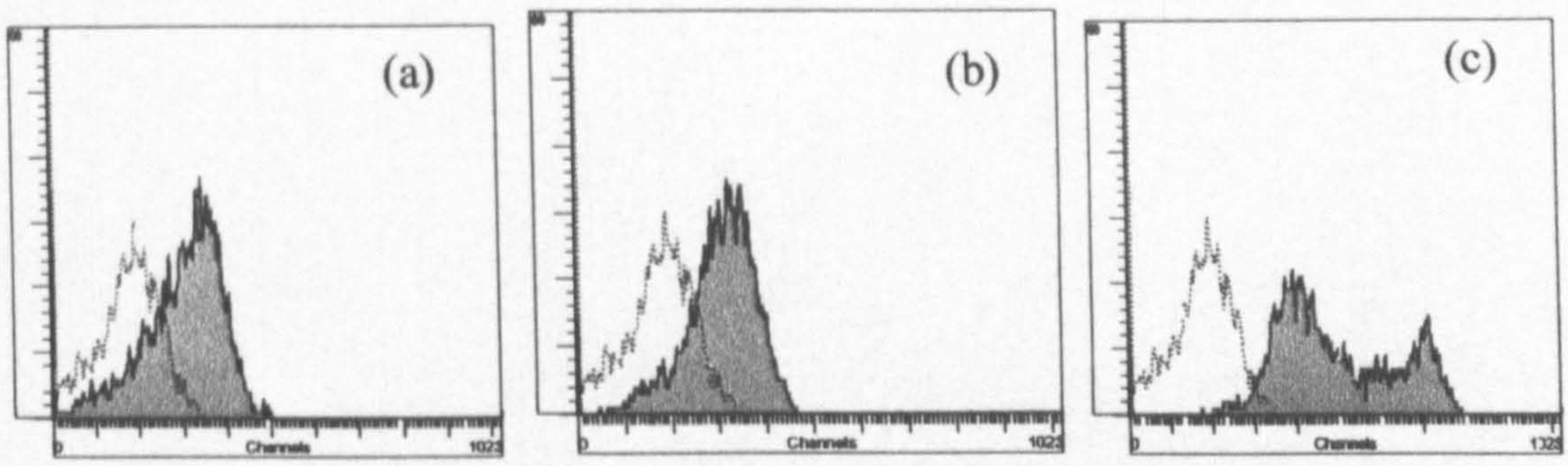


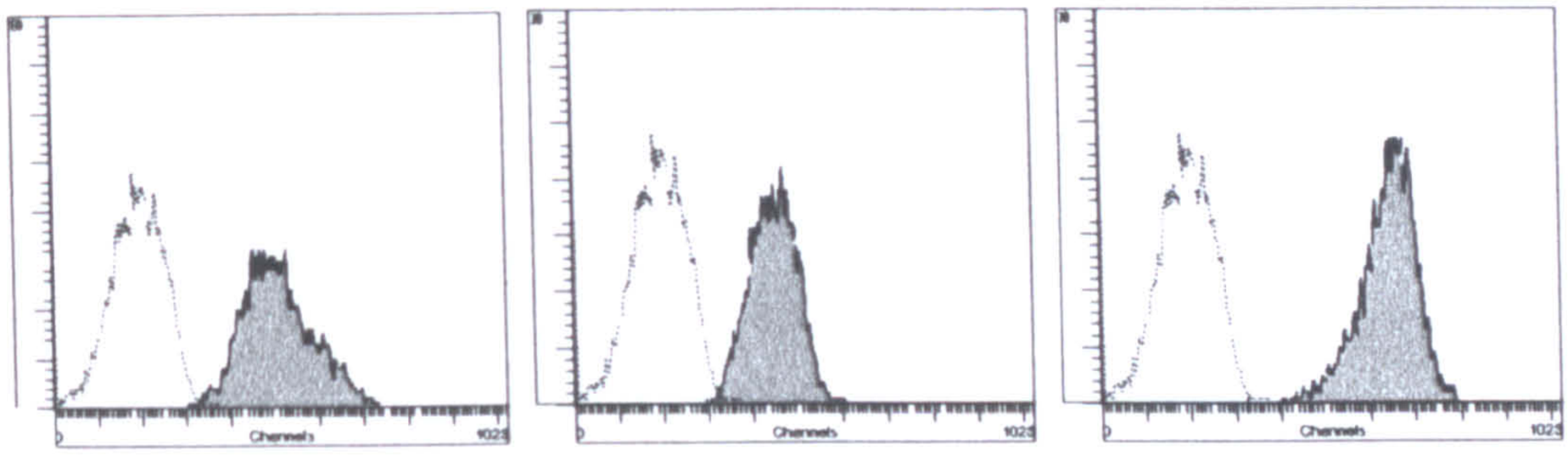

**Figure 4.2.** Flow cytometric profile of a) hsp60, b) hsp72/73 and c) hsp90 expression in mononuclear cells harvested from a patient with CML (patient LM). The dotted line represents the isotype-matched negative control. Broad peaks of protein expression represent heterogenous levels of hsp in these patients.

Table 4.2 summarises the flow cytometry data for the 9 CML patients and shows the mean values and the range of fluorescent staining for each hsp family. Levels of hsp were not significantly different to those seen in normal PBMC's when analysed in terms of the mean peak channel of fluorescence. However, as figure 3.2 illustrates, cells stain as a broad fluorescent peak indicating a heterogeneous pattern of expression. This pattern of expression would be expected in CML if hsp levels are related to the differentiation of

haemopoietic cells, since this disease is typified by the appearance in the peripheral blood of myeloid cells representing all stages of differentiation from immature myeloblasts to mature neutrophils. Indeed, all the CML samples in this study contain myeloid cells at various stages of maturation as can be seen from the haematological data in table 4.2.
It is interesting to note in Fig.4.2 that fluorescence peaks for hsp90 staining demonstrate two distinct cell populations in this patient. One population of cells stained with a lower fluorescence intensity, within the range seen for normal PBMC's. The second distinct population of cells stained more intensely, comparable to the level of staining with anti-hsp90 seen in the cells from AML patients (see below). Cells from other patients (3/8) also showed two peaks of fluorescence for hsp90 staining.

### 4.3.3. Expression of hsps in AML mononuclear cells.

Table 4.3 demonstrates that mononuclear cells from AML patients had a significantly higher level of expression ($p<0.01$) of all three hsp's compared with cells from CML patients or normal PBMC. Within the AML patients as a group, a wide range of hsp expression was evident, with a nine-fold difference in mean fluorescence intensity of hsp72/73 staining observed between individual patients. Hsp60 and hsp90 staining also showed a wide heterogeneity in terms of fluorescence staining, although less pronounced than seen for hsp72/73.
In most AML patients, expression of the hsp in the mononuclear cell fraction was seen as a narrow peak of fluorescence intensity, presumably representing the almost homogeneous nature of the cell populations which were predominantly immature blast cells.




a) b) c)

**Figure 4.3.** Immunofluorescent staining of AML cells (patient SB) with monoclonal antibodies to a) hsp60, b) hsp72/73 and c) hsp90 reveals that these cells express higher protein levels for all three hsp families, compared with either normal mononuclear cells or cells from patients with CML.

Figure 4.3 illustrates the immunofluorescence profile of patient SB whose mononuclear cells were almost entirely myeloblasts (96%). The fluorescence peaks show a narrow window of staining, indicating a uniform pattern of expression in all the blast cells. However, other AML patients demonstrated dual staining peaks as was seen in some of the CML cells. For example, patient KW (figure 4.4) had a much lower proportion of myeloblasts in the peripheral blood, and the presence of both immature blasts and more mature cells was reflected by two distinct peaks of staining intensity for hsp72/73 and hsp90, but not hsp60. The gap between these two peaks is more marked than was seen in the CML cells.

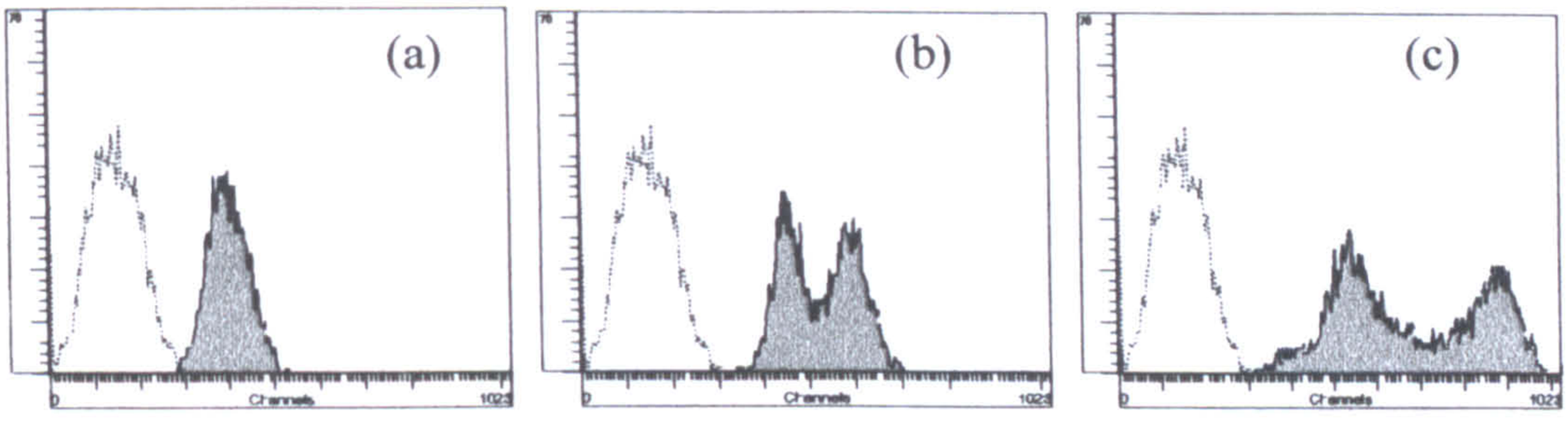



**Figure 4.4.** Immunfluorescent staining profile of AML mononuclear cells harvested from patient SB using monoclonal antibodies to hsp60, hsp72/73 and hsp90. Although analysis of the peripheral blood from this patient revealed that the white cell population consisted almost entirely of myeloblast cells (96%), two staining populations are evident for b) hsp72/73 and c) hsp90, but not a) hsp60.

Patient D.G, whose mononuclear cells consisted entirely of myeloblasts by morphological criteria, also demonstrated two distinct staining peaks for hsp90 expression. This pattern of dual staining peaks for hsp90, but not hsp70 or hsp60, was found in two other AML cases where the cell population appeared homogeneous by morphological examination alone.

| Patient | WBC x$10^9$/l | % Blasts | †Immature myeloid % | Hsp60 (MPC) | Hsp70 (MPC) | Hsp90 (MPC) |
|---|---|---|---|---|---|---|
| LM | 209 | 12 | 74 | 10 | 12 | 32 |
| FB | 184 | 0.5 | 36 | 10 | 17 | 48 |
| TM | 255 | 0 | 69 | 8 | 13 | 25 |
| AL | 213 | 8 | 41 | 16 | 18 | 31 |
| VW | 172 | 35 | 54 | 9 | 10 | 17 |
| SKD | 52.2 | 10 | 24 | 7 | 10 | 65 |
| EO | 171 | 0 | 28 | 9 | 16 | 44 |
| NP | 72.9 | 18 | 58 | 15 | 22 | 30 |
| RW | 46.1 | 9 | 26 | 5 | 18 | 34 |

†Immature myeloid % represents immature cells of the myeloid series, i.e. myeloblasts, promyelocytes, myelocytes and metamyelocytes.

| | | | |
|---|---|---|---|
| Mean peak fluorescence | Hsp60: **9.9** (Range=5-16) | Hsp70: **15.1** (Range=10-22) | Hsp90: **36.22** (Range=17-65) |

**Table 4.2.** Haematological data from CML patients indicating the peripheral blood white cell count and the percentage of myeloblasts in each sample. The expression of members of the hsp60, hsp70 (72/73) and hsp90 proteins has been quantified by immunofluorescent staining and flow cytometric analysis, the level of expression of each being expressed in terms of the mean peak channel of fluorescence (MPC).

| Patient | WBC x$10^9$/l | % blast cells | Hsp60 (MPC) | Hsp70 (MPC) | Hsp90 (MPC) |
|---|---|---|---|---|---|
| LR | 27.9 | 62 | 13 | 180 | 32 |
| BT | 44.1 | 99 | 27 | 54 | 95 |
| KW | 43.7 | 40 | 18 | 20 | 72 |
| MM | 39.8 | 82 | 22 | 44 | 81 |
| MS | 31.0 | 95 | 21 | 60 | 83 |
| MB | 82.9 | 88 | 17 | 30 | 27 |
| CJ | 13.8 | 81 | 16 | 20 | 18 |
| MK | 107 | 26 | 11 | 90 | 38 |
| SB | 121 | 96 | 14 | 39 | 49 |
| DG | 104 | 100 | 16 | 40 | 48 |
| KH | 31.9 | 93 | 10 | 50 | 84 |
| AM | 42.1 | 91 | 27 | 75 | 92 |

| | | | |
|---|---|---|---|
| Mean peak fluorescence | Hsp60: **17.6** (Range=10-27) | Hsp70: **58.5** (Range=20-180) | Hsp90: **59.7** (Range=18-95) |

**Table 4.3.** Haematological data of AML Patients and hsp expression in terms of the mean peak channel (mpc) of fluorescence for the three major hsp families.

## 4.4. Discussion.

### 4.4.1.Cytoplasmic expression of heat shock proteins in primary leukaemic cells.

The expression of hsp60, hsp70 (72/73) and hsp90 can all be detected in normal peripheral blood mononuclear cells (PBMC's) by indirect immunofluorescence using monoclonal antibodies against these three major heat shock protein families.
Fluorescence profiles for these cells demonstrate a relatively homogeneous pattern of hsp expression with no large differences apparent between lymphocytes and monocytes from the same individual, evidenced by a single narrow peak of staining, or between the mononuclear fraction of the ten healthy individuals examined.
The expression of all three heat shock proteins examined is clearly elevated in leukaemic cells and more heterogeneous than is observed in normal PBMC. In AML cells, significantly higher levels of all three hsp families were evident compared with either normal PBMC or CML cells. Both AML and CML cells exhibit a wide range of hsp expression, not only between individual patients, but within the groups as a whole. The significance of these differences in hsp expression is not clear, but several explanations are possible. The first, and possibly most plausible explanation, is that hsp levels are a marker of the differentiation state of these cells. Decreased expression of hsp70 and hsp90 is associated with myeloid differentiation of HL-60 cells (Beere *et al*, 1993, Yufu *et al,* 1989, Shakoori *et al,* 1992), and levels of hsp60 mRNA decrease during TPA-induced differentiation of HL-60 or K562 cells (Lu & Seligy, 1992). A further indication of the association between hsp's and differentiation is the observation that heat shock itself, or chemical agents which elicit a heat shock response, induce differentiation of

HL-60 cells (Richards, 1988). The large differences in hsp levels between AML cells, which are predominantly immature myeloblasts, and CML cells may therefore be due to simple differences in the stages of myeloid differentiation between the cells. If this is the case, the heterogeneity seen in AML cells could imply that some myeloblasts are more mature and the possibility that hsp levels could be a marker of differentiation state in AML demands further investigation. Also, therapy aimed at inducing differentiation, e.g the retinoids, would be expected to drive towards lower levels of hsp expression.

A second explanation is that hsp expression is a marker of cell proliferation. By virtue of their role as molecular chaperones, hsp's perform a perform a variety of roles which could be utilised in a developmentally regulated manner to control proteins necessary for cell growth and proliferation (Pechan, 1991). Hsp expression has been shown to be regulated by mitogenic stimuli in a variety of cell types, including haematopoietic cells (Ferris *et al,* 1988, Fincato *et al,* 1991), and both immunoperoxidase staining and flow cytometric analysis of concanavalin A and pokeweed mitogen-stimulated lymphocytes demonstrated markedly increased levels of hsp70 and hsp90 (see Results Chapter 1).

Immunofluorescent histograms of CML patients demonstrated in some cases two distinct peaks suggesting that the presence of immature and more differentiated myeloid cells expressed high and low levels of hsp respectively. However, in some AML cells where morphologically the cells were homogenous and virtually all immature myeloblasts, two distinct populations were evident for hsp90 expression. This may represent the presence of an actively proliferating population of cells which have a distinct level of hsp90 expression., although this would seem unlikely since the proliferating compartment of leukaemias, like most other cancers, is represented by a very small percentage of the overall population of tumour cells. In these cases, it may be more likely that this dual pattern of hsp90 expression is the result of a biphenotypic leukaemia, a situation which arises when leukaemic transformation gives rise to the

clonal proliferation of two populations which may represent different stages of myeloid differentiation.

Although leukaemic cells from different patients showed marked differences in levels of expression, no correlation was observed in the expression of the different hsp families. This is perhaps not unsurprising, since although all heat shock proteins have similar modes of action, they have exclusive roles to play in terms of specific interactions with cellular proteins.

An important feature of the pattern of hsp expression in leukaemic cells, and in particular those from AML patients, is the wide range of hsp expression in cells from individuals with the same diagnosis. Analysis of the clinical data from these patients, in terms of survival, drug therapy and the stage of the disease, revealed no apparent correlation with the level of hsp expression. At this stage, it is not evident whether the high levels of hsp expression in AML simply represent the malignant phenotype of these cells, i.e. their lack of myeloid differentiation, or whether elevated hsp expression has a biological role, particularly in terms of the response to chemotherapy.

As outlined in the introduction, hsp induction in response to a variety of environmental insults appears to afford cells a protective effect. Do the elevated levels of these proteins therefore provide leukaemic cells with a survival advantage which may relate to altered proliferative potential of the malignant clone? Furthermore, how do levels of hsp expression relate to the susceptibility of cells to drugs used in the standard treatment of these malignant disorders?

