ROLE OF THE CONSTITUTIVE HEAT SHOCK PROTEIN HSC70 DURING DIFFERENTIATION OF HAEMOPOIETIC CELLS

by

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ABSTRACT

The work presented in this thesis investigates the role that the constitutive heat shock protein hsc70 may play during differentiation of haemopoietic cells. It has previously been shown that the decrease in hsc70 expression may play a role during granulocytic differentiation of the human promyelocytic cell line HL-60. The thesis tries to investigate the function of hsc70 during differentiation of the murine multipotent haemopoietic cell line, FDCP-mix (clone A4).

Following differentiation of FDCP-mix (clone A4) cells along the macrophage and erythrocytic lineages, no change in hsc70 expression was observed. On the contrary, differentiation of the same cell line along the granulocytic lineage dramatically decreased hsc70 expression at both protein and RNA levels.

In order to determine if the kinetics of hsc70 expression were truly lineage dependent and not cell line dependent, FDCP-mix (*bcl-2*, clones 1B/2J) cells were differentiated along the granulocytic (clone 1B) or erythrocytic (clone 2J) lineages. In contrast to the parental FDCP-mix (clone A4) cells , *bcl-2* transfected FDCP-mix cells do not die upon withdrawal of IL-3 from the culture medium, but differentiates along the granulocytic (clone 1B) or the erythrocytic lineage (clone 2J). However both clones (clone 1B and 2J) could be differentiated in the presence of growth factors. Following the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) in the presence or absence presence of growth factors, a decrease in hsc70 expression was observed at the protein level but not at the RNA level.

The erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J) cells in the absence of growth factors, led to a dramatic fall of hsc70 protein expression. No change in hsc70 RNA was observed during the erythrocytic differentiation of this cell line. In contrast to the erythrocytic differentiation of the parental FDCP-mix (clone A4) cells, the differentiation of FDCP-mix (*bcl-2*, clone 2J) cells did not lead to mature erythrocytic cells.

It has been shown in this work, that following the differentiation, 2J cells did not synthesise globin proteins. A hypothesis which involves a complex of heat shock proteins is proposed for explaining this absence of globin synthesis during the erythrocytic differentiation of the 2J clone.

This work concludes that the kinetics of changes in hsc70 protein expression are lineage dependent and that the regulation of hsc70 is not controlled at the transcriptional level, but at the translational or post-translational level. Moreover it is proposed that hsc70 protein expression may be a survival marker and reflect the life span of haemopoietic cells.

DECLARATION

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ABBREVIATIONS

ATP	adenosine triphosphate
BAS-CFC	basophil colony-forming cell
BCNU	1,3-*bis*(2-chloroethyl)-1-nitrosourea
BFU-E	erythroid burst-forming unit
BHCNU	1,3-*bis*(*trans*-4-hydroxycyclohexyl)-1-nitrosourea
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CCNU	1-(2-chloroethyl)-3-cyclo-hexyl-1-nitrosourea
CFC-MIX	mixed colony-forming cell
CFU	colony-forming unit
CFU-E	erythroid colony forming unit
CFU-MEG	megakaryocyte colony forming unit
CFU-S	spleen colony forming unit
CHLZ	chlorozotocin
CM	conditioned medium
CSF	colony-stimulating factor
CTP	cytosine triphosphate
dCTP	desoxycytosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPEC	diethylpyrocarbonate
EDTA	ethylenediaminetetra acetic acid disodium salt
EOS-CFC	eosinophil colony-forming cell
EPO	erythropoietin
EPOR	erythropoietin receptor
FCS	fetal calf serum
GM-CFC	granulocyte/macrophage colony-forming cell
GM-CSF	granulocyte/macrophage colony stimulating factor
GM-CSFR	granulocyte/macrophage colony stimulating factor receptor
G-CSF	granulocyte colony stimulating factor
G-CSFR	granulocyte colony stimulating factor receptor
GTP	guanine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulphonate
HSP(s)	heat shock protein(s)
HS	horse serum
IL(1-11)	interleukin(1-11)
IL-3R	interleukin-3 receptor
IMDM	iscove Modified Dubelcco Medium
kDa	kilodalton
LCM	lung conditioned medium
M-CFC	macrophage colony-forming cell
M-CSF	macrophage stimulating factor
M-CSFR	macrophage stimulating factor receptor
MFM	methionine free medium
MIP-1α	macrophage inflammatory protein-1α
mRNA	messenger ribonucleic acid
Mw	molecular weight
MOPS	N-morpholinopropane sulphonic acid

NMF	N-methylformamide
ORF	open reading frame
PBS	phosphate-buffered saline
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride
PEG	polyethylene glycol
SCF	stem cell factor
SDS	sodium dodecylsulphate
TCA	trichloracetic acid
TEMED	N,N,N,N'-tetramethylethylenediamine
TGF-ß	transforming growth factor-ß
TNF	tumor necrosis factor
TPA	phorbol ester 12-0-tetra-decanoyl-phorbol-13-acetate
TRIS	tris (hydroxymethyl) aminomethane
TTP	thymidine triphosphate

OBJECTIVES OF THIS WORK

Stress and hsp70 expression during differentiation of haemopoietic cell lines

Hsp70, in both eukaryotic and prokaryotic cells, is induced following a stress such as heat shock. It has also been shown that hsp70 expression may be associated with cell differentiation. However it is not clear whether stress is the cause and/or the consequence of cell differentiation. The initial objective of this work was to determine if the differentiation of normal non-leukaemic haemopoietic cells was triggered by stress as a response to the change of stimuli in the environment. The consequences of this stress was detected as the expression of the hsp70 gene product. In order to answer this question, an IL-3 dependent cell line, FDCP-mix was differentiated along erythrocytic, granulocytic and macrophage lineages, in the presence of the appropriate growth factors. The differentiation of FDCP-mix cells required a 100 fold reduction in the concentration of IL-3, and thus may stress these cells. It was important therefore to establish whether this decrease in IL-3 concentration increased hsp70 expression in FDCP-mix cells.

Hsc70 expression and cell survival

Hsc70/hsp70 have been shown elsewhere to be involved in protein translocation through endoplasmic reticulum, nucleus and mitochondria. In yeast, deletions of heat shock genes similar to hsc70/hsp70 lead to cell death, suggesting that hsc70/hsp70 are survival gene products. Is hsc70 expression a marker for cell survival in FDCP-mix cells?

Mature haemopoietic cells are programmed to die, and thus have defined half-lives, according to the lineage. If hsc70 promotes cell survival, it should be an appropriate marker to characterise half-lives of haemopoietic cells. Is the level of hsc70 expression specific to the lineage of differentiation, and to the life span of haemopoietic cells?

Growth factors have been associated with survival of haemopoietic cells, suggesting

that growth factors may regulate genes involved in cell survival. Is hsc70 expression regulated by growth factor stimulation?

Transfection of the human *bcl-2* gene into FDCP-mix cells delayed apoptosis upon growth factor withdrawal. Does the human *bcl-2* expression in FDCP-mix cells change hsc70 expression upon withdrawal of growth factors from this cell line?

Regulation of hsc70 expression

During chemically induced differentiation of leukaemic cells, it was shown elsewhere that changes in hsc70 expression was regulated transcriptionally. Is hsc70 expression regulated at the transcriptional (RNA), post-translational or translational (protein) levels in FDCP-mix cells during differentiation?

Chapter 1 INTRODUCTION

Our main concern in philosophy and in science should be the search for truth. Justification is not an aim; and brilliance and cleverness as such are boring. We should seek to see or discover the most urgent problems, and we should try to solve them by proposing true theories; or at any rate by proposing theories which come a little nearer to the truth than those of our predecessors.

Karl R. Popper

Objectives

The first part of this chapter reviews different concepts of haemopoiesis such as proliferation/differentiation and apoptosis/cell death. The role of growth factors in the regulation of haemopoiesis is investigated in this work. In the second part of this chapter, the possible role of heat shock proteins in eukaryotic cells and more specifically in haemopoiesis is also studied.

1.1: HAEMOPOIESIS

1.1.1: TURNOVER OF BLOOD CELLS

The main haemopoietic organ is the bone marrow which produces approximately $4x10^{11}$ blood cells per hour. A small population of primitive stem cells is responsible for maintaining the steady-state haemopoietic cells in mammals. The bone marrow stem cells have an extensive proliferation capacity and possess the ability to give rise to either new stem cells or to more mature and committed cells (Figure 1.1). Murine stem were identified by their ability to form haemopoietic colonies in the spleen of irradiated recipient

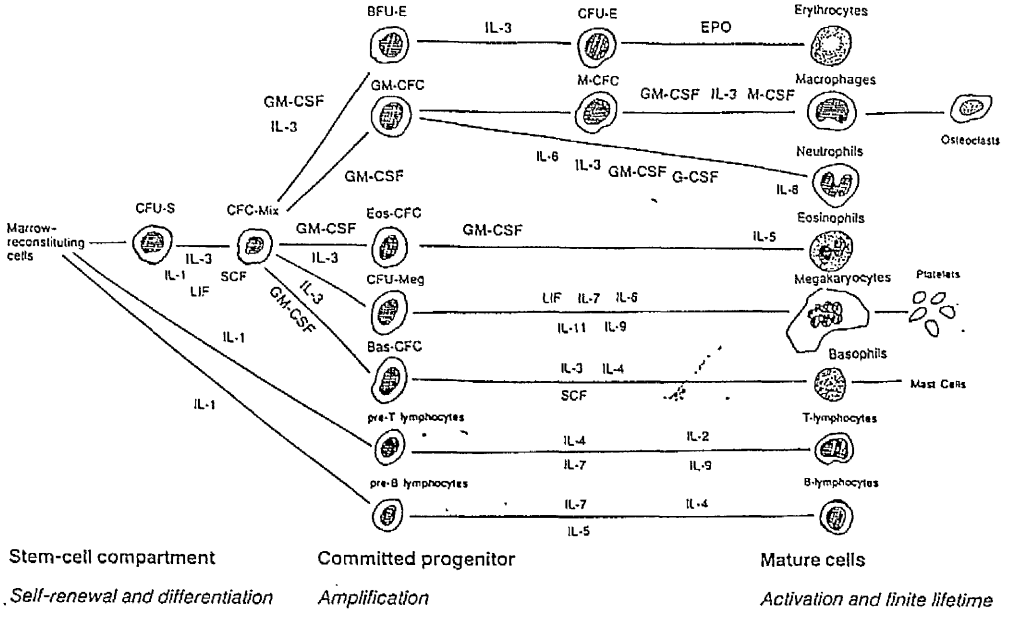


Figure 1.1: Blood cell lineage (authorized by Cowling and Dexter, 1992). In the stem cell compartment, primitive cells are capable of self-renewal and/or to give rise to more committed cells, which can mature along different lineages. Growth factors interact at different stages of cell maturation (see section 1.1.2).

mice (McCullough and Till, 1964). These stem cells were termed colony-forming-unit in spleen (CFU-S). The authors demonstrated, that by injecting bone marrow intravenously into irradiated mice, discrete colonies of precursor cells grew in the spleens of the recipients. Transfer of a single colony into a second irradiated mouse gave rise to secondary spleen colonies, showing that stem cells give rise not only to differentiated cells (committed along different lineages) but also to multipotent cells. The recognition of CFU-S as a minimal-cycling population during steady-state haemopoiesis indicated that the massive expansion in cell numbers between the CFU-S and mature cells occurs via an intermediate population of proliferating cells. These populations are called committed progenitors, consisting of cells with limited proliferative capacity; they can undergo proliferation and development along a specific haemopoietic lineage to give rise to mature cells. Such intermediate progenitor cells have been characterised using **in vitro** assays, which assess the ability of cells to give rise to colonies of mature haemopoietic cells when immobilised in semi-solid media, such as agar or methylcellulose (reviewed by Metcalf, 1984).

By interposing agar layer between the feeder layer and seeded haemopoietic cells, Pluznik and Sachs (1965) showed that the soluble factors required for the formation of macrophage and granulocyte clones were secreted by the feeder layer cells. When cells were washed at various times, after initiating the induction of clones, there was no further development of either macrophage or granulocyte clones unless the inducer was added again (Paran and Sachs, 1968). From this data, it became apparent that the provision of media containing the appropriate growth stimulating factors is an essential element in the formation of such colonies and is necessary for progenitor cells to survive, proliferate and develop. Current culture conditions allow the growth of progenitors of other cell lineages, for example (1) mixed colonies (CFC-mix), consisting of granulocytes, macrophages, erythrocytes and megakaryocytes, (2) erythrocytic colonies: BFU-E (Burst-

forming-unit-erythroid) and the more mature progenitors, CFU-E (colony-forming-unit erythroid) (reviewed by Metcalf, 1984) and (3) B-lymphoid colonies (Paige, 1983).

1.1.2: HAEMOPOIETIC CELL GROWTH FACTORS

1.1.2.1: Introduction

Following the work of Pluznik and Sachs (1965) colony-stimulating factors (CSF) have been detected and purified from different animal or human sources: (1) CSF was detectable in low levels in the serum of mice and humans, and in normal urine. (2) CSF levels were elevated in the serum and urine of animals and patients with acute viral or bacterial infections and in animals and patients with some forms of advanced cancer where secondary infections were possible. (3) Serum CSF levels were low or undetectable in germ-free animals but became elevated when animals were maintained in normal conditions. (4) Damage to haemopoietic populations by irradiation or cytotoxic drugs led to elevations in serum CSF levels in normal animals but not in germ-free animals. (5) CSF was extractable from all major tissues in the mouse, and in concentrations higher than in serum. (6) Serum CSF levels were elevated dramatically by the injection of endotoxin, bacterial antigens and other foreign proteins. Tissue levels of CSF also increase following the injection of endotoxin. The purification of active proteins from medium conditioned by lung cells, L-cells and T-lymphocytes led to the discovery of various murine CSFs, GM-CSF/G-CSF, M-CSF and IL-3 respectively. Their respective cDNAs have been cloned and, recombinant growth factors have been produced. These soluble growth factors (Table 1.1) are interleukins 1-11, the colony-stimulating factors (G-CSF, M-CSF, GM-CSF), erythropoietin (EPO) and stem cell factor (SCF) (Anderson *et al*, 1990; Zsebo *et al*, 1990; Molineux *et al*, 1991). They have been designated according to their effects in specific bioassays.

Negative regulators of haemopoiesis have also been investigated. The best

characterised are transforming growth factor (TGF-ß) (Keller *et al*, 1988; Goey *et al*, 1989) and macrophage inflammatory protein-1 α (MIP-1α) (Graham *et al*, 1990). Both selectively inhibit the proliferation of CFU-S.

These growth factors act on cells via membrane bound receptors either individually or in synergy to induce cell proliferation, development or differentiation to one or more lineages. Haemopoietic growth factor receptor number is generally low on primitive cells but following differentiation of murine bone marrow cells, it has been observed, that the expression of G-CSF receptors is up-regulated. However within any one subpopulation, up to 50-fold heterogeneity exists in the number of receptors on bone marrow cells (reviewed by Nicola, 1989). Most receptors exist in both a high and a low affinity form. Variable numbers of each form are present on different cell types. These high and low affinity receptors seem to be interconvertible (reviewed by Nicola, 1991). An important consequence of receptor affinity conversion is that the ligand-receptor internalization is much more likely for high affinity than for low affinity receptors, because the ligand is less likely to dissociate from high affinity receptors before the internalization process can occur (reviewed by Nicola, 1991). Only the growth factors and their respective receptors (Figures 1.2 and 1.3) that are used in this work are further described.

Cytokine	Activity
IL-1α/ß (1)	Activation of the immune and haemopoietic system
IL-2 (2)	Induces the growth of T-cells, natural killer cells (NK), B-lymphocytes,macrophages
IL-3 (3)	Growth and differentiation of myeloid and erythroid cells, maintenance of cell lines of primitive haemopoietic cells and mast cells
IL-4 (4)	Growth factor for B and T-lymphocytes
IL-5 (5)	Growth and differentiation of eosinophils and B and T-lymphocytes
IL-6 (6)	Induces terminal differentiation of B-cells, activates T-cells,synergizes with IL-3 to induce haemopoietic stem cells
IL-7 (7)	Induces proliferation of B and T-cells, LAK cells and platelets
IL-8 (8)	Activates monocytes, fibroblasts and endothelial cells
IL-9 (9)	Growth of some T cells clones
IL-10 (10)	Supresses cytokine production by type 1 T helper cells
IL-11 (11)	Enhances IL-3 stimulated megakaryocyte growth
GM-CSF (12)	Induces growth and differentiation of neutrophils, eosinophils, monocytes,mast cells and erythroid cells; enhances neutrophil functions;supports growth of normal and leukaemic myeloid cell lines
G-CSF (13)	Induces granulocytic proliferation and differentiation
M-CSF (14)	Induces monocytes and macrophages colonies; induces supports growth of murine cell lines
EPO (15)	Activates erythroid progenitor growth and differentiation

Table 1.1: Haemopoietic cytokines. (1-7) Pierce (1988); (8) Matsushima *et al* (1988); (9) Yang *et al* (1990); (10) McNeil *et al* (1990); (11) Paul *et al* (1990); (12-15) Dexter *et al* (1990).