# Chapter 5

# Heat shock protein expression and apoptosis in myeloid leukaemia cells.

## 5.1. Introduction.

Normal haemopoiesis is dependent upon a complex network of haemopoietic growth factors. These regulatory cytokines are essential for the control of growth and differentiation as well as maintenance of viability, and removal of such factors results in apoptosis (reviewed by Sachs, 1996 ). During normal myeloid cell differentiation, apoptosis plays a regulatory role and myeloid precursor cells which become deprived of haemopoietic growth factors undergo apoptotic cell death. Likewise, leukaemic blast cells from most patients with AML are also dependent upon the presence of these 'survival' factors and cannot be maintained *in vitro* without the addition of haematopoietic growth factors (Lotem and Sachs, 1996). However, myeloblasts from a small proportion of AML patients exhibit independent growth without the requirement for exogenous growth factors. This observation has clinical significance since it has been shown that AML patients whose cells survive *in vitro* independently have a poor prognosis (Lowenburg *et al,* 1993, Hunter *et al,* 1993).

As outlined in the Introduction, apoptosis is a physiological mode of cell death which plays a major role in the maintenance of homeostasis in several systems of the body. It takes place during embryogenesis and metamorphosis, and plays an important role in tissue turnover by maintaining the balance between cell number and cellular proliferation. It is generally accepted that deregulation of the apoptotic process may contribute to the development of the malignant state by permitting the expansion of a population of cells capable of genetic progression to malignancy (reviewed by Allen *et al.*, 1993, Kerr *et al.*, 1994, Lotem and Sachs, 1996). In addition, the susceptibility of tumour cells to apoptosis has important therapeutic implications since cytotoxic drugs have been shown to exert their effects via induction of this mode of cell death (Lotem & Sachs, 1992, Sens & D'Incalci, 1992).

In Chapters 3 and 4 it was demonstrated that hsp expression in myeloid leukaemia cells is heterogenous, which leads one to question whether the levels of hsp expression observed have any biological relevance in terms of susceptibility to apoptosis. The posssible relevance of hsp expression to apoptosis can be approached from two angles. Firstly, hsp induction in response to environmental insults has been shown to have a protective effect; does hsp expression therefore influence the induction of apoptosis by cytotoxic drugs? Secondly, hsp expression is clearly linked to cell growth and differentiation, probably by virtue of hsp interaction with growth regulatory factors, e.g *c-myc*, p53. The expression of these two growth factors is known to be involved in apoptosis (Lotem and Sachs, 1996) which implies that hsp expression may in turn be implicated in the process.

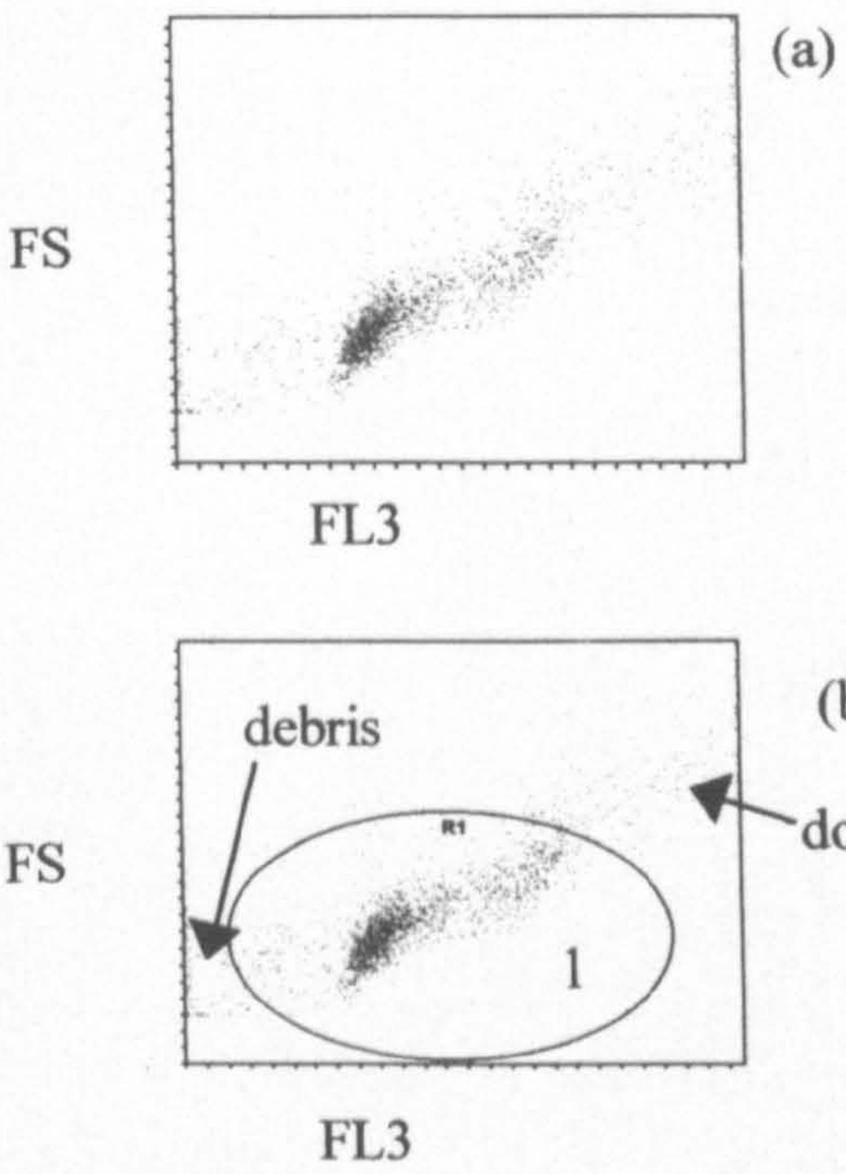
A relationship between hsp expression and apoptosis is suggested by several lines of evidence. The induction of hsp by heat shock or overexpression by introduction of a vector containing hsp gene affords resistance to a variety of agents known to induce apoptosis in haematopoietic cells, e.g heat shock, TNF (Li *et al* , 1991; Jaattela *et al* , 1992). In addition, it has been reported that the cytotoxic drug, bischloronitrosourea (BCNU), will induce hsp expression (Kroes *et al,* 1991). Furthemore, there is evidence that hsp may have a role in the control of apoptosis. As described in detail in the Introduction, members of the hsp70 family will form complexes with p53, a tumour suppressor gene product. It has been found that mutant forms of p53 are preferentially associated with the constitutive hsp70 (hsc70) (Hainaut *et al,* 1992) and the *Escherichia coli* hsp 70 analogue, DnaK, will also bind to p53 and enhances its DNA binding capacity. This implies that chaperones, potentially hsp70, may play a role in regulating tumour suppressor function as suggested by Lane *et al* (1993), and therefore be involved

in escape from apoptosis. Neurones are protected by heat shock from apoptosis induced by transfer into serum free medium, i.e lacking viability factors (Mailhos *et al,* 1993), and thymocytes are similarly protected from glucocorticoid-induced apoptosis (Migliorati *et al*,1993 ). This latter observation is particularly interesting as glucocorticoids can induce apoptosis in thymocytes by a p53-independent mechanism (Clarke *et al*,1993).

I have therefore examined the apoptotic process in cells from AML patients in order to discover whether a difference in susceptibility to cell death exists between samples from different patients, and between samples taken from the same patient during different stages of the disease. The possibility that the *in vitro* susceptibility of malignant cells to apoptosis may relate to clinical factors such as disease aggression and response to chemotherapy requires investigation.

## 5.2 Experimental design

**5.2.1. Assessment of apoptosis by propidium iodide staining of cultured cells.**
Flow cytometry has become a method of choice for analysis of apoptosis in a variety of cell systems. A variety of techniques utilising flow cytometry have been developed with methods based upon changes in cell morphology, function of the plasma membrane, DNA degradation and DNA stability to denaturation (reviewed by Darzynkiewicz *et al*, 1997). The method of choice for identifying and quantifying apoptotic cells was flow cytometric analysis of propidium iodide-stained cells. Propidium iodide (PI) is a highly polar compound which intercalates with nucleic acids in a stoichiometric manner, enabling the quantitation of total nuclear DNA content.




(a) Cell populations are examined by forward scatter (FS), which is a function of cell size, and red fluorescence staining (FL3) which is a function of propidium iodide binding and therefore DNA content.
(b) A bitmap (1) is drawn around the cells of interest to include apoptotic cells plus cells in $G_1$, S and $G_2$/M phases of the cell cycle. This excludes doublets and cell debris from the analysis.
(c) Gated cells are displayed as a histogram of cell number versus FL3 intensity and the use of region analysis software integral to the Coulter Epics flow cytometer permits quantitation of cells within each phase of the cell cycle including the apoptotic population.

Early experiments with normal mononuclear cells and cells from leukaemic patients demonstrated that propidium iodide staining successfully identified apoptotic populations

following culture in the absence of any added growth factors. As described in chapter 2, the data from propidium iodide staining correlated well with morphological analysis of cytospin preparations in which apoptotic cells were counted visually under light microscopy.

## 5.2.2. Patient samples.

Mononuclear cells were harvested by density gradient centrifugation (see Chapter 2, section 2.1) from peripheral blood samples or bone marrow aspirates collected into EDTA from 9 patients with AML. Samples were collected at initial presentation, and when possible, during induction of remission by standard chemotherapeutic regimens and in certain cases upon subsequent relapse (Table 5.1). A total of 20 samples were analysed

Cryopreserved cells from AML patients were rapidly thawed in a 37°C waterbath, and washed in RPMI medium. Cell viability was determined in each case by the trypan blue dye exclusion test and in all samples viability was >88%. An aliquot of cells from each patient sample was analysed for hsp70 and p53 expression (as described in materials and methods), and cells were resuspended in RPMI 1640 medium without any added serum or exogenous growth factors.

| Sample | Comments |
|---|---|
| JJ a) | PRV-AML transformation |
| JJ b) | Relapse 1, drug resistant |
| JJ c) | Relapse 2 |
| EH a) | Presentation *de novo* AML |
| EH b) | Relapse 1 |
| EH c) | Relapse 2 |
| EH d) | Relapse 2 + Tx |
| BT a) | Relapse 1 |
| BT b) | Relapse 2 |
| LM | CML-AML transformation |
| MC a) | Presentation *de novo* AML |
| MC b) | Relapse 1 |
| MM a) | Relapse 1 |
| MM b) | Relapse 2, drug resistant |
| MM c) | Relapse 2 + Tx |
| DG | Presentation *de novo* AML |
| MW | Relapse 1 |
| PC a) | Presentation *de novo* AML |
| PC b) | Relapse 1 |
| PC c) | Relapse 1 + Tx |

**Table 5.1.** Leukaemic mononuclear cells from nine patients with AML were harvested by density gradient centrifugation. Samples were collected at initial presentation, and where possible, during subsequent treatment and/or relapse.

## 5.3. Results

### 5.3.1. AML cells undergo apoptosis in the absence of exogenous growth factors.

AML cells cultured *in vitro* rapidly undergo apoptosis as evidenced by the appearance of a sub-$G_1$ peak with propidium iodide-stained cells. Morphological analysis by light microscopy of giemsa-stained cytospins reveals the presence of significant numbers of apoptotic cells after 48 hours incubation in RPMI 1640 medium alone (Plates 5.1 and 5.2). with the characteristic features of condensed chromatin and formation of membrane-bound apoptotic bodies.

*In vitro* culture of AML cells in the absence of added serum rapidly led to the appearance of apoptotic cells as determined by the presence of sub-diploid peaks during flow cytometric analysis of propidium iodide-stained cells. Figure 5.1 illustrates that AML cells demonstrated a time-dependent increase in apoptotic numbers, with apoptotic cells observed in some samples as early as 6 hours. By 48 hours, some AML cells (5/20) demonstrated greater than 90% apoptosis. By contrast, cells from other samples are more resistant to apoptosis, in one case the proportion of apoptotic cells being just 3% after 48 hours. Figure 5.1 illustrates that AML cells fall generally into two groups, some highly susceptible and others relatively resistant to induction of apoptosis.

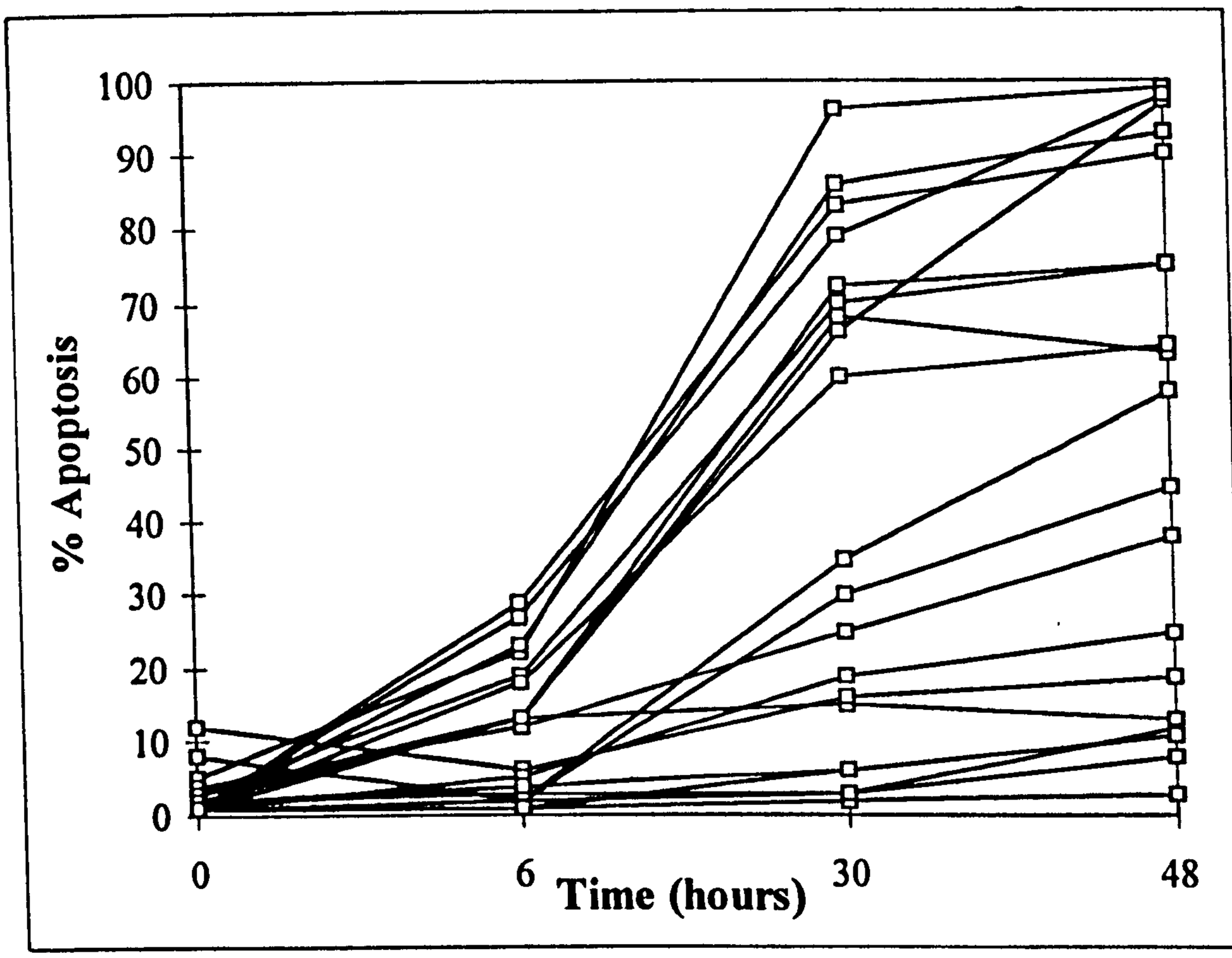



**Figure 5.1.** AML cells undergo apoptosis *in vitro* when cultured in serum-free medium. The percentage of apoptotic cells in mononuclear cells harvesyed from individual patients was determined by propidium iodide staining and flow cytometric analysis to quantify the sub-$G_1$ apoptotic peak. The susceptibility of different sample to apoptosis varies considerably.

### 5.3.2.Susceptibility to apoptosis correlates with hsp70 expression in AML cells.

As was found with the earlier analysis of hsp expression in AML cells (see Chapter 4), a broad range of hsp72/73 expression is evident in samples from different AML patients and between samples from the same patient taken at different stages of the disease, with a six-fold variation in hsp72/73 levels between 'high' and 'low' expressers (Table 5.2). The expression of p53 oncoprotein was also examined in these cells. Cells were scored as positive for p53 expression if the number of positive cells was greater than 10% as compared with the isotype-matched negative control antibody. In table 5.2, patients are arranged in order of the susceptibility of AML cells to *in vitro* apoptosis as determined by the number of apoptotic cells after 48 hours.

A comparison of hsp72/73 expression with the susceptibility of cells to apoptosis, as measured by the percentage of apoptotic cells after 48 hours, demonstrated that there is a significant correlation ($p = 0.009$) between apoptotic numbers and the mean peak channel (mpc) of fluorescence staining with anti-hsp72/73 (Figure 5.2).