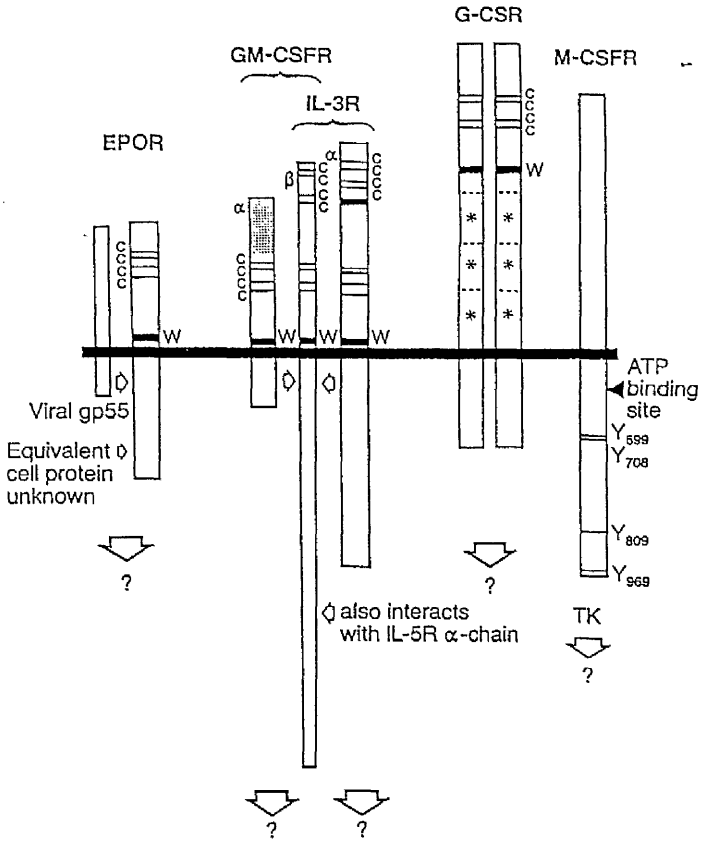


Figure 1.2: Structure of haemopoietic growth factor receptors (authorized by Cowling and Dexter, 1992). In human, there are two forms of IL-3R, α-subunit and ß-subunit which is shared with the ß-subunit of GM-CSFR; GM-CSFR consists of one α and ß-subunits; The common ß-chain of the IL-3R and GM-CSFR interacts with the α-chain of the IL-5R (not reviewed in this work); G-CSFR exists as a dimer; M-CSF is a monomer. The short, solid black lines (W) immediately above the membrane are the conserved WSXWS motif presumed to be involved in protein-protein interactions. The four conserved Cys residues are shown (C). Although members of the cytokine reseptor superfamily are not tyrosine kinases, IL-3R and IN-5R are known to induce tyrosine phosphorylation in response to ligand binding. The ATP binding site and tyrosine residues that are capable of being phosphorylated are shown on the M-CSFR. The subsequent signal-transduction processes are, as yet, unknown (?).

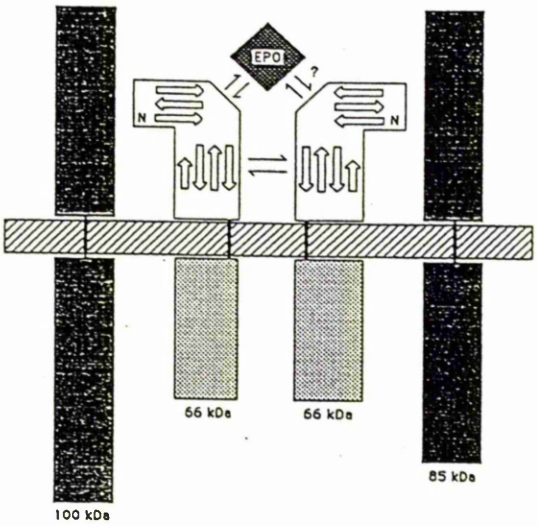


Figure 1.3: Erythropoietin receptor (authorized by Winkelmann, 1992). The ligand-binding EPOR is labeled "66 kDa". The EPOR-associated "100" and "85" kDa proteins identified by crosslinking are shown as transmembrane proteins.

1.1.2.2: Interleukin 3 (IL-3) and IL-3 receptor

The main source of murine IL-3 from normal cells is mitogen-stimulated T lymphocytes (Parker and Metcalf, 1974). Transformed cell lines, WEHI 3BD (murine myelomonocytic leukaemia cell line) and LBRM-33 (T cell leukaemia cell line), also produce IL-3 (Fung *et al*, 1984; Prestidge *et al*, 1984). *In vivo*, the major effect of recombinant IL-3, infused into mice appears to be a redistribution of CFU-S from the bone marrow to the spleen, peripheral blood and liver (Kindler *et al*, 1987). Although progenitor cell numbers increase by as much as 18-fold in the spleen, there is no increase or only a 2-3 fold (Metcalf *et al* 1986) increase in neutrophils and monocytes in peripheral blood. *In vitro*, purified or recombinant IL-3 induces the formation of colonies in semi-solid cultures of bone marrow cells (Hapel, 1985) and IL-3 has therefore been classified as a CSF. In semi-solid cultures, IL-3 supports the development of mixed myeloid/erythroid colonies, including macrophage, neutrophil, eosinophil and basophil colonies as well as colonies containing progenitors of the erythroid and megakaryocyte lineages. Murine and human IL-3 cDNA sequences possess a 54% sequence homology while the proteins present only 29% of homology (Cohen *et al*, 1986). Human and murine IL-3 genes have been localized on the long arm of chromosome 5 in the region of 5q23.3 and 5q32 (LeBeau *et al*, 1986) and 11 (Mason *et al*, 1986) respectively . Murine IL-3 is a glycoprotein with a molecular weight of 28kDa and between 14 and 28KDa in humans (reviewed by Pierce, 1988).

The murine IL-3 cross-links three receptor proteins or subunits, ß1 (120kDa), ß2 (140kDa) (which is unique for IL-3) and a α-subunit (70kDa) (Park *et al*, 1986; Nicola and Metcalf, 1986; reviewed by Schreurs *et al*, 1990). In contrast the human receptor has only one α (70kDa) and one ß (120kDa) subunits (reviewed by Kitamura, 1991). In both the human and mouse receptors, it is the association of the subunits of IL-3R which increases the affinity of IL-3 for the receptor (Walker *et al*, 1985; Kitamura *et al*, 1991). The

number and affinity of IL-3 receptors varies significantly among cell lines (Nicola and Metcalf, 1986). IL-3 downmodulates all CSF receptors upon binding on its receptor (Walker *et al*, 1985). On murine bone marrow cells, the concentration of IL-3 required to down-modulate 50% of IL-3R, GM-CSFR and M-CSFR is approximately the same, but 10 times more is needed to downmodulate 50% of G-CSFR. These results suggest that IL-3 plays a major role in both proliferation and differentiation of haemopoietic cells. In addition, IL-3R has a common ß subunit with GM-CSFR (figure 1.2). It has been shown that IL-3 selectively inhibits GM-CSF binding via the ß subunit of its shared receptor (Taketazu *et al*, 1991).

The signal transduction of IL-3 remains unclear and unknown. However it has been shown, that the ß2 subunit is tyrosine kinase phosphorylated upon the binding of IL-3 (Scheurs *et al*, 1990), and PKC is activated (Whetton *et al*, 1988) and translocated to the plasmic membrane (Pelech *et al*, 1990). Activation of *c-myc* and *c-fos* oncogene has also been observed (Harel-Bellan and Farrar, 1987).

1.1.2.3: Granulocyte-macrophage colony stimulating factor (GM-CSF) and GM-CSF receptor

GM-CSF is produced by many cell types such as T lymphocytes, macrophages and endothelial cells. Murine GM-CSF stimulates the formation of colonies containing pure populations of granulocytes or macrophages as well as colonies consisting of both cell lineages. The granulocytes produced are almost exclusively neutrophilic, but occasional eosinophil colonies are observed (Robinson *et al*, 1987). Megakaryocyte colonies have also been observed at high concentrations of GM-CSF (Robinson *et al*, 1987). Peritoneal injection of GM-CSF into mice leads to an increase in the numbers of macrophages, neutrophils and eosinophils present in the peritoneal cavity (Metcalf *et al*, 1987). GM-CSF can also enhance the functional activity of eosinophils, neutrophils and macrophages

(Stanley *et al*, 1983). In mouse, the GM-CSF gene is located on chromosome 11, (Barlow *et al*, 1987) and in human, it is localized on chromosome 5 at band q21-q31 (reviewed by LeBeau *et al*, 1986). Murine and human GM-CSF have molecular weights of 23kDA and between 14 and 35kDa respectively (reviewed by Pierce, 1988). Moreover, GM-CSF is a glycoprotein which is variably glycosylated (Burgess *et al*, 1986). Murine and human GM-CSF have a 56% amino acid sequence identity. Given the relatively low sequence identity between murine and human GM-CSF, the lack of cross-species activity displayed by this factor is not surprising (reviewed by Metcalf, 1984).

GM-CSFR consists of one ß subunit shared with IL-3R associated with one α subunit. The high affinity receptor or ß subunit has a molecular weight of 120kDa in both human and mouse cells (Figure 1.2). The low affinity receptor or α subunit is a 50-60 kDa protein in mouse (Park, 1992) and 85kDa protein in humans (Baldwin *et al*, 1989). The average number of murine GM-CSFR per positive bone marrow cell is 176 and positive human bone marrow cells have between 200 and 500 (reviewed by Nicola, 1989). In general, the number of GM-CSFR decreases with differentiation but even mature cells display significant numbers of GM-CSFR (up to 100 per cell) (Nicola, 1987). Upon GM-CSF binding to GM-CSFR, down-modulation of GM-CSFR, M-CSFR and G-CSFR has been observed on murine bone marrow cells (reviewed by Walker *et al*, 1985). Walker and Burgess (1987) showed that the ligand bound to GM-CSFR is rapidly internalized. They also showed the recycling of unoccupied receptors to the cell surface. Following the binding of GM-CSF to the receptor, it has been reported that some proteins are phosphorylated on serine and tyrosine residues (Sorensen *et al*, 1989).

Because the GM-CSF receptor does not contain kinase activity, these authors suggested that other subunits (such as kinases) associated with the complex would lead to production of second messengers that activate cellular kinase systems. In response to the proliferative and differentiating effect of GM-CSF, an increase in the expression of *c-myc*

and *c-fos* transcripts occurred upon the binding on GM-CSFR (Harel-Bellan and Farrar, 1987).

1.1.2.4: Granulocyte colony stimulating factor (G-CSF) and G-CSF receptor

G-CSF can be produced by macrophages, fibroblasts, endothelial cells and melanoma cells after bacterial lipopolysaccharide stimulation, however, almost all studied murine tissues can produce active G-CSF **in vitro** (reviewed by Nicola, 1990). At low concentration, G-CSF promotes the proliferation and development of progenitor cells which form colonies consisting of granulocytes and macrophages. At high concentrations of G-CSF, only macrophages are observed. In mice, peritoneal or subcutaneous injection increases neutrophil number in peripheral blood and stimulates myelopoiesis in the spleen. Multipotent stem cells are also released into the blood circulation following G-CSF injection (Kobayashi *et al*, 1987; Molineux *et al*, 1990). The genes for human and murine G-CSF are located on chromosome 17 q21-q22 (Tweardy *et al*, 1987) and on the distal half of chromosome 11 (Buckberg *et al*, 1988) respectively. Human and mouse G-CSF are glycoproteins of 18-22 and 25kDa respectively (reviewed by Pierce, 1988).

G-CSFR (Figure 1.2) consists of a single polypeptide chain but high and low affinity forms of receptor have been detected (Fukanaga *et al*, 1990). This monomeric receptor has a molecular weight of 140kDa and the high affinity receptor consists of oligomer of the same protein (Fukunaga *et al*, 1990). The average number of receptors per positive human marrow cell varies between 50 and 300, and on positive murine bone marrow cells, there are an average of 185 receptors (reviewed by Nicola, 1989). The primary G-CSF interaction with its receptor results in the rapid internalization of receptor and ligand followed by slow degradation of internalized G-CSF (reviewed by Nicola, 1990). Following the binding of G-CSF to G-CSFR, a down-modulation of G-CSFR and M-CSFR have been observed on murine bone marrow cells. However the concentration

of G-CSF required for 50% receptor down-regulation was 10 fold higher for M-CSFR than G-CSFR (reviewed by Walker *et al*, 1985).

G-CSF receptor does not have tyrosine kinase activity (Nagata and Fukunaga, 1991). However, upon the binding of G-CSF on G-CSFR, phosphorylation on the tyrosine residue of cytoplasmic protein, and induction of *c-myc* and *c-fos* transcripts occurred (Farrar *et al*, 1989)

1.1.2.5: Macrophage stimulating factor (M-CSF) and M-CSF receptor

M-CSF induces formation of pure macrophage colonies from individual bone marrow precursors plated in semisolid medium (Stanley *et al*, 1978). M-CSF stimulates the growth of monocyte precursors and can promote the continued growth of mature macrophages. It also potentiates the ability of mature mononuclear phagocytes to perform their differentiated functions by enhancing their ability to kill infectious microorganisms (Karbassi *et al*, 1987) and tumour cells (Wing *et al*, 1982) and by regulating release of macrophage cytokines, such as interferon, tumour necrosis factor, IL-1, and G-CSF (Moore *et al*, 1980). M-CSF is normally produced by various cell types, including stromal cells and macrophages and is present in urine. Murine L-cells are a convenient source of M-CSF for experimental work (Stanley and Heard, 1977). The human M-CSF gene is located on chromosome 5q33. The location of the murine gene is not known. Human macrophage colony-stimulating factor (CSF-1) exists in two forms, both are homodimers which do not retain activity if dissociated. The long form (70-90kDa) exists as a glycoprotein complex of two 35-45kDa subunits each, whilst the short form (40-50kDa) is a dimer of 20-25 kDa subunits each (reviewed by Kawasaki and Ladner, 1990).

The average number of human receptor number per positive bone marrow cell for M-CSF is 4600 (reviewed by Nicola, 1989). Upon binding of M-CSF to M-CSFR (Figure 1.2), the ligand-receptor complex is rapidly internalized, targeted to lysosomes, and

degraded (Guilbert and Stanley, 1986). Following the binding of M-CSF to M-CSFR, GM-CSFR is also downregulated, but a very high concentration of M-CSF is needed (reviewed by Walker *et al*, 1985).

The cytoplasmic domain of M-CSF receptor has tyrosine kinase activity (reviewed by Miyajima *et al*, 1992). The *c-fms* proto-oncogene has similar distribution and biological properties of the M-CSFR (Sherr *et al*, 1985). Activating mutations within the *c-fms* gene in myeloid cells might contribute to human haematopoietic malignancies (for reviewed in Sherr, 1990; Dubreuil *et al*, 1988). The *v-fms* gene of the Susan McDonough strain of feline sarcoma virus (SM-FeSV) was derived by recombination between a feline leukaemia virus and *c-fms* protooncogene sequences of the domestic cat (Donner *et al*, 1982). Upon transfection of *v-fms* in CSF-dependent cell lines, the requirement for growth factor is abrogated (Wheeler *et al*, 1987). After M-CSF stimulation, the signal transduction pathway remains unclear but involves activation and translocation of PKC (Datta, 1992), an increase in the expression *c-fos* and *c-myc* genes (Muller *et al*, 1985).

1.1.2.6: Erythropoietin (EPO) and EPO receptor

The kidney is the major source of EPO in adult mammals. EPO is a glycoprotein and is the primary physiological factor responsible for the generation of erythrocytes (Koury al, 1984). In early seventies, EPO-dependent assays were developed for committed erythroid progenitor cells. EPO stimulates both the proliferation and differentiation of immature erythroid, the burst-forming unit-erythroid (BFU-E) and their more-mature progeny, the colony-forming unit-erythroid (CFU-E). The human EPO gene has been localized on chromosome 7 q11-q22 (Watkins *et al*, 1986) and in mouse it is located on chromosome 5 (Lacombe *et al*, 1988). Both human and murine EPO have a molecular weight of 39kDa (reviewed by Pierce *et al*, 1988). Amino-acid sequence homology between mouse and human EPO is significantly higher than for other haemopoietic growth

factors. The mature murine and human EPO proteins share 81% similarity in which large regions are 100% conserved.

The cell surface of normal erythroid progenitor cells presents between 200 and 1000 EPORs (Figure 1.3) with high or/and low affinity (reviewed by D'Andrea and Zon, 1990; Cowling and Dexter, 1992). Binding of EPO to its cell surface receptors is followed by internalization of the ligand-receptor complex, and EPO is degraded and released into the medium (Koury *et al*, 1984). EPOR has been identified as a 66kDa protein by SDS polyacrylamide gel. Cross-linking studies showed two proteins of 105 and 65-kDa (D'Andrea *et al*, 1989), however other molecular weights have been reported, 100 and 85kDa (reviewed by Winkelmann, 1992). It has been proposed that high affinity EPO receptor may result from the association of two 66kDa, 100 and 85kDa proteins (Figure 1.3). Scatchard analysis revealed that certain erythroid cells express only either low-affinity or high-affinity receptors. It is suggested that the two affinities may account for different cellular responses to EPO. Friend virus-infected MEL cells respond to EPO by proliferating and differentiating, but a variant of these cells (clone 745) presents only a low affinity to EPOR and do not respond to the hormone (D'Andrea *et al*, 1990). COS cells transfected with a recombinant plasmid containing the EPO receptor cDNA from a MEL cell cDNA library demonstrated both low and high affinity for EPO. These workers suggested that a second subunit may be endogenous to COS cells (D'Andrea *et al*, 1989). In cells transfected with Friend spleen focus forming virus, EPOR binds to a retrovirus encoded, membrane bound protein, gp55 (section 1.1.5.2). Through direct binding of the EPOR, gp55 can stimulate the receptor and bypass the normal requirement for EPO, causing a prolonged proliferation of infected erythroid cells. This interaction is observed in all MEL cells and probably accounts for the absence of high-affinity binding sites on MEL cells and for their EPO unresponsiveness of these cells (Li *et al*, 1990).

In EPO-dependent cell lines, EPO induces rapid phosphorylation of tyrosine residues

in some common and several unique cellular proteins (Miura *et al*, 1991). Upon binding of EPO to EPOR, an increase of expression of *c-myc* transcript has been reported in erythroid cells (Chern *et al*, 1991).

1.1.3: MODELS OF HAEMOPOIESIS

1.1.3.1: Commitment

It has been suggested that commitment and differentiation of multipotential progenitors to individual lineages is regulated by either a stochastic, inductive or hybrid mechanisms.

The stochastic model suggests that differentiation of multipotent cells occurs by chance, without the influence of the environment. This model has been explained by two hypotheses. In the first hypothesis, it is proposed that multipotent cells can give rise to other multipotent cells, which upon several divisions become more and more committed along a lineage. Therefore in this hypothesis, one multipotent cell give rise to several types of mature cells. In an alternative hypothesis, the multipotent stem cells may be randomly committed to only one cell lineage (Till *et al*, 1964; Ogawa, 1993).