### 5.3.3. Expression of p53 is associated with high levels of hsp70.

The expression of the p53 tumour suppressor gene has an established role in the process of apoptosis, . Analysis of p53 expression in these cells by flow cytometry reveals that the majority of the AML samples showed no specific p53 staining, and that where specific staining did occur, this was weak (figure 5.3). However, it does appear that AML samples exhibiting high apoptotic rates score positive for p53 expression (see table 5.2).

| Patient | hsp70 (mpc) | p53 | % Apoptosis (48 hrs) |
|---|---|---|---|
| EH a) | 122 | + | 99 |
| MC a) | 240 | + | 98 |
| JJ a) | 170 | + | 97 |
| MM c) | 131 | - | 93 |
| MM a) | 156 | + | 90 |
| LM | 212 | + | 76 |
| MW | 176 | + | 64 |
| PC b) | 130 | - | 63 |
| EH d) | 251 | - | 48 |
| EH c) | 198 | - | 45 |
| BT a) | 92 | - | 35 |
| PC c) | 113 | - | 35 |
| MM b) | 44 | + | 28 |
| BT b) | 66 | - | 23 |
| JJ b) | 139 | - | 13 |
| PC a) | 121 | - | 12 |
| MC b) | 58 | - | 11 |
| JJ c) | 144 | - | 11 |
| EH b) | 90 | - | 7 |
| DG | 43 | - | 3 |

**Table 5.2.** Leukaemic cells from nine AML patients collected at various stages of the disease (see Table 5.1) demonstrate variable degrees of apoptosis after 48 hr *in vitro* culture. Samples are arranged in order of susceptibility to apoptosis and show the level of hsp72/73 expression. Samples were scored as positive for p53 expression if the number of positive cells was greater than 10%.

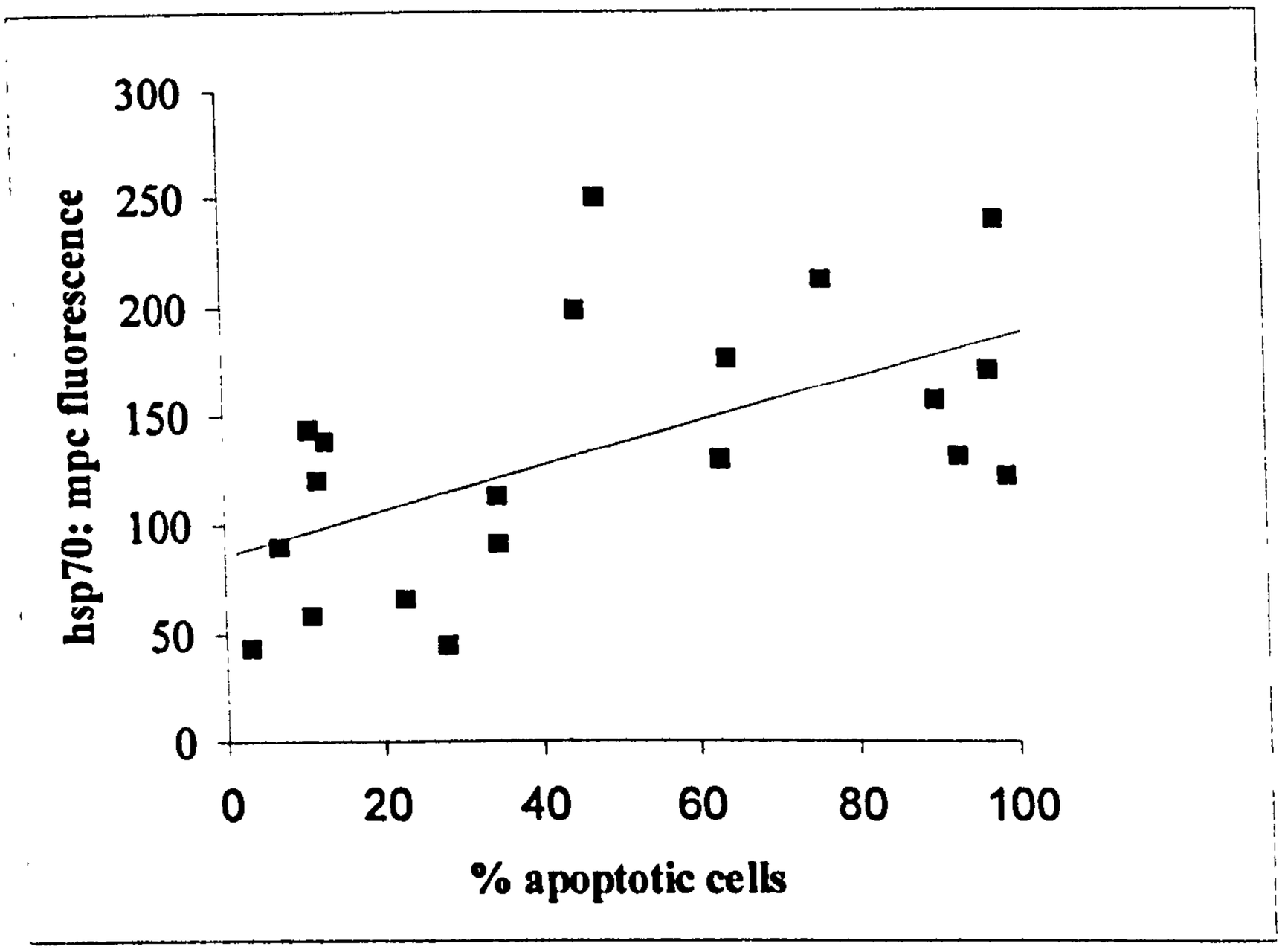



**Figure 5.2.** The percentage of apoptotic cells from 20 AML patients after 48hr in vitro culture correlates significantly (P = 0.009) with hsp72/73 expression.

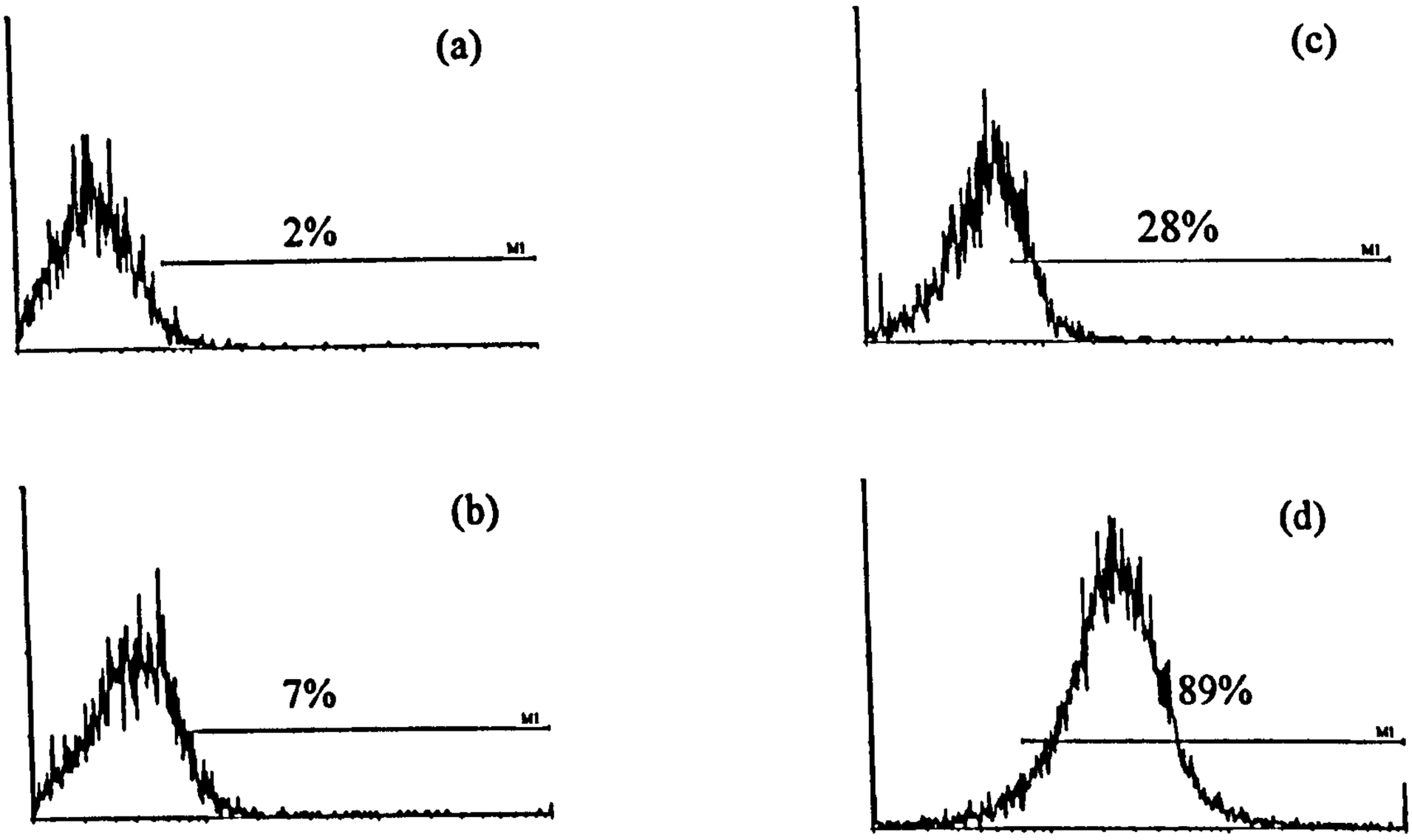



**Figure 5.3.** p53 expression in AML cells was measured by flow cytometric analysis of patients cells stained with a mononclonal antibody recognising both the wild-type and mutant forms of p53. Specific staining was compared with an isotype-matched negative control antibody (a), and analysis region M1 was set so that 98% of these cells were negative. Specific staining with p53 antibody was measured by the number of positively staining cells within this region as indicated. In table 5.2, positive expression of p53 in cells of patients with AML was taken if the percentage of cells within analysis region M1 was 10% or greater.

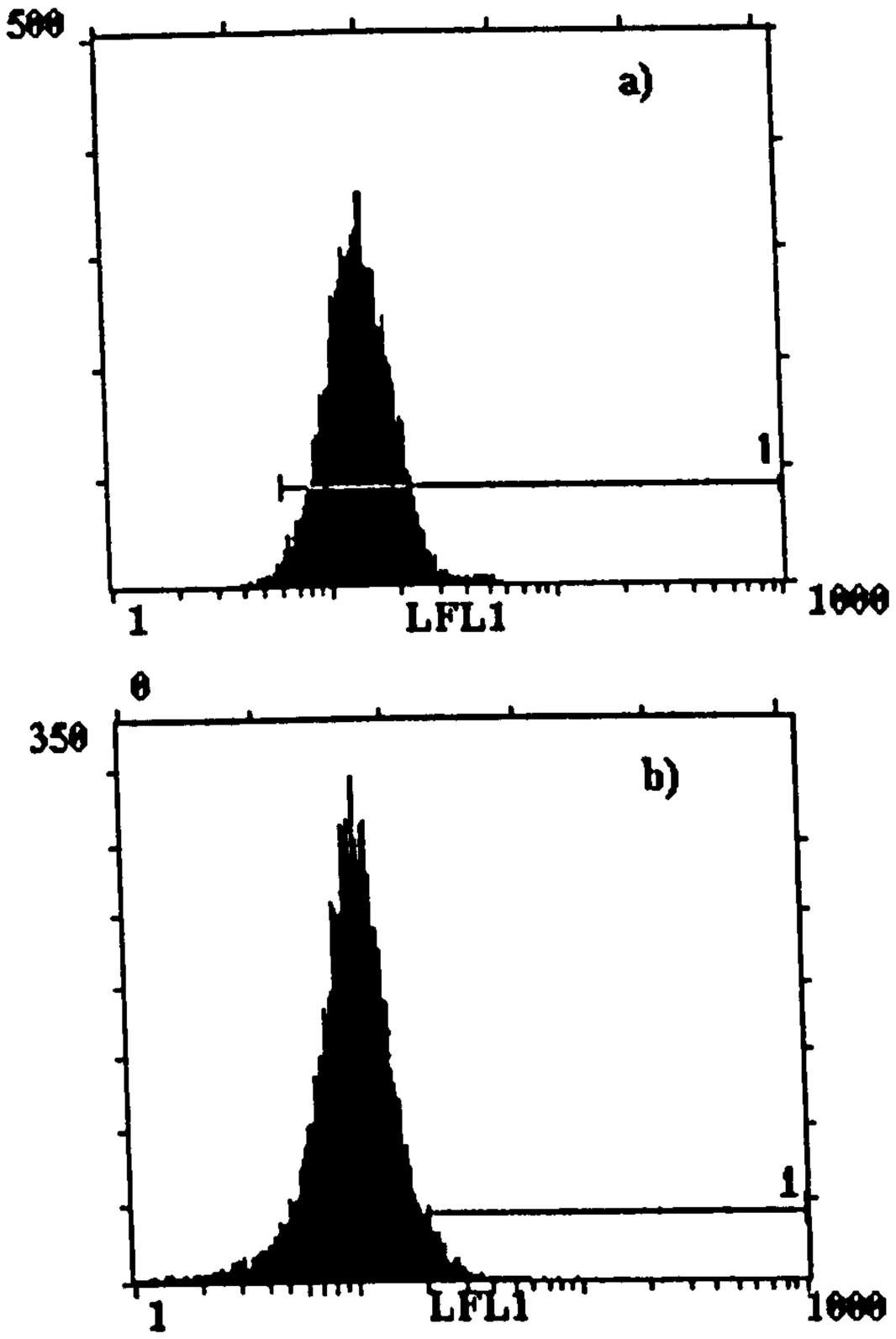



**Figure 5.4.** Variable but low levels of Bcl-2 expression were demonstrable in AML cells. Fixed cells were stained with a primary antibody to bcl-2 followed by a secondary FITC-labelled goat anti-mouse antibody. Specific bcl-2 protein levels were expressed in terms of the mean peak channel (mpc) of fluorescence.

### 5.3.4.Correlation of hsp90 and bcl-2 with susceptibility to apoptosis.

In order to determine whether hsp90 demonstrated a similar correlation with the susceptibility of AML cells to apoptosis induced by serum withdrawal, the expression of this hsp was determined in a second series of AML cell samples (table 5.3) as there was insufficient material from the first series for these additional determinations. In addition, hsp70 expression was also measured as well as bcl-2, a recognised inhibitor of apoptosis in lymphoid cells.

| Sample | Comments |
|---|---|
| RS | CML-AML transformation |
| MM | Relapse 2 + Tx |
| KH | AML presentation |
| MM | Relapse 2 |
| BT | Presentation + Tx |
| EH | Relapse 2 + Tx |
| JJ | PRV-AML |
| JJ | PRV-AML bone marrow |
| DG | AML Presentation |
| LR | AML Presentation |
| AM | AML Presentation |
| LR | AML bone marrow |
| PC | Relapse 1 + Tx |

**Table 5.3.** *In vitro* apoptosis of AML cells was examined in a second series of patients

As before a strong correlation was found between hsp70 expression and apoptosis ($p = 0.0004$: data not shown). Both bcl-2 and hsp90 could be reliably quantified in these cells (figures 5.4 and 5.5 respectively). The data indicated that there was a negative but not significant correlation between apoptosis and bcl-2 (figure 5.6) expression, and there was no correlation with hsp90 expression (figure 5.7).

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### 5.3.5. Susceptibility to apoptosis and patient prognosis in AML.

Susceptibility to apoptosis was analysed in relation to patient survival. Patient cells included in this study were taken from some patients at initial disease presentation, and in other cases from patients previously in remission whose disease had relapsed. There was no significant correlation ($p=0.67$) between the susceptibility of myeloid leukaemic cells to apoptosis and the survival rate of the patients included in this study.

## 5.4. Discussion.

The results presented in this chapter demonstrate that AML cells undergo apoptosis when cultured *in vitro* in the absence of added serum reflecting the requirement of haemopoietic cells for viability factors which inhibit the induction of apoptosis. The results demonstrate that there is a marked variation in the susceptibility to apoptosis of AML cells from different patients, and indeed there are differences between samples from the same patients taken at different stages of the disease. However, I was unable to demonstrate any correlation between the rate of apoptosis and clinical factors such as patient survival or the development of drug resistance.

Examination of the stage of disease does initially appear to suggest that there is some relationship between the stage of the disease and the levels of hsp70 and, following on from this, the susceptibility of these cells to apoptosis.Examination of the results in table 5.2 shows that there was a difference in hsp70 and susceptibility to apoptosis between cells harvested from patients at initial presentation compared with cells taken from the same patients at relapse and second relapse. For example, mononuclear cells from patient JJ (see table 5.1) were highly susceptible (97%) to apoptosis at initial presentation of the disease, but the cells were more resistant (13%) at first relapse and highly resistant (7% apoptosis after 48 hours serum starvation) at second relapse. Also, cells from patients EH and MC were highly susceptible to apoptosis at initial presentation, whilst cells collected during subsequent relapse demonstrated a greatly increased resistance to apoptosis induced by serum starvation. This is a very interesting observation which suggests that leukaemic cells during relapse have undergone alterations in the susceptibility to apoptosis which may play a role in the response of

these cells to cytotoxic therapy. However, patient PC showed a relative increase in susceptibility to apoptosis between cells harvested at initial presentation and cells from samples taken later during disease relapse. It is therefore impossible to draw any conclusions from these results, especially when based upon such a small sample of patients. This aspect therefore requires further investigation in order to establish whether there is a relationship between disease state and apoptosis.

When the rate of apoptosis *in vitro* was compared with the expression of hsp70 in the AML cells upon isolation, it is clear that there is a highly significant correlation between the two in two independent series of AML cell samples. Thus, although hsp70 expression protects cells against apoptosis induced by a variety of agents , paradoxically it appears that there is an inverse relationship between the level of hsp70 and the rate of apoptosis induced by withdrawal of growth factors in these AML cells. This finding is in contrast to the initial expectations at the outset of this investigation. Two factors which are widely regarded as playing important roles in apoptosis are p53 and bcl-2. The former is thought to initiate apoptosis, at least where this is induced by DNA damage, and the latter to protect. In these studies, p53 was weakly and inconsistently expressed so that although p53 expression was not reliably quantified, there did appear to be some correlation between p53 and apoptosis. Expression of bcl-2 did demonstrate a negative relationship with the susceptibility to apoptosis suggesting that bcl-2 may play some role in the protection of AML cells against apoptosis, although this correlation was not statistically significant.