The inductive model suggests that growth factors are responsible of the commitment and differentiation of stem cells. Cells are directed either to proliferate or undergo some lineage-specific response by specific receptor-coupled signalling molecules (Nicola and Metcalf, 1991). Some of these soluble factors seem to play an important role at specific stages of the differentiation pathway of stem cells. It has been established that there are early and late acting growth factors (figure 1.1). For example, it has been shown that IL-3 is an early acting growth factor on stem cells (Suda *et al*, 1985). On the contrary, M-CSF and IL-5 are considered to be specific and late acting for macrophage/monocyte and eosinophil lineages, respectively (Sanderson, 1992). Kinetic studies have shown that under conditions of accelerated haemopoiesis the stem cell populations are rarely called upon to

divide and output is maintained by elevated proliferative activity within the more primitive progenitor populations. This suggests that some inhibitors would regulate haemopoiesis in a classical inhibitory "feed back" loop mechanism. These inhibitors have been mentioned previously (section 1.1.2.1).

The hybrid model (stochastic and inductive) was suggested by Just *et al* (1991). They showed that FDCP-mix cells, transfected with a retrovirus containing GM-CSF gene, demonstrated a high variability in the proportion of granulocyte and macrophage differentiation within the same clone. The expression of GM-CSF in FDCP-mix cells did not necessary differentiate all cells to granulocytes or macrophages. This experiment suggests that commitment to a particular lineage occurs very early, and in a stochastic manner, and that growth factors are not the only requirement for differentiation of haemopoietic cells (see later). Addition of IL-3 to GM-CSF transfected FDCP-mix cells, prevented differentiation to either granulocytes or macrophages, probably by competing with the shared ß-subunit between GM-CSF and IL-3 receptors. This result may show that the balance between differentiation and self-renewal of haemopoietic stem cells is regulated by growth factors.

1.1.3.2: Apoptosis

Programmed cell death is a functional term, used to describe cell death that is a normal part of the life of a multicellular organism, and apoptosis describes a type of cell death exhibiting a distinct set of morphological features (reviewed by Martin *et al*, 1994). For example, cell death with all the morphological and biochemical features of apoptosis can be induced by a wide variety of cytotoxic drugs and physical stimuli. These instances of apoptosis are not programmed, but represent the cell's responses to changes in its environment (reviewed by Martin *et al*, 1994).

Apoptotic cell death is characterized by plasma membrane blebbing, cell volume

loss, nuclear condensation, and endonucleolytic degradation of DNA at nucleosomal intervals (Wyllie *et al*, 1980). Haemopoietic cells die by apoptosis if growth factors are not present (Williams *et al*, 1990).

1.2.3.3: Survival

Bcl-2 is a oncogene product which is found at low concentrations in quiescent lymphocytes (Reed *et al*, 1987) or other cell types (Negrini *et al*, 1987). It is most often associated with malignancies of mature B cells (Korsmeyer, 1992). It is thought that deregulation of the expression of this oncogene product, leading to its overexpression, is responsible directly or indirectly of the long-term survival of non-dividing cells normally destined to undergo apoptosis (Vaux, 1993).

Bcl-2 gene is normally located on chromosome segment 18q21.3. Following deregulation of *bcl-2*, there is a translocation of the *bcl-2* gene to the immunoglobulin heavy chain in 85% of follicular B-cell lymphomas. Recently, Boise *et al* (1993) demonstrated that *bcl-2* expression may be regulated by other proteins of the same family, *bcl-*x_s and *bcl-*x_l. The protein coded by the cDNA *bcl-*x_s differs from the *bcl-*x_l by a stretch of 63 amino acids. These workers observed that *bcl-*x_l when transfected into growth factor-dependent cell lines, played the same role as *bcl-2*: Following removal of the growth factor, they are resistant to cell death. On the contrary, *bcl-*x_s appears to make transfected cell lines dependent upon growth factor, rendering them resistant to the ability of *bcl-2* to delay apoptosis upon growth factor withdrawal. Therefore the *bcl-*x_s protein appears to negatively control *bcl-2* and *bcl-*x_l products.

In leukaemia, it has been shown that a high level of expression of *bcl-2* was associated with high resistance to various apoptosis-inducing cancer chemotherapy compounds (reviewed by Lotem and Sachs, 1993). Moreover during differentiation of haemopoietic cells along the granulocytic lineage, the level of *bcl-2* expression is

downregulated (Hockenbery *et al*, 1991; Sachs and Lotem, 1993). It has been reported that mature granulocytes have a very short life span (Rubinson *et al*, 1976). Therefore, Lotem and Sachs (1993) suggested that *bcl-2* is linked with haemopoietic life span.

Transfection of growth factor-dependent cell lines with a *bcl-2* gene delays the apoptotic death of cells when the growth factor is removed from the culture medium. For example, after three days of culture without IL-3, 98% of untransfected *bcl-2* FDCP-1 cells were dead, whereas 90% of *bcl-2* transfected FDCP-1 cells were still viable (Vaux *et al*, 1988; Nunez *et al*, 1990; Borzillo *et al*, 1992).

1.2.3.4: The decision to differentiate or die

Recently, Fairbairn *et al* (1993) transfected a mouse multipotent cell line, FDCP-mix (clone A4) with a retrovirus in which it was introduced the human *bcl-2* gene. FDCP-mix (clone A4) cells is a interleukin-3 dependent cell line, upon withdrawal of this factor, they rapidly die by apoptosis (Williams *et al*, 1990). FDCP-mix cells which express *bcl-2* die much slower upon withdrawal of IL-3 from the culture medium and differentiate along either the granulocytic or erythrocytic lineages, depending on the cell clone.

These results indicate that growth factors are not absolutely required either for differentiation of multipotent cells or the production of mature cell progeny and that there is an existing differentiation programme in primitive stem cells. *Bcl-2* gene expression is acting as a survival signal allowing the cells to remain alive prior to preprogrammed differentiation. Such results favour the stochastic and for hybrid models of haemopoiesis reported previously.

1.1.4: HAEMOPOIETIC CELL LINES

1.1.4.1: Cell lines

In order to understand and treat leukaemias, leukaemic or non-leukaemic cell lines

have been cloned. The advantage of using haemopoietic cell lines to study biochemical changes is that they are monoclonal. Biochemistry results obtained from a monoclonal cell population which undergoes a differentiation into a specific lineage is therefore representative of the biological events. On the contrary, long term bone marrow culture contains a mixed population. Even after a selection of clones from the bone marrow, there is always a small amount of cells which does not correspond to the cells wanted. It is very convenient to test the efficiency of different drugs as inducers of differentiation of various leukaemic cell lines which are blocked at different stage of the differentiation pathway (see Table 1.3). While working on a non leukaemic monoclonal cell line, it is easy to control different parameters, for example the concentrations of growth factors to differentiate the cell line. Polyclonal LTBMc stroma cells produce different growth factors and therefore it is more difficult if not impossible to understand the role of each growth factor during differentiation. Cell lines can be cultured easily in standardised conditions and large scale of cells can be prepared at anytime for the experimentation.

1.1.4.2: Leukaemia

It has been shown in a previous section (1.1.1) that normal human myelopoiesis has the ability to produce large, but tightly regulated number of cells in the steady state, yet it can increase the production of particular lineages in response to infection or trauma (Bagby, 1989). In myeloid leukaemias, haemopoietic cells blocked at various stages of the differentiation pathway giving rise to large numbers of more primitive cells that are still capable of further proliferation.

All **chronic myeloid leukaemias** (CML) are to be considered as the result of a defect arising late in the maturation of myeloid cells, with progressive accumulation of cells blocked near to their final mature step. There are two major classes of CML:

First, chronic granulocytic leukaemias (CGL), found at all ages and are characterized in

90% of patients by a clonal development of Philadelphia positive (Ph +) stem cells. In Philadelphia positive cells, a translocation involving chromosome 9 band q34 and chromosome 22 band q11 is found. This translocation involves the transfer of the *c-abl* gene (tyrosine kinase) from chromosome 9 to the long arm of chromosome 22. It is the formation of the *bcr/abl* chimaeric gene which leads to the production of p210 *bcr/abl* protein with enhanced *abl* tyrosine kinase activity (Wiedemann *et al*, 1988; Kurzrock *et al*, 1989). CML can be induced in irradiated mice following the transplantation of bone marrow from a syngeneic donor after the marrow had been infected with a retrovirus encoding p210kDa (*bcr-abl*) (Daley *et al*, 1990). Transfection of IL-3-dependent cell line 32D a temperature sensitive p210 *bcr-abl* gene has been shown to decrease the rate of cell death in the absence of IL-3 (Carlesso *et al*, 1994). Spooncer *et al* (1994) also showed that at permissive temperatures, the expression of a temperature-sensitive *v-abl* in transfected FDCP-mix cells does not lead to growth autonomy, but only to a modest effect in delaying the onset of apoptosis, when cells are deprived of IL-3. However, it leads to a delay in maturation with a concomitant increase in cell production. Thus, a combination of delayed apoptosis and enhanced proliferative ability of cell populations in response to reduced growth factor levels may be the mechanism that provides human CML cells expressing *bcr-abl* with a selective advantage over their normal counterparts.

Second, the chronic myelomonocytic leukaemia (CMML) is found in older patients (>50 years old) and is Philadelphia negative.

Acute myeloid leukaemias (AML) affect patients of all ages and are characterized by numerous chromosome anomalies. AML has been divided into subcategories by using both biological and clinical data (Table 1.2).

Subtype	Features	Karyotype abnormality
M0	undifferentiated myeloblasts	-
M1	myeloblastic without maturation	t(9;22); inv(3); +8, -7;7q-, -5;5q-
M2	myeloblastic with maturation	t(6;9); +8, -7;7q-, -5;5q-
M2 baso	M2 with basophil blasts	t(12p); +8, -7;7q-, -5;5q-
M3	hypergranular promyelocytic	t(15;17); +8, -7;7q-
M3 variant	micro or hypogranular bilobed promyelocytes	+8, -7;7q-
M4	myelomacrocytic with both granulocytic and monocytic differentiation	subclass eosinophil/inv(16); +4, +8, -7;7q-
M5a	monocytic:monoblastic	t(9;11), +8, -7;7q-,
M5b	promonocytic:monocytic	t(8;16); +8, -7;7q-
M6	erythroleukaemia, with >50% erythroblasts and ≤30% or >30% blasts	+8, -7;7q-, -5;5q-
M7	megakaryoblastic	?

Table 2: FAB (French, American, British) classification of chromosome changes in AML (revised table from Rees, 1990) 1.2

Cell line	Source	Proliferation	Inducers of differentiation	Lineage of differentiation
UT-7	human megakaryoblastic	spontaneous	HGFs, e.g. GM-CSF, EPO	megakaryocytic
K562	human erythroleukaemia	spontaneous	retinoic acid hemin* phorbol esters, TGFß1	erythrocytic* granulocytic megakaryocytic
MEL	Friend virus transformed murine erythroleukemia	spontaneous	EPO, DMSO	erythrocytic
HL-60	human promyelocytic leukaemia	spontaneous	DMSO, NMF*, TPA, retinoic acid	granulocytic* monocytic
U937	human monoblastic leukaemia	spontaneous	phorbol diesters retinoic acid DMSO,IL-6 interferon-gamma	monocytic
ML-1	myelomonoblast	spontaneous	phorbol diesters IL-6,interferon-gamma,G-CSF	macrophage
WEHI-3B D+	murine promyeloleukemia	spontaneous	G-CSF	granulocytic
KG-1	myeloblast	spontaneous	phorbol diesters	macrophage

Table 1.3: Characteristics of leukaemic cell lines (revised table from Kan et al, 1993). (1) Miura *et al* (1990); (2) Cioe *et al* (1981); Luisi-Deluca *et al* (1984); Ohlsson-Wilhelm *et al* (1987); Leary *et al* (1987); Chen *et al* (1989); Larsson *et al* (1989); (3) Friend *et al* (1971); (4) Collins (1987); Leglise *et al* (1988); Denburg *et al* (1989); Fisckoff and Rossi (1990); (6) Stockbauer *et al* (1985); Takuma *et al* (1987); Guan and Bloch (1988); (7) Nicola *et al* (1983); (8) Sugimoto *et al* (1984); Kiss *et al* (1987). (*) Particular differentiating inducer which is used to differentiate the cell line along a specific lineage, also indicated by *.

1.1.4.3: Experimental models using leukaemic cell lines

1.1.4.3.1: Human promyelocytic leukaemia cell line HL-60

This cell line was isolated from a patient with an acute promyelocytic leukaemia (APL) (Collins *et al*, 1977). It can be induced to differentiate into monocytes in response to TPA (Rovera *et al*, 1979) and cytosine arabinoside (Griffin *et al*, 1982), and into granulocytes with N-methylformamide (Langdon and Hickman, 1987), retinoic acid (Breitman *et al*, 1980) and DMSO (Collins *et al*, 1980). A range of inducing agents, testedby Langdon and Hickman (1987) on HL-60, showed that there is no apparent structural characteristic requires to induce differentiation in either lineages. For each inducer, the concentration which gives optimal HL-60 differentiation, is found to be marginally below a cytotoxic concentration. This concentration is defined as that which prevents a single doubling of cells and reduces viability by >70% (Langdon and Hickman, 1987). It has been observed that during granulocytic differentiation, HL-60 cells undergo one or two divisions in the presence of the inducer before proliferation ceases, the nuclear to cytoplasmic ratio decreases and the nucleus begins to segment (Fontana *et al*, 1984). It is interesting to note that commitment to differentiation is irreversible. When HL-60 cells are incubated in the presence of a chemical inducer such as DMSO for 24 hours, washed free of drug and cultured again in medium with or without drug, develop in both cases 70 to 90% of cells develop into granulocytes after 48 hours (Yen, 1985). Thus, it appears that the cells retain a memory of exposure to the inducer. Differentiation along the granulocyte lineage is not cycle regulated. HL-60 cells synchronised by a thymidine block and then exposed to DMSO at different stages of the cell cycle, are equally sensitive to the inducer and require the same length of incubation, in the drug, to commit them to differentiate, regardless of their cell cycle phase (Tarella *et al*, 1982).

The human *c-myc* gene is amplified approximately 16-fold in HL-60. Differentiation into granulocytes with inducers such as 12-myristate 13-acetate (Shima *et*

al, 1989) or DMSO (Gailani *et al*, 1989) or NMF (Vass *et al*, 1990; Beer *et al*, 1993a) results in a loss of *c-myc* expression after 1 hour of treatment. However it has been shown that following 24 hours of treatment with retinoic acid (monocytic lineage), the level of expression of *c-myc* increases and at 120 hours starts to decrease (Yen and Guernsey, 1986). These results suggest that the kinetics of *c-myc* expression may vary in regard to the differentiation lineage. It was shown that the activity of PKC in HL-60 cells was increased following treatment with retinoic acid (Zylber-Katz and Glazer, 1985; Fontana *et al*, 1986). It is not known whether PKC plays a role during monocytic differentiation or is simply associated with the decrease of cell growth.

1.1.4.3.2: Murine erythroleukaemic cells MEL

Friend virus was isolated from the leukaemic spleen of a 14-month-old Swiss mouse that was inoculated at birth with a cell-free filtrate obtained from an Ehrlich ascitic tumour. There are two variants of Friend virus: one, FV-A, that induces anaemia and another, FV-P, that induces polycythemia (elevated erythrocytes in the blood). The primary target of Friend virus is committed (EPO)-responsive erythroblasts consisting of CFU-E and late BFU-E (Peschle *et al*, 1980; Kost *et al*, 1981).

Following infection with Friend virus, these infected target erythroblasts begin to proliferate, migrate from the bone marrow, and are sequestered in the spleen (Tambourin and Wendling, 1981). MEL cells derived from FLV-A-infected mice have EPO receptors and therefore are induced with EPO along the erythrocytic lineage. On the contrary, MEL cell lines derived from FLV-P infections do not differentiate in response to EPO treatment but are still able to bind EPO on their plasma membrane (Rifkind and Marks, 1978; Chern *et al*, 1991). MEL cells EPOR are single affinity receptors for EPO (D'Andrea *et al*, 1989). EPO receptors bind to retrovirus encoded, membrane bound protein (gp55) of the Friend spleen focus forming virus (SFFV). An IL-3-dependent lymphoid cell line becomes

IL-3-independent when these cells are co-transfected with EPO receptor and gp55 (reviewed by Showers and D'Andrea, 1992; Wang *et al*, 1993). When these cells are transfected with gp55 alone, they remain IL-3-dependent. The interaction between the EPOR and gp55 also observed in MEL cells may account for the absence of high-affinity binding sites on MEL cells and for the EPO-independent growth of these cells. MEL cells are differentiated along the erythroid pathway by exposure to a concentration of DMSO varying from 1.5 to 2% (v/v). Friend *et al* (1971) showed that MEL cells induced along the erythrocytic lineage with 2% (v/v) DMSO, cycle three times before the end of their maturation. Cells exposed to DMSO for 24 hours or longer will go on to differentiate even if transferred to inducer-free medium; therefore these cells are described as committed (Gusella *et al*, 1976). At a concentration of 3% (v/v) DMSO prevents the growth of these cells (Friend *et al*, 1971).