The question which arises from these results is whether hsp70 itself plays a role in apoptosis, or if high levels of hsp70 simply reflect that the most susceptible cells are already 'stressed' and therefore more susceptible to a further insult such as removal of viability factors. However, if this were the case it would be expected that hsp90, another stress protein, would also correlate with apoptosis, and this did not appear to occur. If the association of hsp70 elevation with apoptosis is not merely coincidental, either its importance is through interaction with the p53 pathway or else through an independent pathway.

Of particular interest in this context is the observation that constitutive members of the hsp70 family (hsc70) interact with the product of the p53 tumour suppressor gene. The biological significance of hsp70-p53 complexes is poorly understood, although it has

been shown that binding of hsp70 could be part of a regulated process leading to changes in p53 conformation. According to this hypothesis, hsp70 regulates folding from mutant , with promoter function, to wild-type conformation which has suppressor function. (Hainaut & Milner, 1992). Further evidence of this relationship between hsp70 and p53 is suggested by the report that mutant p53 proteins can transactivate the hsp70 gene promoter (Tsutsumiishii *et al.* 1995).

Hsp70 appears to play a role in the balance between control of cell growth and differentiation, and loss of cells via apoptosis. Our finding that AML cells which express high levels of hsp70 are more susceptible to apoptosis is paradoxical in view of the protective nature of hsp, although it has been shown that HL60 cells treated with N-methylformamide (NMF) will differentiate or undergo apoptosis according to the concentration of NMF used, and that differential expression of hsp70 correlates with the path taken (Beere *et al,* 1993). Differentiation is associated with decreased levels of hsp70, while the induction of apoptosis by higher levels of NMF correlates with maintainance of hsp70 levels. The authors of this report suggest that hsp70 acts, similar to p53, as a cellular 'sensor' permitting the cell to discriminate between different levels of cellular damage, and is therefore a key mediator in the response to drug-induced damage.

# Chapter 6

# Analysis of the heat shock response and apoptosis in leukaemic cell lines

## 6.1. Introduction.

In chapter 5, I reported a significant correlation between hsp70 protein levels and the susceptibility to apoptosis of primary AML cells. Paradoxically, this predisposition to apoptosis induced by serum withdrawal was greater in those cells expressing higher levels of hsp70, suggesting that either these cells were already 'stressed', or that high levels of hsp70 actually provide a pro-apoptotic signal to the cell.

In order to analyse whether hsp70 overexpression does influence the apoptotic process in leukaemic cells, myeloid leukaemia cell lines were used to study the relationship between hsp expression and the susceptibility to apoptosis. Heat shock of cultured cells was used to increase the expression of the major hsp, and the heat shock response was studied by measuring both the kinetics and quantitative expression of the major hsp following hyperthermic shock. By such an approach, the influence of hsp overexpression on the susceptibility of cells to apoptosis induced by serum starvation could be studied. In addition, the enhancement of hsp expression by hyperthermic shock provides a model to determine whether increased hsp expression protects leukaemic cells against the deleterious effects of cytotoxic drugs.

## 6.2. Analysis of the kinetics of the heat shock response in myeloid leukaemia cell lines.

Four myeloid leukaemia cell lines were used, THP-1 (myelomonocytic), KG1a (myeloid), HL-60 (promyelocytic) and KU812 (myeloid). These were maintained as described in the Methods and Materials section. Cell lines were subjected to hyperthermic conditions by incubation at 42°C for periods of 15, 30 and 60 minutes, prior to returning the cells to the incubator at 37°C. The expression of the major hsp and cell cycle analysis was then determined by double immunofluorescence staining and flow cytometric analysis of cells removed from culture at various time intervals.

### 6.2.1. Hyperthermic shock rapidly induces hsp70 and hsp90 expression.

Hyperthermia resulted in increased expression of hsp25, hsp70 and hsp90 in all cell lines studied. Hsp70 and hsp90 induction were the most pronounced in all four lines, both in terms of the magnitude and the rapidity of the response (see figure 6.1). As shown in figure 6.1, heat-shock of HL-60 cells results in an increase in hsp90 expression which is evident after only 4 hours. The increase in hsp90 levels was more rapid in cells exposed to a longer heat shock, but in all experiments, similar maximum levels of hsp90 were reached (after ~16 hours) irrespective of the duration of the hyperthermic treatment. Finally, the kinetics of hsp90 decay varied according to the duration of heat shock. Higher levels of hsp90 expression were maintained for longer in those cells incubated at 42°C for 60 minutes than in those exposed to the higher temperature for 30 and 15 minute periods.

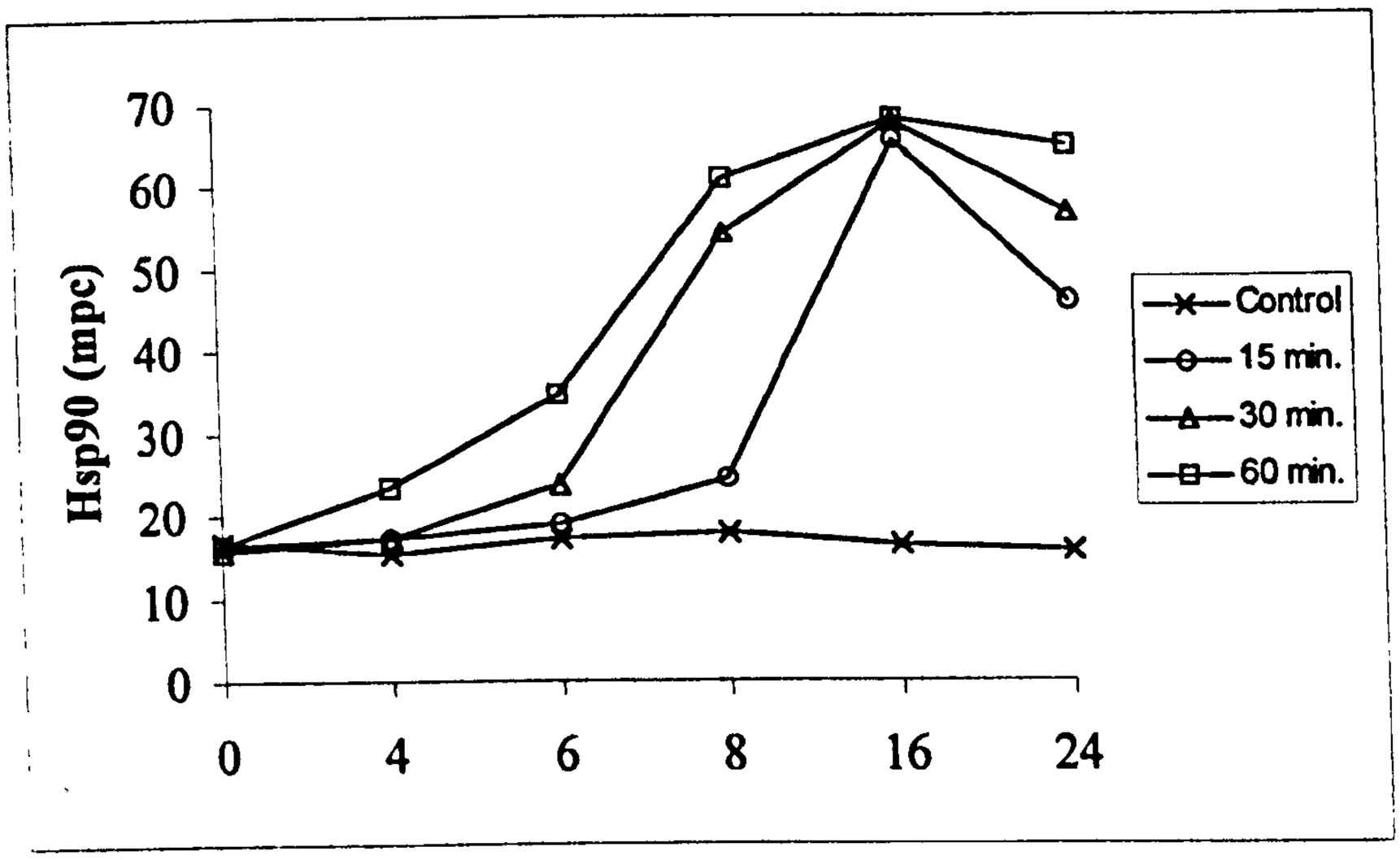
As expected, the highly heat-inducible hsp72 demonstrated the most significant increase in expression following hyperthermia, a 15-17 and a 10-12-fold increase in hsp72 protein levels being seen 16 hours post-heat shock in the KU812 and HL-60 cell lines respectively. For hsp72 induction, the duration of hyperthermic treatment was directly proportional to the maximum levels of protein expression seen, with higher levels being reached in those cells subjected to a 60 minute heat shock. In terms of the kinetics of the response, the time interval before detectable induction of hsp72 expression did not vary significantly according to whether a 15, 30 or 60 minute heat shock was used. However, at 24 hours, levels of hsp72 had decreased more rapidly in cells incubated at 42°C for shorter periods. These distinctive patterns of hsp90 and hsp72 induction, to a maximum at approximately 16 hours followed by a decrease dependent upon the duration of hyperthermic treatment, were similar in the other three cell lines studied.

The kinetics of hsp25 induction varied slightly between the cell lines (results not shown), but in all cases the magnitude of the response was less than that seen for hsp90 and hsp70. The most rapid response for the induction of hsp25 was seen in HL-60 cells, with an increase in protein expression evident 4 hours post-heat shock, and reaching a maximum at 16 hours (3.2-fold increase compared with control cells). In the

KU812, KG1a and THP-1 cell lines, induction of hsp25 was not detected until 8 hours post-treatment, with maximum induction reaching between 2.4 (in the KU812 cells) and 3.7-fold (in THP-1 cells) the protein levels seen in the control cells which were maintained throughout at 37°C.

Although the magnitude and rapidity of hsp25 induction was not as great as seen for hsp70 and hsp90, hsp25 levels remained elevated for longer than any of the other hsp families examined. In THP-1 and KU812 cells the levels of hsp25 were still maximal at 24 hours post-heat shock.

Hyperthermic treatment at 42° resulted in no detectable change in levels of hsp60 in three of the myeloid cell lines tested, even in cells exposed to the elevated temperature for 60 minutes (results not shown). A significant increase in hsp60 was evident only in the THP-1 cell line, with levels reaching a maximum at 16 hours post heat shock which was 4-5-fold higher than control cells.



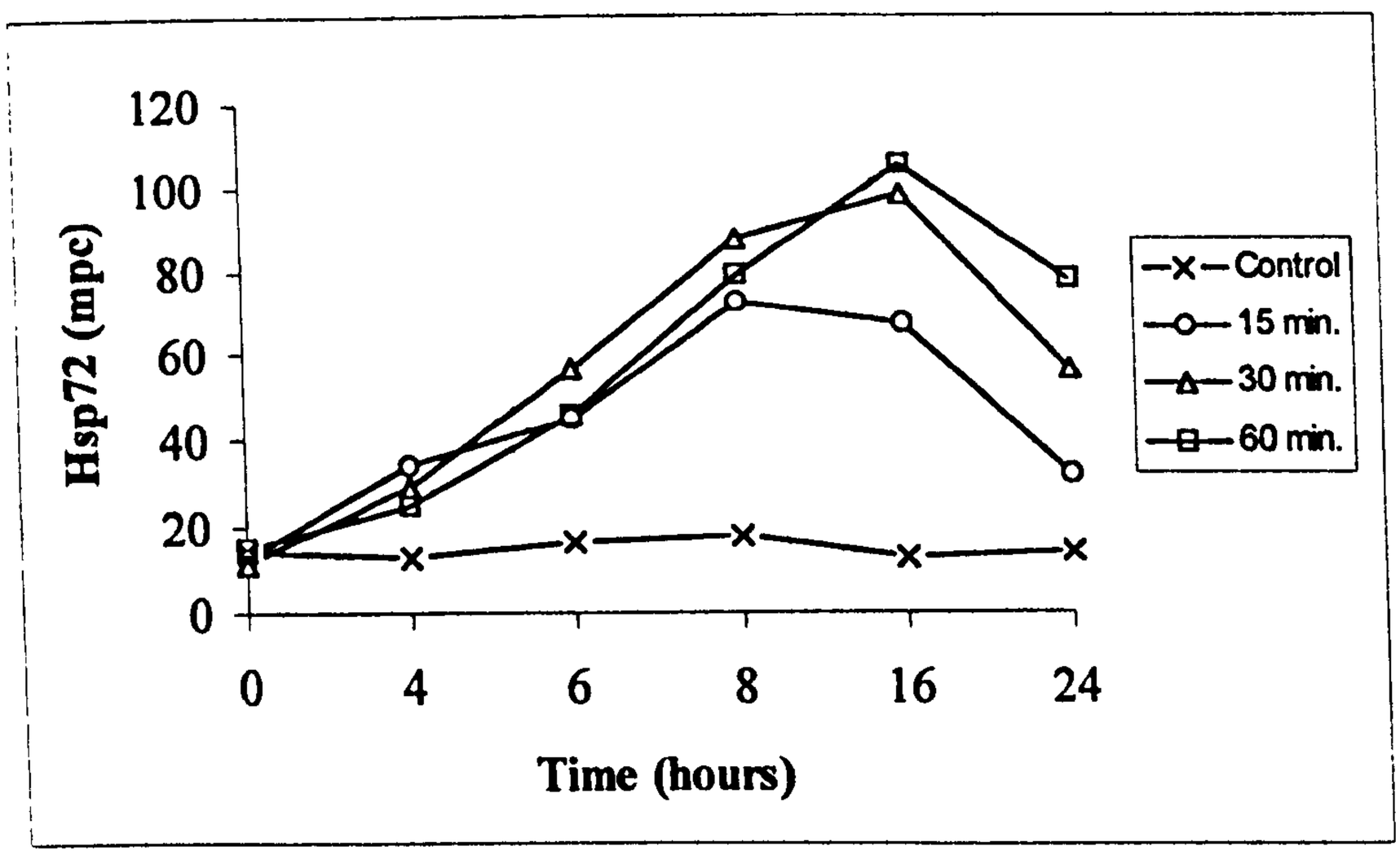




**Figure 6.1.** Induction of hsp90 and hsp72 in HL-60 cells by hyperthermic exposure (42°C) for various time intervals. Cells were incubated in a 42°C waterbath for 15, 30 and 60 minutes and the kinetics of hsp90 and hsp72 induction measured by comparison with control cells maintained at 37°C. Hsp90 and hsp72 are shown in terms of the mean peak channel of fluorescence (mpc). Results plotted are the means of triplicate experiments.

## 6.2.2. Hyperthermia induces the expression of p53 in some myeloid cell lines.

Expression of p53 was examined in leukaemic cell lines following hyperthermic shock (42°C for 30 min.). Immunofluorescent staining with an antibody recognising both wild type and mutant p53 revealed that acumulation of p53 protein ocurred in the KG1a and KU812 cell lines, this increase being evident 8 hours post-heat shock.

Double immuno-fluorescence staining of ethanol-fixed cells using a FITC-labelled p53 antibody and propidium iodide to stain DNA, revealed that the induction of p53 occurred independently of the cell cycle, with levels increased throughout in $G_1$, S and $G_2$/M (figure 6.2). These flow cytometry histograms of propidium iodide staining (FL3) versus p53 levels (LFL1) show that there is a slight increase in LFL1 in cells in the $G_2$/M phase of the cell cycle, but this is non-specific staining as shown by a similar pattern with the isotype-matched negative control antibody. This artifact is probably due to the larger size of cells in the $G_2$/M phase.

However, in both KG1a and KU812 cells, hyperthermia-induced expresion of p53 was associated with a small, but reproducible, accumulation of cells in the $G_2$/M phase (Table 6.1). In exponentially growing KU812 cells, the proportion of cells in the $G_2$/M phase was consistently measured as being between 12-14 % of the total population and this increased to 20-28 % following incubation at 42°C for 60 minutes. A similar increase in the $G_2$/M population was evident in KG1a cells following hyperthermia, and in both cases this was concomitant with a reduction in the number of cells in the $G_0/G_1$ phase of the cell cycle. This accumulation of cells at the $G_2$/M boundary was detectable at a time interval coincident with the measurable induction of p53, i.e at 6-8 hours post-heat shock.

No increase in p53 levels was evident in the THP-1 line following hyperthermic treatment, and, as expected, HL-60 cells (which have large deletions in the p53 gene) expressed no detectable p53 in either the control or heat-shocked cells.

| | Control, 37°C | | | Heat shock, 42°C, 30' | | |
|---|---|---|---|---|---|---|
| | $G_0/G_1$ | S | $G_2/M$ | $G_0/G_1$ | S | $G_2/M$ |
| HL-60 | 65.4 | 9.5 | 25.1 | 62.9 | 11.5 | 25.6 |
| THP-1 | 55.6 | 25.0 | 19.4 | 52.4 | 28.3 | 19.3 |
| KU812 | 65.0 | 18.8 | 13.2 | 51.2 | 21.2 | 27.6 |
| KG1a | 61.4 | 10.1 | 28.5 | 45.4 | 15.6 | 39.0 |

**Table 6.1.** Cell cycle analysis of myeloid cell lines in exponentially growing cells and following hyperthermia (42°C, 30 min.). Distribution of cells in each phase of the cell cycle was measured by flow cytometric analysis of propidium iodide-stained cells, and in the heat-shocked cells at an interval of 8 hours after hyperthermic treatment.