DMSO-induced differentiation has been observed to dramatically reduce the expression of the *c-myc* oncogene at the RNA level following two hours of exposure to the compound; this is followed by a return to normal levels with the next 24 hours (Lachman and Skoultchi, 1984). Coppola and Cole (1986) transfected viral-promoter-driven *c-myc* genes into MEL cells and showed that constitutive *c-myc* expression blocked DMSO-induced differentiation. Wingrove *et al* (1988) demonstrated, by simultaneous measurements of *c-myc* mRNA and protein levels in differentiating MEL cells, that the early decline in *c-myc* transcript has no significant effect on the cellular concentration of *c-myc* protein. They showed a constancy of the *c-myc* protein levels during differentiation with DMSO. Therefore they suggested that *c-myc* has not an immediate regulatory function in the differentiation process. They also reported an increase in *c-myc* protein half-life during differentiation and that may explain why the level of *c-myc* protein does not decrease following the decrease of *c-myc* transcription during DMSO-induced differentiation. *In vitro* translation of *c-myc* transcripts resulted in the synthesis of two

isoforms. One of these isoforms is preferentially synthesised following differentiation. These workers proposed that one isomer may play a role during cell growth and the other during differentiation. MEL cells treated with EPO and cycloheximide, a protein synthesis inhibitor, resulted in accumulation of *c-myc* transcript (Chern *et al*, 1991). This suggests that the accumulation of *c-myc* RNA transcript does not require protein synthesis, and EPO upregulates the level of expression of this gene. The transduction pathway leading to DMSO-induced differentiation of MEL cells is unknown. No cell membrane receptor for DMSO has been found on MEL cells but it has been observed that the activity of protein kinase C (PKC) reaches a maximum, when MEL cells are treated with the DMSO concentration optimal for cell differentiation (Chakravarthy *et al*, 1992). These workers suggest that differentiation of MEL cells with DMSO is caused by an increase in PKC activity rather than cytosolic redistribution. The activation of membrane PKC without the translocation of cytosolic PKC has also been observed in 3T3-L1 fibroblast cells (Halsey *et al*, 1987).

1.1.4.3.3: Chronic myelogenous leukaemic cell line K562

This human leukaemic cell line has a positive Ph^+ (Philadelphia) chromosome which has persisted after prolonged *in vitro* cultivation. The cells were initiated in culture from a pleural fluid specimen obtained from a patient with CML in blast crisis (Lozzio and Lozzio, 1975). The cell line can also be differentiated into different lineages depending on the agent used; into megakaryocytes with phorbol esters (Lumelsky and Forget, 1991), monocytes-macrophages by TPA (12-0-tetradecanoyl phorbol-13-acetate) (Kubota *et al*, 1991) and erythrocytes with haemin (Anderson *et al*, 1979; Cioe *et al*, 1981; Singh and Yu, 1984). Anderson *et al* (1979) reported 48% of benzidine positive cells following four days of exposure to sodium butyrate.

The *c-myc* expression is down regulated during erythroid differentiation by

tiazofurin (Weber *et al*, 1991). This synthetic nucleoside acts as an inhibitor of inosine monophosphate dehydrogenase which has been used for clinical treatment of leukaemia (Weber, 1989). The expression of *c-myc* was also down regulated when K562 cells were differentiated with DMSO (Darling *et al*, 1989). During the erythrocytic differentiation of haemin-treated cells, the decrease in *c-myc* mRNA expression was not found to be linked to the differentiation programs, but to the loss of replicative activity (Toffoli *et al*, 1989). K562 cells induced with *cis*-diamminedichloroplatinum II (CDDP) along the erythrocytic lineage, decreased dramatically the level of expression of *c-myc* following 1 hour of incubation, to increase above the basal level following 5 hours, and decrease again progressively after 24 hours, to reach a dramatic level of expression by 72 hours of incubation (Marazzi *et al*, 1991). This suggests that the level of expression of *c-myc*, during the erythrocytic differentiation of K562 cells varies with the chemical reagent which is used to differentiate the cell line. No role of PKC has been observed during the erythrocytic differentiation of K562 with adriamycin (Hoffman and Newlands, 1991). These workers reported that the PKC inhibitor H-7 did not block adriamycin-induced erythrocyte differentiation.

1.1.4.3.4: Conclusion

Although the use of leukaemic cell lines (Table 1.3) has been useful in establishing a number of genetic changes in leukaemic cells, and in identifying drugs to treat leukaemic cells, they have three major limitations:

Firstly, such cells are partially or completely blocked in differentiation. The molecular events that occur during chemically induced differentiation may not be the same as those seen in normal haemopoiesis. In the bone marrow, rapid proliferation of the cell populations appeared to be coupled with differentiation whereas leukaemic cells undergo limited self renewal (perhaps 1-2 divisions) before phenotypic changes are effected.

Secondly, drugs used to induce leukaemic cells to differentiate are either non-physiological or are used at non-physiological doses.

Thirdly, leukaemic cell lines are usually committed into specific lineages. For example, the promyelocytic leukaemic cell, HL-60 is arrested along the granulocytic or macrophage lineages; no growth factor or chemical compound can induce differentiation of this cell to another lineage such as the erythrocytic pathway (Yen, 1985).

1.1.4.4: Experimental models using non-leukaemic haemopoietic cells

1.1.4.4.1: 32D cell lines

The original clone 32D is a diploid IL-3-dependent cell line established from murine LTBMc cultures infected with the Friend murine leukaemia virus (Greenberger, 1983). The cells express proteins immunologically related to the gag and env proteins of the ecotropic (rodent) viruses (Migliaccio *et al*, 1989). However, the cells do not induce tumours when injected into histocompatible recipients (Greenberger *et al*, 1983). A variety of growth factor-dependent subclones of the murine interleukin-3-dependent cell line 32D have been isolated. Two versions of the IL-3 dependent cell line have been described: 32D cl 23 and 32D cl 3. 32D cl 23 clone was originally described to form colonies composed only of basophil and mast cells in the presence of IL-3 (Greenberger, 1983). Migliaccio *et al* (1989) selected several subclones by culturing, in the absence of IL-3, 32D cl 3. with EPO (32D EPO), G-CSF (32D G) and GM-CSF (32D GM).

The subclone 32D EPO produced erythrocytic cells (50-60% of benzidine) when cultured in the presence of EPO. The 32D GM clone when cultured without IL-3, in the presence of GM-CSF, consisted of myeloblasts, with 44% of positive cells for chloroacetate esterase. Following incubation of 32D cl3 with G-CSF, Valtieri *et al* (1987) reported that after 72 hours, less than 50% of cells were found to form colonies on semi-solid agar (when compared to day 0). By day 14, no clonogenic cells were observed. This suggests

that all the cells were committed along the granulocytic lineage. From day 4 to day 12, the cell population increased 10 fold: No cell growth was reported between day 0 and day 4 of the granulocytic differentiation. Morphology shows that by day 14 of the granulocytic differentiation the cell population was composed of 80% of neutrophils and 20% metamyelocytes. They also demonstrated that addition of IL-3 to the differentiating culture medium containing 30U/ml G-CSF prevents 32D cells to differentiating along the granulocytic lineage. Similar observations have been made for the haemopoietic cell line FDCP-mix (see following section). 32D EPO cloned cell line does not grow in the presence of GM-CSF or G-CSF and 32D G/32D GM clones do not grow in the presence of EPO.

The 32D cell lines do not produce detectable spleen colonies (CFUs) *in vivo*, nor do intravenous inoculation of these cells protect lethally irradiated mice from bone marrow failure (Greenberger, 1983). Collectively this data suggest that these subclones of 32D are committed towards either erythrocytic or granulocytic lineages but are not multipotential.

1.1.4.4.2: FDCP-mix cell lines

FDCP-Mix cells is a multipotential cell line (Spooncer *et al*, 1986; Heyworth *et al*, 1989). It was originally isolated from murine LTBMC infected with a molecular recombinant of Rous sarcoma virus and murine amphitropic leukaemia virus, *src* (MoMuLV). The infected cultures displayed an altered balance in the accumulation of cells in different compartments of granulocytic differentiation. There was a drastic increase in the stem cell (CFU-S) and the committed progenitor cell (GM-CFC) compartments and a decrease in mature granulocytes (Boettiger, 1984). The method used to select FDCP-Mix, consists of plating the supernatant arising from the long term marrow culture on semi-solid medium with Fisher's medium and horse serum [10% (v/v)] supplemented with IL-3 (1500U/ml). Then, each colony is mixed in Fisher's medium, horse serum and IL-3 in 24-

well cluster plates. Cells are examined and the culture is gradually expanded (Spooncer and Dexter, 1990).

FDCP-Mix cell lines have a normal karyotype and are non-leukaemic. These cells grow continuously *in vitro* in the presence of IL-3; in the absence of IL-3, they die by apoptosis (Williams *et al*, 1990). Differentiation of FDCP-Mix cells can be induced in semi solid culture or in liquid systems by using growth factors. When GM-CSF is used alone, it allows differentiation into macrophages and granulocytes but it is a poor proliferation stimulus. Cells proliferate and differentiate when they are incubated in the presence of a concentration of 1.5U/ml in IL-3, 50U/ml in GM-CSF and 5000U/ml in G-CSF [or 10% (v/v) of lung conditioned medium]. Cells which are incubated with GM-CSF (50U/ml) and a high concentration of IL-3 (100U/ml) proliferate. At a low concentration of IL-3 (1U/ml) and a high concentration of GM-CSF and G-CSF, a rapid translocation of protein kinase C from the membrane to the cytosol is observed (Shearman *et al*, 1993). It has been also observed that a high concentration of IL-3 suppresses the developmental response elicited by GM-CSF (Heyworth *et al*, 1990). Erythrocyte differentiation with the same cell line is performed with EPO (3U/ml), haemin (0.2mM) and also a low concentration of IL-3 (2U/ml). FDCP-Mix cell line can also differentiate when cultured in association with bone marrow stromal cells in the absence of IL-3: Cells differentiate and develop into mature myeloid cells (Spooncer *et al*,1986).