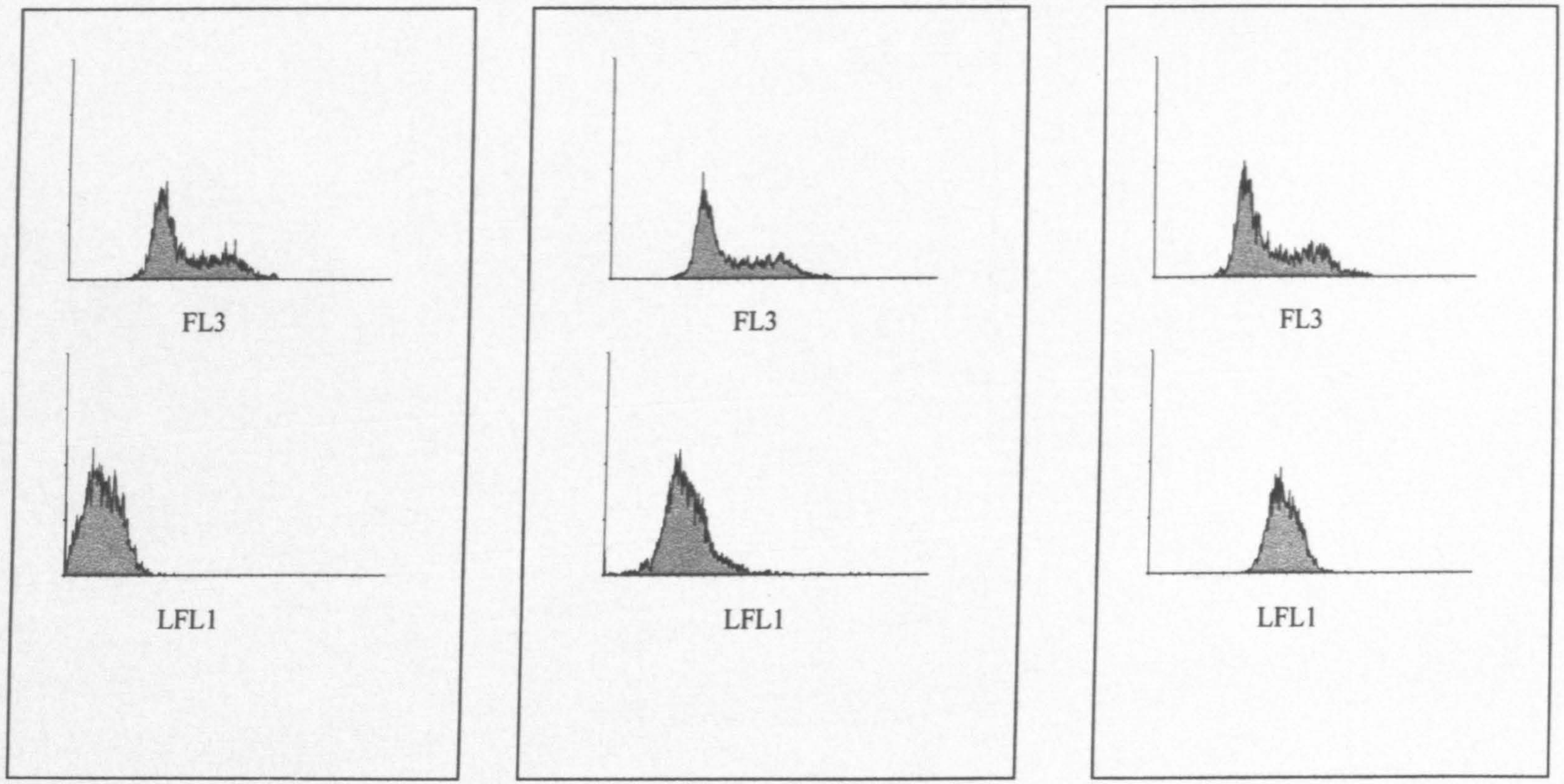



Figure 6.2. Induction of p53 protein expression by hyperthermia in the KU812 cell line. Flow cytometric analysis of heat shocked cells (42°C, 30 min.) demonstrates increased FL1 staining (c) compared with (b) a non-heat shocked control. Specific staining of p53 was compared with (a) an isotype matched control

### 6.2.3. Induction of hsp by hyperthermia does not protect against apoptosis induced by serum withdrawal.

KU812 and KG1a cells were exposed to a hyperthermic shock (42°C for 15 min.), and allowed to recover at 37°C for 3 hours. Control cells were maintained throughout at 37°C. Cells were then washed and resuspended in RPMI 1640 medium containing no foetal calf serum (FCS), and cell cycle analysis was performed by propidium iodide staining of cells removed at subsequent intervals of 4, 18, 24 and 48 hours. As shown in table 6.2, withdrawal of serum from exponentially growing KU812 and KG1a cells resulted in the appearance of apoptotic cells, evident at a time interval of 18 hours after the resuspension of cells in growth medium lacking FCS. As expected, the proportion of apoptotic cells increased with time. This was demonstrated by the number of propidium iodide-stained cells appearing as a sub-$G_1$ peak in the samples removed at 24 and 48 hour intervals. KU812 and KG1a cells exposed to hyperthermic treatment expressed higher levels of hsp25, hsp72/73 and hsp90, as described in section 6.2.1. When the rate of apoptosis following removal of growth factors was examined, there was no decrease in the susceptibility of cells to apoptosis compared with control cells maintained at 37°C.

| | Control cells | (37°C) | Heat shocked | (42°C, 30 min) |
|---|---|---|---|---|
| Time (hrs.) | KU812 | KG1a | KU812 | KG1a |
| 0 | 3.5 | 2.1 | 5.6 | 2.3 |
| 4 | 3.7 | 1.7 | 4.9 | 4.2 |
| 18 | 11.3 | 4.6 | 17.3 | 8.7 |
| 24 | 24.7 | 19.9 | 35.2 | 23.4 |
| 48 | 75.6 | 45.3 | 85.2 | 48.1 |

**Table 6.2.** Culture of KU812 and KG1a cell lines in the absence of added FCS results in the induction of apoptosis. Hyperthermia (42°C, 30 min.) does not appear to protect against apoptosis withdrawal. Figures shown are the means of triplicate experiments.

## 6.3. Induction of apoptosis by chemotherapeutic agents.

A range of chemotherapeutic agents, with differing modes of action (table 6.3) were used to investigate the stress response of leukaemic cell lines. As described in the introduction, at least one cytotoxic drug (bis-chloronitrosourea) will induce hsp70 expression and so the response of leukaemic cell lines to a range of concentrations of this drug was investigated. In addition, cells were exposed to the drugs, vincristine, cytosine arabinoside and doxorubicin at the concentrations indicated in figure 6.3.

| Drug | Mode of action |
|---|---|
| Vincristine | A Vinca alkaloid whose primary mode of action is inhibition of mitosis. Inhibits formation of mitotic spindle by blocking the polymerisation of tubulin. Widely used in treatment of leukaemias and lymphomas. |
| Cytosine arabinoside | A pyrimidine nucleoside analogue. Incorporation into DNA as ara-CTP leads to termination of DNA strand synthesis. One of the mainstays in the treatment of AML. |
| Bischloronitrosourea (BCNU) | An alkylating agent, forms covalent bonds with biologic nucleophiles changing their chemical structure and activity. Results in damaged DNA and also altered protein structures. |
| Doxorubicin | An anthracycline antibiotic which intercalates with DNA and inhibits DNA-dependent DNA polymerase and DNA-dependent RNA polymerase. Widely used in treatment of acute leukaemias and certain solid tumours. |

**Table 6.3.** Four classes of cytotoxic drug widely used in the treatment of leukaemias were utilised in the analysis of apoptotic induction and hsp expression in myeloid leukaemia cell lines.

### 6.3.1. Cytotoxic drugs induce apoptosis in myeloid cell lines.

Treatment of all four myeloid leukaemia cell lines with BCNU, cytosine arabinoside, vincristine and doxorubicin (table 6.2) resulted in the appearance of apoptotic cells as determined by the presence of a sub-diploid peak in propidium iodide-stained cells. A linear response of increasing apoptosis in myeloid cells exposed to increasing concentrations of BCNU was evident in all cell lines in terms of the induction of apoptosis (figure 6.3). Similar results were obtained with the other three classes of cytotoxic drug, demonstrating that chemotherapeutic drugs with different primary modes of action all exert their cytotoxic effects via the induction of apoptosis.

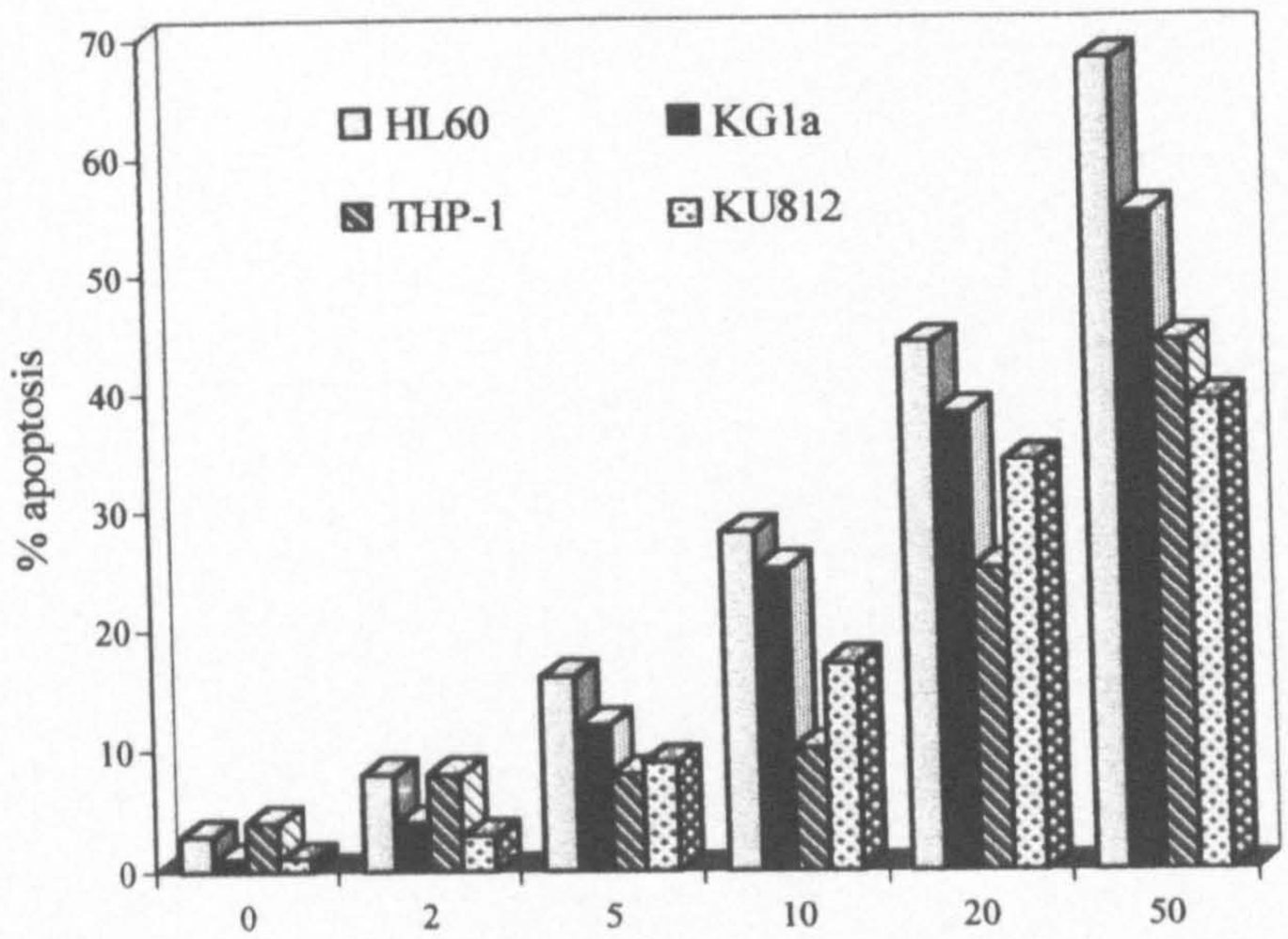



**Figure 6.3.** Apoptosis induced by the alkylating agent, bischloronitrosourea (BCNU) in myeloid cell lines.

### 6.3.2. BCNU induces hsp70 (72/73) expression in leukaemic cell lines.

Cytotoxic concentrations of BCNU induced transient levels of hsp72/73 overexpression, detectable 4-6 hours after exposure to the cytotoxic drug, and this preceded the onset of apoptosis. Levels of hsp70 induction by BCNU were not as great as the levels which can be induced by heat shock (hyperthermia). Despite relatively high levels of hsp70 being induced in the cells treated with the highest concentrations of BCNU, this stress response did not protect against apoptosis as evidenced by the higher levels of apoptosis seen in these cells.

Analysis of hsp70 expression 4 hours after exposure to BCNU demonstrated that the induction of hsp70 occurred in all cells rather than in a small number. Cell cycle analysis of hsp70 expresion in these drug-exposed cells revealed that there was no cell cycle-specific response, with all cells expressing similarly elevated hsp70 levels in all phases of the cell cycle (see figure 6.4).

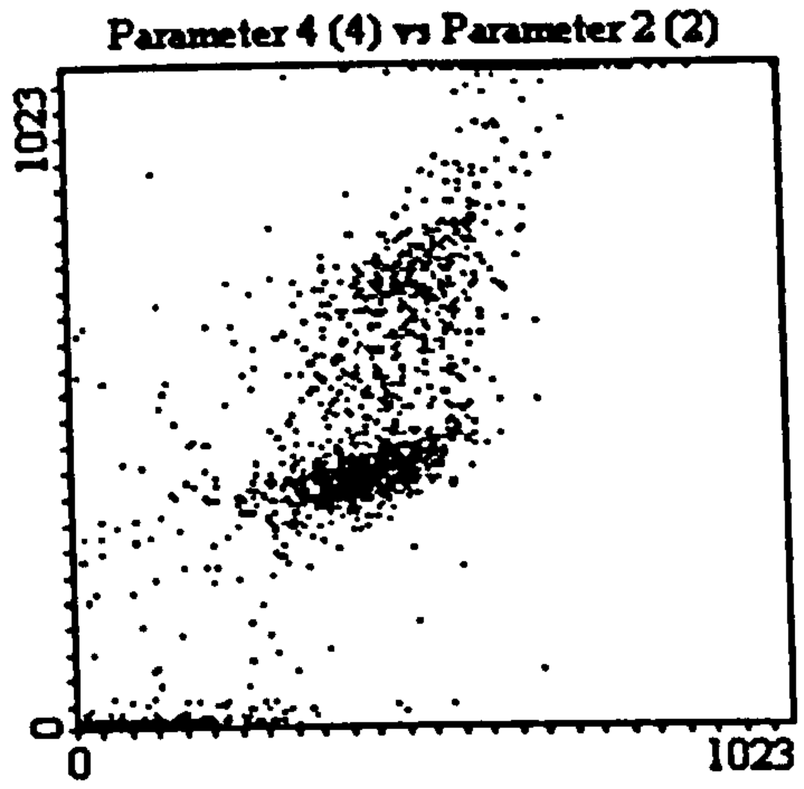



**Figure 6.4.** Increased Hsp70 expression in KU812 cells demonstates that hsp70 induction occurs is not cell-cycle-specific and occurs in all phases of te cell cycle.

Exposure of these myeloid cell lines to the three other drugs described in table 6.2 had variable effects on the stress response in terms of hsp70 induction. Vincristine at cytotoxic concentrations led to an increase in hsp70 levels, although these were not as great as those seen with BCNU, while doxorubicin also induced hsp70 expression to levels which were similar to those seen with BCNU, and similarly this preceded the onset of apoptosis. However, cytotoxic concentrations of cytosine arabinoside did not alter the detectable levels of hsp70 despite inducing apoptosis within a similar time range to the other three drugs tested.

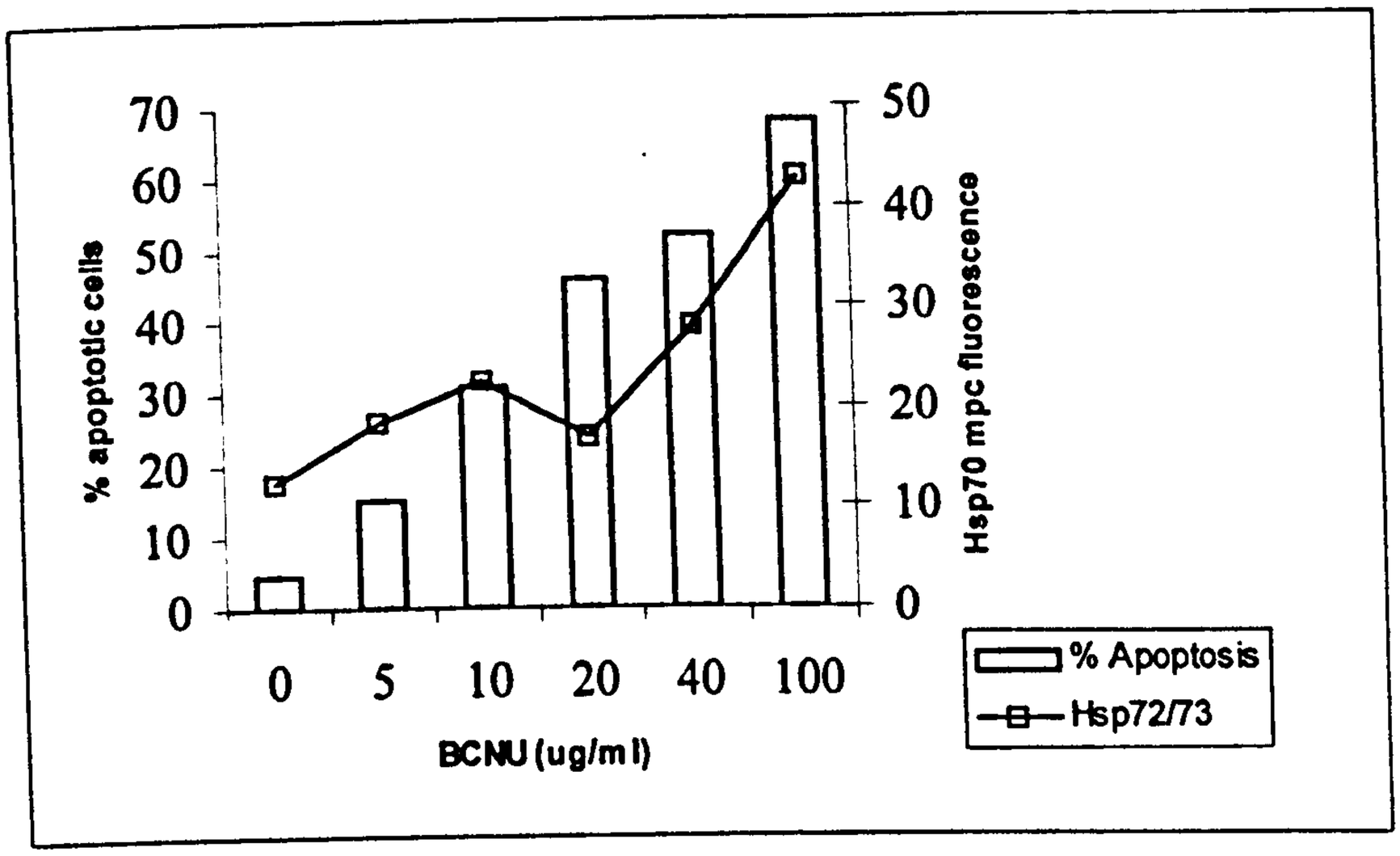



**Figure 6.5.** Effect of the alkylating agent BCNU on hsp72/73 expression and apoptosis in HL60 cells. Hsp70 expression was analysed in these cells using an antibody to both the constitutive (hsp73) and inducible (hsp72) forms of hsp70 at a time interval of 6 hours post addition of BCNU. The percentage of apoptotic cells was determined at 24 hours. .

## 6.3. Mild hyperthermia protects cells aginst the lethal effects of cytotoxic drugs.

### 6.3.1. Hyperthermic shock protects myeloid cell lines against the cytotoxic effects of BCNU.

Prior exposure of KG1a and KU812 cells to a sub-lethal heat shock leads to elevated levels of heat shock proteins as disussed earlier. Whwn these cells were exposed to the alkylating agent, bischloronitrosourea, BCNU, it was found that these cells with higher levels of hsp were partly resistant to the cytotoxic effects of BCNU as evidenced by a decrease in the number of apoptotic cells after 24 hours compared with control cells which have been maintained throughout at 37°C. (Figure6.6)

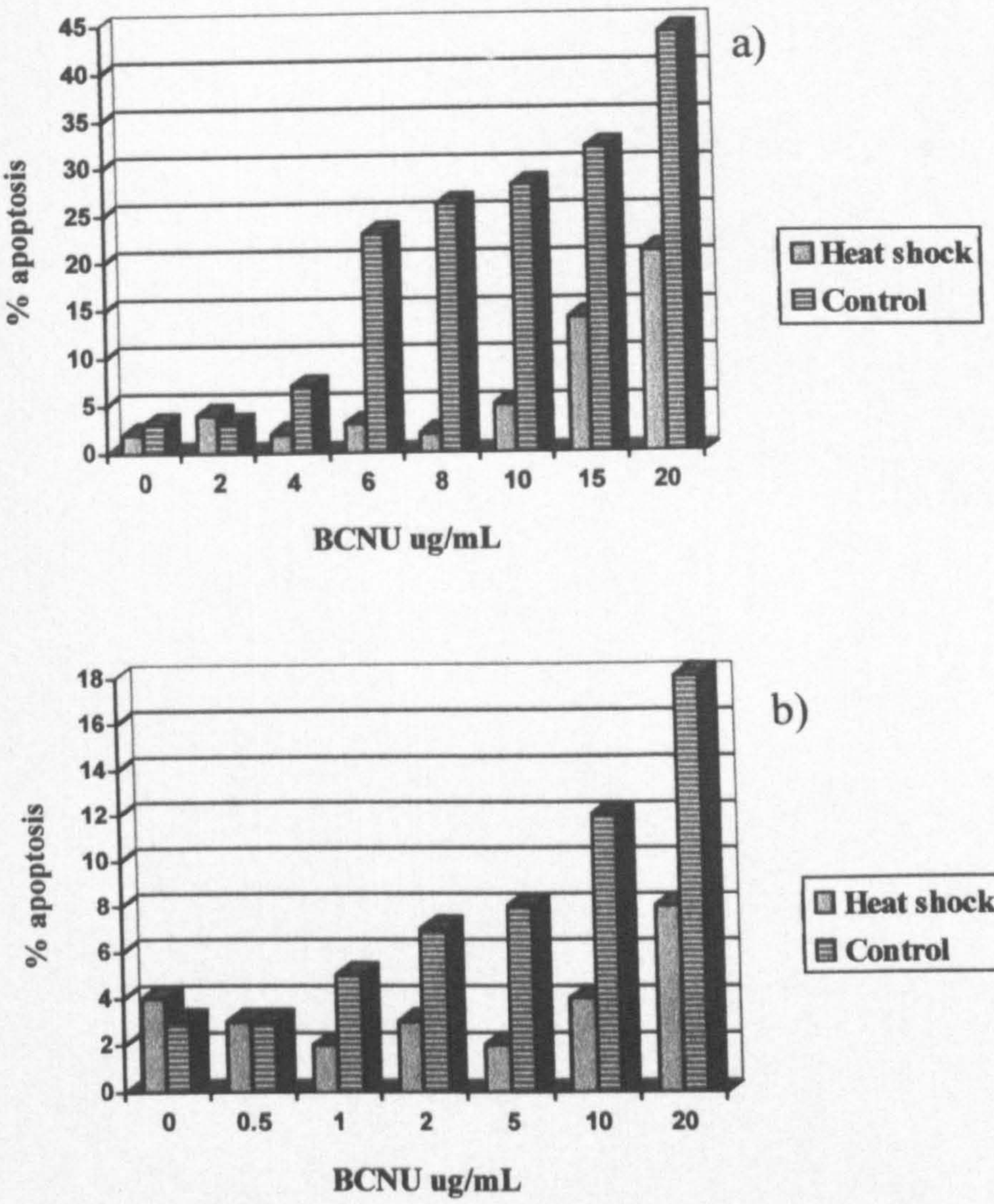



**Figure 6.6.** Mild hyperthermic shock (42°C, 30 min.) partly abrogates the apoptotic response of a) HL-60 and b) KG1a myeloid cell lines following exposure to the alkylating agent, bischloronitrosourea.

## 6.3.2. Resistance induced by hyperthermia is independent of bcl-2.

The expression of bcl-2 protein in the myeloid cell cell lines was determined by immunofluorescent staining and flow cytometry. All four cell lines expressed detectable, but low levels of bcl-2. Following heat shock at 42°C for 30 min., bcl-2 expression in KG1a and KU812 cells was unchanged when compared with control cells mainatained throughout at 37°C (Figure 6.7). Similar results (not shown), were obtained for THP-1 and HL-60 cells.

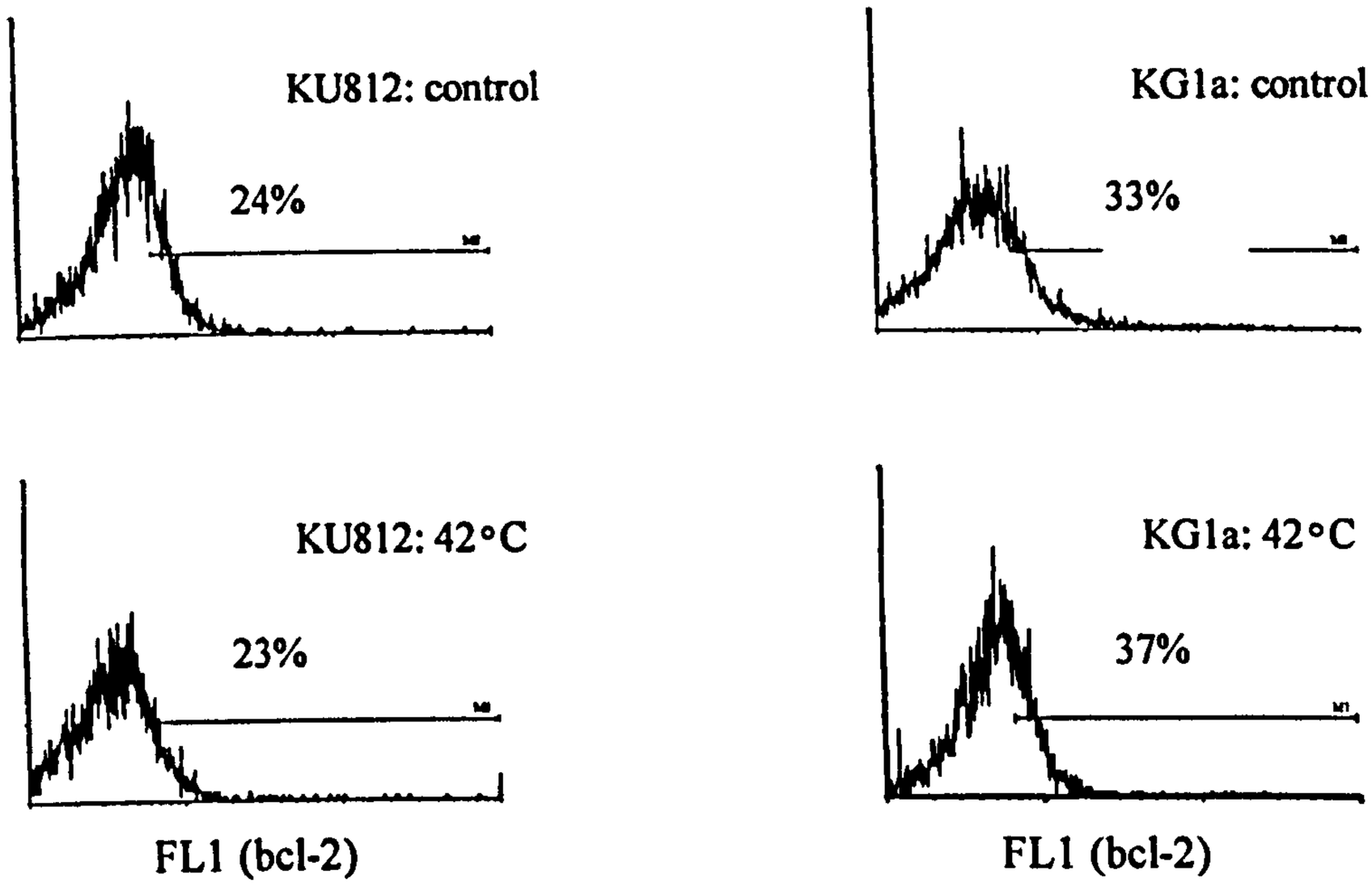



**Figure 6.7.** Expression of bcl-2 protein in KU812 and KG1a cells is unchanged by hyperthermia. Histograms of fluorescence intensity are shown as % positivity within analysis region M1. This was set up using an isotype-matched control antibody so that 98% of cells are outside the analysis region.

## 6.4. Degree of heat shock is either protective or enhances drug-induced apoptosis.

The apoptotic response of myeloid cell lines to BCNU following prior hyperthermic treatment was found to depend upon the severity of the heat shock. As described, heat shock at 42°C for 30 minutes induced protection against the cytotoxic effects of BCNU in all four cell lines, but exposure to higher temperatures had the opposite effect and actually enhanced BCNU-induced apoptosis (Table 6.4).

Heat shock of myeloid cell lines using temperatures above 42°C increases both the basal rate of apoptosis in all four cell lines, and has a synergistic effect on the susceptibility of cells to apoptosis induced by BCNU. Thus at higher temperatures (43°C and 44°C), which are associated with levels of hsp72/73 even higher than those produced at 42°C, overexpression of hsp fails to offer a protective effect.

| | HL-60 | | | THP-1 | | | KU812 | | | KG1a | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BCNU µg/mL | | | | | | | | | | | | |
| | 42°C | 43°C | 44°C | 42°C | 43°C | 44°C | 42°C | 43°C | 44°C | 42° C | 43° C | 44° C |
| 0 | 3.8 | 5.6 | 32.6 | 2.2 | 3.6 | 16.2 | 2.1 | 2.5 | 6.9 | 4.8 | 4.5 | 10.8 |
| 10 | 6.2 | 11.4 | 75.1 | 15.3 | 14.9 | 25.6 | 5.3 | 9.8 | 18.5 | 4.7 | 5.6 | 15.6 |
| 20 | 22.5 | 35.6 | 78.2 | 25.9 | 26.8 | 32.1 | 15.6 | 25.2 | 35.6 | 7.9 | 21.2 | 29.6 |

**Table 6.4.** Effect of different degrees of heat shock on the apoptotic response of myeloid cell lines to bischloronitrosourea (BCNU). Cells exposed to varying hyperthermic temperatures for 30 min. were exposed to BCNU (0, 10 and 20 µg/mL) after 6 hours recovery at 37°C. The percentage of apoptotic cells was measured 16 hours after the addition of the cytotoxic drug.

# Chapter 7

# Discussion and concluding remarks.

## 7.1. Introduction.

The initial aim of my research was to examine heat shock protein (hsp) expression in primary human leukaemia cells, and to establish whether this expression was abnormal either in terms of protein levels or the cellular localisation of hsp. At the outset of this work, a growing body of evidence from a variety of cell systems, including haematopoietic cells, suggested that hsp expression is linked to cellular growth and differentiation. For example, hsp70 and hsp90 expression is induced in T-lymphocytes following mitogenic stimulation (Ferris *et al.*, 1988; Haire *et al.*, 1988; Fincato *et al.*, 1991), whilst the differentiation of HL-60 and K562 (myeloid leukaemia) cell lines is associated with changes in hsp60, hsp70 and hsp90 expression (Mivechi *et al.*, 1994). It was also established at this time, that hsp70 expression is regulated throughout the cell cycle in normal cells and involves interaction of hsp70 with specific proteins at specific points in the cell cycle (Pelham, 1986). The identity of these proteins associated with hsp70 was unknown, and this remains largely unclear today, although it appeared likely that these were cell cycle-specific growth regulatory factors. One such factor, which was known to interact with hsp70, is the protein product of the tumour suppressor gene, *p53*. Research into the biology and function of p53 protein has proliferated over the last decade, not least because it has been found that mutations within the *p53* gene represent the most common genetic abnormalities found in human cancers to date (Lane, 1994). Roles for p53 have been identified in a number of central cellular processes, including cell cycle regulation, DNA repair, and control of apoptosis following genetic injury (reviewed by Ko and Prives, 1996, and see Introduction, section 1.2). What was most exciting in terms of this project, is the fact that the formation of hsp70/p53 complexes is an association which modulates the function of p53 (Hupp *et al.*, 1992). It was intriguing that abnormal levels of hsp expression had been reported in virally- and chemically-transformed cells (Finlay *et al.*, 1988), as well as in some human tumour cell lines (Ferrarini *et al.*, 1992). One of the hypotheses upon which this project developed was therefore the possibility that hsps play an indirect role in the control of cellular differentiation via their ability to interact with proteins which have a direct regulatory role. Thus, could the abnormal levels of

hsp expression seen in some tumour cells play a role in tumourigenesis via dysregulation of the normal growth/differentiation processes, or are they simply a reflection of the malignant phenotype of these cells? In order to address this question, the expression of three major hsp families, hsp60, hsp70 and hsp90 was analysed by immunochemical means in leukaemic cells collected from patients presenting with a variety of haematopoietic malignancies. This analysis determined the localisation of hsp expression as well as the levels of each of these hsp families in normal and leukaemic cells.

The second major aspect of hsp expression in leukaemia addressed in this work concerns the relationship between hsp expression and apoptosis. Apoptosis (programmed cell death) is a homeostatic mechanism inherent to all cell types, which is responsible for the maintenance of cell numbers within a tissue. There is a fine balance between the opposing forces of growth/proliferation and cell death via apoptosis, and a number of genetic lesions have been identified which impinge upon both these processes. Many of these lesions are strongly implicated in carcinogenesis. Apoptosis is also a critical component of the cellular response to injury in both normal and malignant cells, and this clearly has implications towards the treatment of cancers since most, if not all, chemotherapeutic drugs exert their effects via the induction of apoptosis (reviewed by Lyons and Clarke, 1997).

What is the relevance of hsp expression in terms of the regulation of apoptosis? The evidence available during the early stages of my work suggested that hsp expression may be integrally linked to apoptosis in two ways. Firstly, the protective nature of hsps may prevent apoptosis in response to cellular injury, and this may have implications for the unwanted survival of leukaemic cells during chemotherapy. Secondly, the balance between cell growth/differentiation and apoptosis may be controlled by the interaction of hsps with regulatory factors involved in both processes, a point which was emphasised earlier in this section with relation to the roles of p53 protein. The role of hsp expression in the cellular response to stress is well established; over-expression of hsps protects cells against the deleterious effects of a variety of cellular insults, and this includes both environmental and pathophysiological agents which are known to induce apoptosis. For example, hyperthermia induces apoptosis in a variety of cell systems, and this can be inhibited by overexpression of hsps either by a prior sub-lethal heat

shock, or by transfection of cell lines with hsp-encoding genes, including both hsp70 and hsp25 (Li *et al.*, 1996). Other reports in the literature which suggest that hsps may influence apoptosis include the observation that hsp70 expression affects apoptosis induced by quercetin, a bioflavanoid, in leukaemic cell lines ( Wei *et al.*,1994), hsp70 protects cells against apoptosis induced by tumour necrosis factor (Jaatela *et al.*, 1992), and the induction of hsps by mild heat shock protects thymocytes against glucocorticoid-induced apoptosis (Miglioroti *et al.* 1992). These observations suggest that hsp expression in tumour cells may be extremely important in protecting them against apoptosis, induced for example by hypoxia or by cytolytic immune mechanisms. Furthermore, the induction of hsp expression may protect cells against cytotoxic drugs used in the treatment of cancers. It is recognised that most, if not all, cytotoxic drugs used in the treatment of cancers exert their effects via the induction of apoptosis, and the inherent propensity of a malignant cell to undergo apoptosis is therefore a major determinant of the success of chemo-therapeutic regimens. The hypothesis upon which I worked was that leukaemic cells may be able to mount a stress response following exposure to cytotoxic drugs, involving the induction of hsps. This may protect them against the deleterious effects of cytotoxic drugs, and would therefore be an important mechanism leading to the development of chemoresistance. During the early stages of this project it was known that exposure of human tumour cell lines to at least one cytotoxic agent, bischloronitrosourea (BCNU), induces hsps (Kroes et al., 1991), suggesting that they may indeed be an important mechanism of resistance to standard chemotherapy.

To summarise, the study of hsp expression in relation to apoptosis in leukaemic cells was considered from two mutually exclusive standpoints: (1) dysregulation of apoptotic pathways, or failure to activate apoptosis in response to genetic injury, is generally accepted to be a route towards carcinogenesis, and (2) hsp-mediated inhibition of apoptosis induced by cytotoxic drugs may be a factor in the development of chemoresistance.

In this brief introduction, I have attempted to outline how the aims of this work were formulated in the context of the available knowledge concerning the possible relationship between hsp expression and apoptosis. This clearly has associated implications for leukaemogenesis and the treatment of haematological malignancies. At

the outset of these investigations, no publications existed in the literature which examined the expression of hsp in primary leukaemia cells, only a few reports which described patterns of hsp expression in haematopoietic cell lines, particularly in terms of the differentiation of these cells. It was therefore an original line of investigation which, as will become clear during the discussion of my results, evolved via an elementary examination of hsp expression in leukaemic cells towards an investigation of the possible relevance of hsp overexpression.

## 7.2 Localisation of hsp in normal and leukaemic haematopoietic cells

### 7.2.1. Cell surface expression of heat shock proteins.

The cellular localisation of hsp is generally considered to be intracellular, confined to the cytoplasmic compartment under normal conditions of growth. The specific localisation of major hsps presumably reflects their role as molecular chaperones (reviewed by Scwarz *et al.*, 1996; Lund, 1995). However, at the outset of these investigations, conflicting reports existed in the literature regarding the existence of hsp expression on the cell surface membrane. Some authors had found no evidence of surface expression (Ferm *et al.*, 1989), whilst others had reported surface expression on certain cells. For example, human γ/δ T cells express a cell surface antigen which is recognised by a monoclonal antibody to hsp58 (P1), the human homologue of the *Escherichia coli* groEL protein (Jarjour *et al.*, 1990). Also, murine bone marrow-derived macrophages have been shown to express an epitope which is recognised by ML30, a monoclonal antibody which has specificity for mycobacterial hsp60 (Wand-Württenberger *et al.*, 1991), and surface expression of hsp72, the inducible form of hsp70, has been reported on some tumour cell lines and fresh tumour cells (Ferrarini *et al.*, 1992). At this time, the significance of surface hsp expression in these cells remained unclear, but the possibility that cell surface expression of hsp may be a marker of the malignant phenotype in leukaemic cells required investigation, since

there were no reports in the literature concerning the specific localisation of hsps in primary haematopoietic cells.

As presented in Chapter 3, repeated experiments based upon immunofluorescent staining and flow cytometric analysis of a variety of primary leukaemia cells revealed no detectable expression of any of the major hsp families on the surface of these cells. I examined cells from 16 patients with acute myeloid leukaemia (AML), as well as 6 patients with acute lymphoblastic (ALL) and 4 patients with B-cell chronic lymphocytic leukaemia (B-CLL), a total of 28 individuals. Even using the most sensitive avidin-biotin procedure, I found no evidence of specific cell surface staining for hsp90, hsp72/73 or ML30-reactive epitopes. Having carefully analysed cells from such a relatively large number of leukaemic patients, it was decided that cell surface expression of hsp is not a feature of leukaemia cells. However, I also considered the possibility that cell surface expression of hsp may be a marker of stress. The effect of a hyperthermic shock on leukaemic cells was therefore analysed in order to determine whether such a cellular stress could induce hsp expression on the surface of AML cells. Cells from 6 patients with AML, as well as normal peripheral blood mononuclear cells from 4 healthy individuals were exposed to hyperthermic stress (42°C, 30 min.), and stained as before using monoclonal antibodies to ML30, hsp90 and hsp72/73. In addition, they were also stained with an antibody specific to the highly heat-inducible hsp72 protein. Again, repeated experiments failed to demonstrate the induction of hsp expression at the cell surface. In addition to primary leukaemia cells, I also analysed four haemopoietic cell lines, KU812 (myeloid), KG1a (myeloid), THP-1 (myelomonocytic), and HL-60 (promyelocytic) for evidence of hsp expression on the cell surface. Again, no specific staining was evident using any of the 4 monoclonal antibodies described, either under conditions of normal growth or following heat shock. The data indicates that hsps are expressed infrequently, if ever, on the surface of leukaemic cells.

During the course of these investigations, several new reports have appeared in the literature which suggest that hsp expression occurs on the cell surface membrane of some tumour cells, and the possible biological significance of this has been investigated by one group of investigators in particular. Multhoff *et al.* (1995) have reported that the highly stress-inducible hsp72 antigen is found on the surface of some tumour cells,

including Ewing's sarcoma and osteosarcoma cells, following a non-lethal heat shock. Similar treatment of normal cells, including peripheral blood lymphocytes and fibroblasts derived from healthy volunteers, did not result in accumulation of hsp72 at the cell surface. The authors suggested that this difference between tumour cells and normal cells may provide a means by which to elicit a stress-inducible immune response which specifically targets tumour cells. Recent reports from the same laboratory have described cell surface expression of hsp72 on a variety of tumour cells following non-lethal heat shock. These include a human lung carcinoma cell line (Botzler *et al.*, 1996), a colonic carcinoma cell line (Multhoff *et al.*, 1997) and also, interestingly, on a myeloid leukaemia (K562) cell line (Multhoff, 1998). In the latter case, they demonstrated that heat shock-induced cell surface expression of hsp72 was associated with an increased sensitivity of K562 cells to lysis mediated by natural killer cells, thus reinforcing their earlier assertion that cell surface expression of hsp may be a tumour-specific immunogenic determinant.

This final observation leads me on to an interesting postscript regarding the possibility of hsp expression on the surface of leukaemic cells. Although I have been unable to demonstrate any evidence of surface hsp expression in either normal or leukaemic haemopoietic cells, I have recently examined the role of hsp expression and mechanisms of apoptosis in myeloma cell lines. These experiments have investigated the influence of hsp expression on the susceptibility of myeloma cell lines to apoptosis, induced by both cytotoxic drugs and a bacterial toxin, verocytotoxin. As part of this investigation, I have looked for surface expression of hsp, and preliminary experiments have indicated that apoptotic cells do in fact express both hsp70 (the specific heat-inducible hsp72) and hsp90 on the cell surface. In these experiments, the induction of apoptosis by the cytotoxic agent, bischloronitrosourea (BCNU), was demonstrated by increased annexin V binding, which was evident on myeloma cells at approximately 6 hours after the addition of BCNU. Double labelling of these cells, revealed that early apoptotic cells, which were identified by annexin V binding, expressed hsp72 and hsp90 on the cell surface. At this stage, the significance of these observations remains unclear, although it is feasible that hsp may have a functional role in the apoptotic process. It is possible that these hsp function as direct antigenic determinants which act as an apoptotic 'flag' and a signal to neighbouring phagocytic cells. There is a precedent for this hypothesis in the expression of ubiquitin, which itself can be

classified as an hsp since it is induced by hyperthermia, and is well known to act as a flag to phagocytic cells. Alternatively, hsp may be functioning as molecular chaperones by interacting with other cellular protein(s) which are transported to the cell surface during the apoptotic process. The observations by Multhoff's group concerning stress-induced surface hsp expression along with my observations that apoptotic cells express hsp72 and hsp90 on the cell surface, demands a re-investigation of the potential role of hsps in leukaemia cells. It would therefore be interesting to re-analyse leukaemic cells following stresses known to induce apoptosis.

## 7.2.2.Localisation of hsp by immunocytochemical staining.

Expression of all the major hsp families appears to be generally confined to the cytoplasmic compartment of cells, and their specific sub-cellular localisation under conditions of normal growth presumably reflects their role as molecular chaperones. Following heat shock, members of the hsp family are rapidly translocated to the nucleus where their major role is to prevent the aggregation of damaged and unfolded proteins within the nucleus. This is an example of how hsp distribution may be altered in response to specific cellular circumstances. At the outset of this project, there was no evidence to suggest that the intracellular distribution of hsps altered during different phases of the cell cycle. However, the original observation by Pelham (1986) that hsp70 interacts with a variety of different proteins during different phases of the cell cycle, suggests that hsp localisation during growth and proliferation may change according to the nature of hsp: protein interactions. Again, the established finding that hsp70 can bind to p53 protein suggests an important mechanism by which it could regulate the accumulation or localisation of this tumour suppressor protein. Thus, it was hypothesised that the distribution of hsp, in particular hsp70, may reflect the malignant phenotype or growth characteristics of the leukaemic cell.

In Chapter 3, the results of an immunocytochemical analysis of both normal and malignant haematopoietic cells is presented. Examination of immunoperoxidase-stained cytospins of normal mononuclear cells demonstrates that these cells express detectable levels of hsp60, hsp70 and hsp90 in the absence of stress. No nuclear localisation was evident in any of the preparations from ten normal individuals. In each case, hsp60,

hsp70 and hsp90 were immunolocalised within the cytoplasm, as evidenced by a diffuse cytoplasmic staining pattern. The expression of the tumour suppressor gene product, p53, was also analysed in these cells and no evidence of cytoplasmic or nuclear p53 accumulation was evident by immunoperoxidase techniques. The finding that normal mononuclear cells constitutively express detectable levels of hsp, even in the absence of any stress, reflects the importance of these molecules as molecular chaperones.

Stimulation of normal peripheral blood lymphocytes with the mitogens concanavalin A or pokeweed mitogen induced transformation and proliferation with an associated upregulation of hsp70 and hsp90. As outlined in the Introduction (Section 1.1.6), a link between hsp expression and cell growth and differentiation is suggested by a number of reports which describe changes in hsp expression during the growth and differentiation of haemopoietic cell lines (e.g Galea-Lauri *et al.*, 1996, Mivechi *et al.*, 1994). In my analysis of mitogen-stimulated lymphocytes, the expression of hsp in these cells was predominantly cytoplasmic, although hsp72/73 was also localised within the nucleus. It is therefore interesting to note that p53 staining of mitogen-stimulated lymphocytes revealed nuclear accumulation of this tumour suppressor gene product. This finding may be significant in terms of the relationship between hsp70 and p53, and may have relevance to the proliferation of haemopoietic cells. For example, it has been reported that wild-type p53 protein in AML cells switches to a mutant conformation in response to growth factor stimulation, leading to the suggestion that conformational changes in normal p53 protein represent an important mechanism for the regulation of cell proliferation (Zhang and Deisseroth, 1994). Further evidence to support this theory comes from the observation that different conformations of p53 protein in AML cells relate to the *in vitro* growth characteristics of the cells, and this can be regulated by either exogenous or autocrine haemopoietic growth factors (Zhu *et al,* 1993).

Leukaemic cells from AML patients express significant levels of hsp60, hsp70 and hsp90 in the absence of stress. Light microscopy of immunperoxidase-stained cytospins revealed that the expression of all three hsp was predominantly cytoplasmic. In fifteen of sixteen cases, no nuclear staining was evident with any of the three hsp monoclonal antibodies. However, in one patient, a strong pattern of nuclear staining for hsp72/73 was evident in the myeloblasts. These cells also exhibited cytoplasmic

accumulation of hsp72/73, but the nuclear staining was more intense. Furthermore, immunoperoxidase staining with an antibody to p53 of cytospins from the same patient revealed significant nuclear expression of this tumour suppressor gene product, whilst immunostaining of the cells from all fifteen other AML patients revealed no significant staining for p53, either cytoplasmic or nuclear. This nuclear co-localisation of hsp72/73 and p53 is interesting since these cells were from a patient whose disease had undergone a second relapse and had become particularly resistant to chemotherapy. It is tempting to speculate that this co-localisation of hsp70 and p53 has some significance in terms of the profound clinical drug resistance which was observed in this one patient. It is well established that p53-dependent mechanisms of apoptosis are involved in cytotoxic drug-induced cell death (reviewed by Bellamy, 1997). Could the co-expression of high levels of nuclear hsp70 and p53 reflect an interaction between the two which is modulating the normal function of p53 protein and therefore affecting the induction of apoptosis by chemotherapeutic agents? The relevance of this observation is uncertain, and since it was only apparent in this one patient, it is impossible to definitively ascribe a clear association between nuclear co-localisation of hsp72/73 and p53 with any clinical parameters such as drug resistance or tumour aggressiveness.

At the time of writing, no reports exist in the literature which describe localisation of hsp70, or any other hsp, in leukaemia cells. However, there are publications in which the prognostic significance of hsp70/p53 expression in a variety of human tumours has been analysed. A significant correlation between hsp70 expression and high levels of p53 has been reported in endometrial carcinomas, and this was associated with poorly differentiated carcinoma (Nanbu et al., 1996), whilst a significant number of malignant oral lesions are characterised by the presence of hsp70/p53 complexes as determined by co-immunoprecipitation (Kaur et al., 1996). In a large study of 169 patients with breast tumours, nuclear accumulation of p53 by immunocytochemistry was shown to be associated with a poor clinical outcome, but there was no association between either cytoplasmic or nuclear hsp70 staining and accumulation of p53 protein. In fact, hsp70-positive tumour cells predicted a better overall survival (Elledge *et al.*, 1994). The prognostic significance of hsp70/p53 interactions obviously still remains unclear. Clearly, it would be desirable to investigate this further, and a much larger sample of AML patients including a larger number with drug resistant malignant cells would be

required. In order to address this, cells from AML patients continue to be harvested at Warwick Hospital so that a larger study may shed light on any potential association between hsp/p53 localisation and drug resistance.

## 7.3.Cytoplasmic expression of heat shock proteins in haematopoietic cells.

Having established that cellular localisation of hsp expression in haematopoietic cells is, with the one exception in the patient with drug-resistant AML, confined to the cytoplasm, the next stage in my investigation was to quantify levels of the major hsp families in both normal and leukaemic haematopoietic cells. In Chapter 4, the results of an analysis of hsp expression in normal mononuclear cells and cells from both chronic and acute myeloid leukaemia patients is presented. In normal peripheral blood mononuclear cells (PBMC), the expression of hsp60, hsp70 (72/73) and hsp90 can all be detected by indirect immunofluorescence using monoclonal antibodies against these three major heat shock protein families. Fluorescence profiles for these cells demonstrate a relatively homogeneous pattern of hsp expression with no large differences apparent between lymphocytes and monocytes from the same individual, evidenced by a single narrow peak of staining, or between the mononuclear fraction of the ten healthy individuals examined.

The expression of all three heat shock proteins examined is clearly elevated in leukaemic cells and more heterogeneous than is observed in normal PBMC. In AML cells, significantly higher levels of all three hsp families were evident compared with either normal PBMC or CML cells. Both AML and CML cells exhibit a wide range of hsp expression, not only between individual patients, but within the groups as a whole. The significance of these differences in hsp expression is not clear, but several explanations are possible. The first, and possibly most plausible explanation, is that hsp levels are a marker of the differentiation state of these cells. Decreased expression of hsp70 and hsp90 is associated with myeloid differentiation of HL-60 cells (Beere *et al*, 1993, Yufu *et al,* 1989, Shakoori *et al,* 1992), and levels of hsp60 mRNA decrease during TPA-induced differentiation of HL-60 or K562 cells (Lu & Seligy, 1992). A further indication of the association between hsps and differentiation is the observation

that heat shock itself, or chemical agents which elicit a heat shock response, induce differentiation of HL-60 cells (Richards, 1988). The large differences in hsp levels between AML cells, which are predominantly immature myeloblasts, and CML cells, may therefore be due to simple differences in the stages of myeloid differentiation between the cells. If this is the case, the heterogeneity seen in AML cells could imply .hat some myeloblasts are more mature and the possibility that hsp levels could be a marker of differentiation state in AML demands further investigation. Also, therapy aimed at inducing differentiation, e.g the retinoids, would be expected to drive towards lower levels of hsp expression.

A second explanation is that hsp expression is a marker of cell proliferation. By virtue of their role as molecular chaperones, hsps perform a perform a variety of roles which could be utilised in a developmentally regulated manner to control proteins necessary for cell growth and proliferation (Pechan, 1991). Hsp expression has been shown to be regulated by mitogenic stimuli in a variety of cell types, including haematopoietic cells (Ferris *et al,* 1988, Fincato *et al,* 1991), and my investigations revealed that immunoperoxidase staining and flow cytometric analysis of concanavalin A and pokeweed mitogen-stimulated lymphocytes demonstrated markedly increased levels of hsp70 and hsp90 (see Results, Chapter 4).

Immunofluorescent histograms of CML patients demonstrated in some cases two distinct peaks suggesting that the presence of immature and more differentiated myeloid cells expressing high and low levels of hsp respectively. However, in some AML cells where morphologically the cells were homogenous and virtually all immature myeloblasts, two distinct populations were evident for hsp90 expression. This may represent the presence of an actively proliferating population of cells which have a distinct level of hsp90 expression., although this would seem unlikely since the proliferating compartment of leukaemias, like most other cancers, is represented by a very small percentage of the overall population of tumour cells. In these cases, it may be more likely that this dual pattern of hsp90 expression is the result of a biphenotypic leukaemia, a situation which arises when leukaemic transformation gives rise to the clonal proliferation of two populations which may represent different stages of myeloid differentiation.

Although leukaemic cells from different patients showed marked differences in levels of expression, no correlation was observed in the expression of the different hsp

families. For example, those cells expressing the highest levels of hsp70 did not have the highest levels of hsp60 or hsp90. This suggests that individual hsp families play different roles with relation to the control of cell growth and differentiation, probably via their interaction with distinct protein substrates within the cell. Although all heat shock proteins have similar modes of action in terms of their 'molecular chaperone' function, they have exclusive roles to play in terms of specific interactions with cellular proteins.

An important feature of the pattern of hsp expression in leukaemic cells, and in particular those from AML patients, is the wide range of hsp expression in cells from individuals with the same diagnosis. Analysis of the clinical data from these patients, in terms of survival, drug therapy and the stage of the disease, revealed no apparent correlation with the level of hsp expression. At this stage, it is not evident whether the high levels of hsp expression in AML simply represent the malignant phenotype of these cells, i.e. their lack of myeloid differentiation, or whether elevated hsp expression has a biological role, particularly in terms of the response to chemotherapy.

As outlined in the introduction, hsp induction in response to a variety of environmental insults appears to afford cells a protective effect. Do the elevated levels of these proteins therefore provide leukaemic cells with a survival advantage which may relate to altered proliferative potential of the malignant clone? Furthermore, how do levels of hsp expression relate to the susceptibility of cells to drugs used in the standard treatment of these malignant disorders?

In order to address these questions, apoptosis of AML cells was studied following the withdrawal of growth factors, i.e by culturing cells in the absence of serum.

## 7.4. Hsp expression and the susceptibility of AML cells to apoptosis induced by serum withdrawal.

The results presented in Chapter 5 demonstrate that AML cells undergo apoptosis when cultured *in vitro* in the absence of added serum, i.e. in the absence of exogenous viability factors. There is however, a marked variation in the rate of apoptosis of AML cells from different patients, and indeed variation between samples taken on different occasions from the same patients. Analysis of the results found no correlation between

the susceptibility of AML cells to apoptosis and patient survival, nor is there a relationship with clinical drug resistance. In addition, there is no obvious correlation between the rate of apoptosis and whether the cell sample was taken at presentation or at relapse.

However, when the rate of apoptosis *in vitro* was compared with the expression of hsp70 in the AML cells upon isolation, it is clear that there is a highly significant correlation between the two in two independent series of AML cell samples. Thus, although hsp70 expression protects cells against apoptosis induced by a variety of agents , it appears that there is an inverse relationship between the level of hsp70 and the rate of apoptosis induced by withdrawal of growth factors in these AML cells. This paradoxical finding is in contrast to the initial expectations at the outset of this investigation.

Two factors which are widely regarded as playing important roles in apoptosis are p53 and bcl-2. The former is thought to initiate apoptosis, at least where this is induced by DNA damage, and the latter to protect. In my studies, p53 was weakly and inconsistently expressed in AML cells. Although p53 expression was not reliably quantified, there did appear to be some correlation between p53 and apoptosis. Those cells expressing the highest levels of p53, as determined by flow cytometric analysis, also appeared to express high levels of hsp70, and in turn be more susceptible to apoptosis. Expression of bcl-2 did demonstrate a negative relationship with the susceptibility to apoptosis suggesting that bcl-2 may play some role in the protection of AML cells against apoptosis, although this correlation was not statistically significant.

A large number of studies have examined the expression of both bcl-2 and p53 in a variety of human tumour types, including haematopoietic cells. For example, the susceptibility of a myeloid leukaemia cell line (M1) to apoptosis induced by the chemotherapeutic agents, methotrexate and verapamil, is associated with high levels of bcl-2 expression (Lotem and Sachs, 1993), whilst bcl-2 protein expression and p53 gene mutation in chronic lymphocytic leukaemia (CLL) is associated with *in vitro* sensitivity to chemotherapeutic agents and disease progression (Morabito *et al.*, 1997; Agelar-Santelises *et al.*, 1996). Banker *et al.*, 1997, recently analysed spontaneous and drug-induced apoptosis in AML cells, demonstrating variable levels of spontaneous apoptosis in cells from a large sample of 56 *de novo* AML patients. Immunofluorescent analysis of bcl-2 protein expression revealed that cells expressing the highest levels of

bcl-2 protein show low levels of apoptosis, but reduced apoptosis was seen in both the presence and absence of bcl-2 overexpression. No association between levels of bcl-2 and the susceptibility of cells to drug-induced apoptosis was noted, and there was no correlation between the susceptibility of cells to spontaneous or drug-induced apoptosis and disease progression. These results, plus those I present in this thesis, suggest that there is no clear association between *in vitro* measurements of apoptotic susceptibility and clinical parameters in AML cells.

I also analysed hsp70 expression and susceptibility to apoptosis in a longitudinal study examining cells harvested from three patients with chronic myeloid leukaemia (CML) who eventually transformed to acute AML. In most patients with CML, the leukaemia presents as two distinct clinical phases: the chronic phase during which there is a proliferation of the myeloid lineage, but there is a normal differentiation capacity resulting in the appearance of cells in the peripheral blood representing all stages of myeloid differentiation, from immature myeloblasts through to mature granulocytes. All patients eventually transform to an acute phase of the disease, and this is associated with a differentiation block and therefore accumulation of myeloblasts (reviewed by Guinn and Mills, 1997). It was therefore interesting to examine whether hsp expression and levels of *in vitro* apoptosis changed during disease progression. My results demonstrated that in all three patients, levels of hsp60, hsp70 and hsp90 were higher in individual patients' cells following transformation to the acute disease. I found that p53 expression was unaltered in all three patients in the CML to AML transformation, but *in vitro* susceptibility to apoptosis increased in 2/3 patients following transformation. These results suggest that hsp expression is higher in AML cells due to the undifferentiated state of these cells in comparison to CML cells, and, again, higher levels of hsp expression were associated with enhanced susceptibility to apoptosis. Although I demonstrated no change in p53 protein levels in these three patients, it has been reported that p53 mutations and/or accumulation of p53 protein is associated with the progression of CML (Guinn and Mills, 1997; Guerrasio *et al.*, 1997).

The question which arises from these results is whether hsp70 itself plays a role in apoptosis, or if high levels of hsp70 simply reflect that the most susceptible cells are already 'stressed' and therefore more susceptible to a further insult such as removal of viability factors. However, if this were the case it would be expected that hsp90, another stress protein, would also correlate with apoptosis, and this did not appear to

occur. If the association of hsp70 elevation with apoptosis is not merely coincidental, either its importance is through interaction with the p53 pathway or else through an independent pathway.

Of particular interest in this context is the observation that constitutive members of the hsp70 family (hsc70) interact with the product of the p53 tumour suppressor gene. The biological significance of hsp70-p53 complexes is poorly understood, although it has been shown that binding of hsp70 could be part of a regulated process leading to changes in p53 conformation. According to this hypothesis, hsp70 regulates folding from mutant , with promoter function, to wild-type conformation which has suppressor function. (Hainaut & Milner, 1992). Further evidence of this relationship between hsp70 and p53 is suggested by the report that mutant p53 proteins can transactivate the hsp70 gene promoter (Tsutsumiishii *et al.* 1995).

Hsp70 appears to play a role in the balance between control of cell growth and differentiation, and loss of cells via apoptosis. My finding that AML cells which express high levels of hsp70 are more susceptible to apoptosis is paradoxical in view of the protective nature of hsp, although it has been shown that HL60 cells treated with N-methylformamide (NMF) will differentiate or undergo apoptosis according to the concentration of NMF used, and that differential expression of hsp70 correlates with the path taken (Beere *et al,* 1993). Differentiation is associated with decreased levels of hsp70, while the induction of apoptosis by higher levels of NMF correlates with maintainance of hsp70 levels. The authors of this report suggest that hsp70 acts, similar to p53, as a cellular 'sensor' permitting the cell to discriminate between different levels of cellular damage, and is therefore a key mediator in the response to drug-induced damage.

In order to examine how hsp expression influences the response of the leukaemic cell to potentially lethal damage, the next stage of my research was to examine the response of myeloid leukaemia cell lines to heat shock and the induction of apoptosis by cytotoxic drugs used in the treatment of leukaemia. This analysis includes the question of how levels of hsp relate to the induction of apoptosis in myeloid cells, and how high levels of hsp induced by hyperthermia influence the susceptibility of these cells to apoptosis induced by other means, in particular via chemotherapeutic agents.

## 7.5. The heat shock response and apoptosis in myeloid leukaemia cell lines.

### 7.5.1. Hyperthermia induces hsp expression in myeloid cell lines.

In Chapter 6, the results of an analysis of the heat shock response in myeloid leukaemia cell lines is presented. Analysis of the kinetics and magnitude of the heat shock response following hyperthermic treatment revealed that in all cell lines, hsp25, hsp70 and hsp90 were induced by sub-lethal heat shock (42°C, 30 min.). Furthermore, in two myeloid cell lines, KG1a and KU812, hyperthermia induced an increase in the levels of p53 protein. This is interesting because hypoxia, a recognised inducer of the heat shock response, also induces accumulation of p53 protein, (Graeber *et al.*, 1994), demonstrating that mechanisms other than DNA damage can affect the expression of p53. This, together with my finding that heat shock induces p53 expression, suggests that both the heat shock response and p53 induction share, at least in part, some common signalling pathway.

Having elucidated the kinetics of the heat shock response, the effect of serum withdrawal on myeloid cell lines was investigated. My earlier results with primary AML cells showed that high levels of hsp70 expression correlated with increased susceptibility to apoptosis following culture in the absence of exogenous growth factors, i.e. no added foetal calf serum (FCS). Four myeloid leukaemia cell lines, KU812, KG1a, THP-1 and HL-60, were used to investigate the effect of hyperthermia-induced overexpression of hsps on the susceptibility of cells to undergo apoptosis during culture in the absence of FCS. As expected, all four cell lines were induced to undergo apoptosis following resuspension in RPMI 1640 culture medium with no added FCS, but it was found that high levels of hsp expression following hyperthermia had no effect on the rate of apoptosis as compared with control cells maintained throughout at 37°C. Thus, overexpression of hsp does not protect against this model of apoptosis. Interestingly, a mild sub-lethal heat shock protects neuronal cells against apoptosis induced by subsequent severe thermal stress, but it does not protect the same cells against apoptosis induced by withdrawal of nerve growth factors

(Wyatt *et al.*, 1996). The mechanism of apoptosis effected by removal of viability (growth) factors therefore appears to differ from apoptosis induced by a variety of other agents where hyperthermic induction of hsp expression is protective. These include the protection of thymocytes against radiation-induced apoptosis (Gordon *et al.*, 1997), inhibition of apoptosis induced by both TNF-α and monocyte cytotoxicity in Wehi-s fibrosarcoma cells (Jaatela and Wissing, 1993), and the protection of murine L929 cells against apoptosis induced by Fas/APO-1 (Mehlen *et al.*, 1996).

### 7.5.2. Overexpression of hsp induces chemoresistance in myeloid leukaemia cell lines.

One of the most important aims of this research was to establish whether hsp overexpression in tumour cells could play a role in the development of chemoresistance by protecting these cells against apoptosis induced by chemotherapeutic agents. Hyperthermic induction of hsps was therefore used as a model to investigate the influence of hsp lexpression on susceptibility to cytotoxic drugs. Apoptosis was induced in all myeloid leukaemia cell lines by the cytotoxic agents, bischloronitrosourea (BCNU), vincristine, cytosine arabinoside, and doxorubicin, four drugs with different primary modes of action. These experiments also revealed that exposure of myeloid cell lines to BCNU, vincristine and doxorubicin results in an increase in hsp72/73 expression, agreeing with the earlier reported observation that BCNU induces hsp70 expression (Kroes *et al.*, 1991). This finding suggests that leukaemic cells mount a stress response following exposure to chemotherapeutic agents, which because of the established protective nature of hsp may suggest an important role in protecting leukaemic cells and therefore counteracting the efficacy of chemotherapeutic regimens. However, as described in Chapter 6, the induction of hsp expression by BCNU only appeared to occur following exposure to lethal concentrations of the cytotoxic drug, suggesting that rather than affording a demonstrable degree of protection, these elevated levels of hsp72/73 observed represent an abortive stress response prior to the onset of apoptosis. As demonstrated It was therefore interesting that induction of the heat shock response induced resistance to BCNU in all cell lines tested. Although it is known that p53

overexpression plays a role in the apoptotic response to cytotoxic drugs, my finding that resistance to apoptosis occurred following hyperthermia in THP-1 cells, in which p53 expression remained unaltered, and in HL-60 cells which have a large deletion in the p53 gene, suggests that this resistance occurred via p53-independent mechanisms. In these myeloid cell lines, hyperthermia-induced resistance to apoptosis also appeared to be independent of bcl-2 expression, since hyperthermia did not alter the levels of bcl-2 protein expression.

The potential involvement of hsp in protecting tumour cells against apoptosis induced by chemotherapeutic agents is suggested by data which shows that elevated hsp expression is associated with chemoresistance both in human tumours (Ciocca, 1993). Similar observations have been made in experimental models; in heat-shocked rat tumour cells the induction of hsp70 is associated with vincristine resistance (Lee *et al*, 1992) while Chinese hamster cells over-expressing the human hsp70 gene are more resistant to a variety of cytotoxic agents (Huot *et al*,1991).

During the course of this research, a number of reports have also highlighted the potential role of hsp as mediators of chemoresistance. Hyperthermic treatment of Ehrlich ascites tumour cells resulted in resistance to vinblastine. Cycloheximide prevented the acqisition of the resistance suggesting that de novo synthesis of hsp is involved in this resistance (Gabai *et al.*, 1995). Transfection of Wehi-s fibrosarcoma cell line with the human hsp25 or hsp70 gene conferred resistance to chemotherapeutic agents including camptothecin, etoposide and actinomycin-D (Samali and Cotter, 1996)

Elevated hsp25 levels in CHO cells and a melanoma cell line associated with resistance to cisplatin (Wachsberger *et al.*, 1997), hsp70 and hsp25 overexpressed in a cisplatin-resistant human ovarian cancer cell line . Much lower levels of these hsp in non-resistant parental line. showing cross-resistance to adriamycin, vincristine and etoposide Hyperthermia further enhanced the drug resistance and induced resistance in parental cell line (Kamishima *et al.*, 1997)

Taken together with these observations, my finding that high levels of hsp following hyperthermia protect myeloid cells against cytotoxic drug-mediated apoptosis suggests

that hsp expression expression in myeloid cells influences their susceptibility to apoptosis . In view of the variable levels of hsp70 found in AML cells, as reported in Chapter 4, this suggests that hsp expression in leukaemia may have some prognostic significance. Although I have been unable throughout the course of this project to demonstrate a clear relationship between hsp expression and clinical factors such as patient survival or drug resistance, as discussed earlier other workers have reported a correlation between hsp expression (particularly hsp70 and hsp25) and prognosis, in a variety of malignancies. Clearly, the question of whether hsp levels influnce the efficacy of chemotherapy and/or the aggressiveness of the disease in leukaemia demands further investigation. Analysis of a larger group of patients with myeloid malignancies would therefore be worthwhile.

## 7.6. Summary: What is the relationship between heat shock protein expression and apoptosis?

In Chapter 5, it was demonstrated that hsp70 expression correlated with the susceptibility of cells from patients with AML to apoptosis induced by serum starvation. This suggests that hsp70 may play a role in the regulation of apoptosis, either as a direct factor influencing the outcome of pro-apoptotic signals, or indirectly via interaction with other factors involved in the regulation of this process.

I have found that hsp70 can protect against apoptosis in myeloid cells exposed to chemotherapeutic agents, as demonstrated by the fact that mild hyperthermia protects cells against the alkylating agent, BCNU. This finding dos not implicitly suggest that one hsp species in particular is responsible for this protective effect. Rather, it could be members of several hslp families including hsp70, hsp90 and hsp25 which are acting in cohort to inhibit apoptosis. It may also be possible that another factor, one which is induced by heat shock, is responsible.

The finding that the degree of hyperthermia influences the protective nature of the heat shock response suggests that hsp70 may play a more integral role in control of the cell cycle and apoptosis than simply as a protective factor. I found that at higher temperatures (43-44°C) which are associated withhigher levels of hsp70, the protective capacity of heat shock is diminished. Thus, very high levels of hsp70 appear to be a

signal that the cell is irretrievably damaged. The fact that high levels of hsp70 correlate with susceptibility to apoptisis induced by serum starvation also suggests that hsp70 may be a marker of cellular damage. It is possible that hsp70 may itself be directly involved in the control of apoptosis via its capacity to interact with other proteins known to be involved in apoptosis.

A unifying theory concerning hsp70 and apoptosis, is that hsp70 acts as a 'cellular sensor' of damage. At low levels of expression it is capable of providing a protective role, either directly through its role as a molecular chaperone which involves the re-folding and repair of damaged proteins, as well as the denaturation and removal of unwanted polypeptide structures. At higher levels of expression, hsp70 is associated with the onset of apoptosis; this may simply reflect the fact that hsp70 is a marker of cellular damage, or it is possible that hsp70 plays a direct role in the induction of apoptosis via the ability of hsp70 to interact with cell cycle-regulatory proteins. These possibilities demand further investigation, and it will be interesting to discover which proteins hsp70 is associated with during the processes involved in the conflicting balance between celluar proliferation and apoptosis.

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