During the granulocytic differentiation of FDCP-mix cells, the expression of a number of genes including the T cell protease, CCP1, decrease at both transcriptional and protein levels (Hampson *et al*, 1992). It was suggested that this serine protease (CCP1) may have autocrine growth stimulatory activity for the FDCP-mix cells and a role in cell death. Fusion of the promoter and upstream sequences of CCP1 reveals the presence of both positive and negative regulatory sequences to which transcription factors can interact. The identification of the regulatory interactions which play either passive or active roles

in the down regulation of CCP1 expression during differentiation is currently under investigation (Cross *et al*, 1993).

The effects of ectopically expressed GM-CSF in FDCP-mix cells resulted in the differentiation along the granulocyte lineage, when IL-3 was removed from the culture medium (Just *et al*, 1991). These authors also showed that the addition of IL-3 to the culture medium containing FDCP-mix cells transfected with GM-CSF gene, blocks the differentiation of these cells along the granulocyte lineage, confirming that IL-3 has a proliferative stimulus favouring self-renewal over differentiation. Transfection into FDCP-mix cells with IL-3 gene renders it IL-3 independent (Spooncer *et al*, 1989). However, addition of exogenous IL-3 to these cells further enhanced their growth, suggesting that IL-3-transfected FDCP-mix cells produce IL-3 in rate-limiting quantities.

The advantages of FDCP-mix cell line are considerable: (I) It is multipotential (Table 1.4). (II) Differentiation does not require chemical inducers but growth factors (Whetton and Dexter, 1989); and (III) Cell differentiation and proliferation are coupled.

In the following sections, the possible role of heat shock proteins (hsps) in eukaryotic cells during cell growth and differentiation of haemopoietic or non-haemopoietic cell lines are reviewed. It emphasises the role of hsps as chaperones and house keeping proteins.

Cell line	Source	Proliferation	Inducers of differentiation	Lineage of differentiation
(1) FDCP-mix (A4)	murine LTBMC	IL-3 dependent	HGFs, e.g. GM-CSF*, G-CSF*, M-CSF, EPO**	Granulocytic* macrophage erythrocytic**
(2) FDCP-mix (1B)	murine LTBMC (bcl-2)	IL-3 dependent	HGFs, e.g. GM-CSF*,G-CSF*, M-CSF, EPO** removal of IL-3 from culture medium*	granulocytic* macrophage erythrocytic**
(3) FDCP-mix (2J)	murine LTBMC (bcl-2)	IL-3 dependent	HGFs, e.g., GM-CSF, G-CSF, M-CSF**, EPO* removal of IL-3 from the culture medium*	erythrocytic* granulocytic macrophage**
(4) 32D (subclones)	murine bone marrow	IL-3 dependent	HGFs, e.g., GM-CSF, G-CSF, EPO*	erythrocytic* granulocytic
(5) LyD9	murine bone marrow	IL-3 dependent	HGFs, e.g. GM-CSF G-CSF, EPO	neutrophil granulocytic macrophage

Table 1.4: Characteristics of non-leukaemic cell lines. (1) Spooncer *et al* (1986), Heyworth *et al* (1990); (2-3) Fairbairn *et al* (1993); (4) Greenberger *et al* (1983), Valtieri *et al* (1987); (5) Kinashi *et al* (1991), *Lee et al* (1991).

1.2: HEAT SHOCK PROTEINS

1.2:1: CELLULAR RESPONSE AFTER A HEAT SHOCK

1.2.1.1: Introduction

The concept of cellular stress is not well defined. Thomas *et al* (1982) summarized some presumed targets of stress which may trigger the increase of the level of expression of heat shock proteins (hsps): There are (1) changes in protein structure following heat shock or cold shock, (2) treatment with heavy-metal ions and ionophores, (3) respiratory inhibitions of oxidative phosphorylation and electron transport (Lindquist, 1986; Subjeck and Shyy, 1986; Yost *et al*, 1990). If stress is chemically induced, the type and toxic concentration of the compound appear to be important.

Further sections of this chapter therefore review changes occurring following cellular stress and the possible role of hsps.

1.2.1.2: Thermotolerance

Most prokaryotic and eukaryotic cells increase the expression of heat shock proteins (hsps) at both RNA and protein levels following stress. The response often allows the cell to escape death, whereas, cells unable to express hsps show less tolerance when challenged by heat or chemical stress (Mattei *et al*, 1988). Microinjection of rat fibroblasts with neutralising monoclonal antibodies specific to the hsp72 stress proteins have been shown to negate thermotolerance (Riabowol *et al*, 1988). A comparative analysis, measuring the thermotolerance of leukaemic cell lines K562, HL-60 and KG-1 suggests a possible link between the period of induction of hsp following heat shock and thermotolerance (Mivechi, 1989). This study also showed that K562 is three times more thermotolerant than the other cell lines. K562 expresses the inducible heat shock protein hsp70 for 24 hours following hyperthermia; on the other hand, both cell lines HL-60 and KG-1 induced hsp70 for only

2 or 4 hours respectively. Cells which are treated with a sublethal heat shock, severe enough to increase the levels of the hsp, and then returned to their normal growth temperature, exhibit significantly higher survival rates to a second heat shock challenge (which would otherwise be lethal without pre-treatment) (Gerner and Schneider, 1975). Such pretreated cells also show resistance to drug-induced stress.

Cells subjected to chemically induced stress (for example, a previous exposure to Amphotericin B, a drug which interacts with cholesterol to form ionophores in plasma membranes) can also protect against heat shock at 43°C (Hahn and Li, 1990).

1.2.1.3: Biochemical changes in stressed cells

It has been suggested that plasma membrane fluidity and lipid composition play a role in governing how sensitive a cell is to hyperthermia (Anderson and Parker, 1982; Cress *et al*, 1982). Heat shock and other forms of stress produce changes in the activity and/or regulation of a number of plasma membrane components. For example, (1) changes in the Na+/K+ ATPase activity resulting in the increase of K^+ and decrease of Na^+ inside the cell (Anderson and Hahn, 1985), (2) the overall decrease of growth factor binding activity, apparently due to a reduced numbers of receptors. For example, EGF (epidermal growth factor) binding to Rat-1 cells (Magun and Fennie, 1981) and insulin binding activity in HA-1 Chinese hamster ovary cells following heat shock (Calderwood and Hahn, 1983), (4) a reduced number of cell surface histocompatibility antigens (Medhi *et al*, 1984), (5) a reduction in the ability of the lectin (concanavalin A) to induce patching and capping of cell-surface glycoproteins (Stevenson *et al*, 1981), and (6) an increase in hexose transport into the cell (Warren *et al*, 1986). After hyperthermia, a drop in intracellular pH and an increase in cytosolic calcium levels is observed (Drumond *et al*, 1986; Stevenson *et al*, 1986). It is interesting to note that manipulation of mammalian cells at their normal temperature by external agents that either lower intracellular pH or result in increased

intracellular Ca^{2+} levels does not result in the activation of the heat shock response. Moreover, preventing intracellular pH decreases after heat shock does not inhibit the activation of heat shock gene transcription or the increase of hsp synthesis (reviewed by Welch, 1990). Another early effect of hyperthermia is a rapid reduction in intracellular ATP levels (Findly *et al*, 1983). This reduction in ATP is most likely correlated with the observed alterations in the integrity of the mitochondria. Denaturation of various intramembranous mitochondrial proteins is observed after hyperthermia (Lepock *et al*, 1983). The presence of hsps suggests that they may stabilize the constituents of cells by forming macromolecular complexes with them (Welch, 1987; see section 1.2.3.1).

1.2.1.4: Biochemical properties of heat shock proteins

Two major families of stress proteins have been identified in eukaryotic cells, the heat shock proteins (hsps) and the glucose regulated proteins (grps) (Table 1.5). The expression of hsps is increased following different types of cellular stress (section 1.2.1.1) and the grps are stimulated by deprivation of glucose or oxygen, agents perturbing calcium homeostasis such as the calcium ionophore A23187 and inhibitors of glycosylation.

1.2.1.4.1: Hsp110

A group of proteins referred to as hsp110 have been analyzed principally in mammalian cells. They are expressed both constitutively, and after heat shock. Following hyperthermia, hsp110 is concentrated in the granular region of the nucleolus, the location of pre-ribosomes. It has been suggested that hsp110 expression is induced to protect ribosomes (Welch and Suhan, 1985).

Name	Modification	37°C	42°C	Characteristics
Hsp28 (c, i)	phosphorylation	cytoplasm near golgi	nucleus,cytoplasm	increased phosphorylation in response to stress, repressed during cell growth
Hsp47 (c, i)	phosphorylation	ER	ER	bind collagen
Hsp58 (c)	?	mitochondria	mitochondria	synthesized as a precursor
Hsp70 (i)	methylation		cytoplasm nucleus, nucleoli	low or non-existant level at 37°C; binds ATP
Hsc70 (c)	methylation	cytoplasm	cytoplasm nucleus nucleoli	involved in clathrin-coated vesicle assembly/disassembly; protein translocation through membrane; protein degradation; regulation of steroid receptor,heme kinase (HRI);binds to other proteins:p53,c-myc, actin and others.
Hsp90α (c) Hsp90ß (i)	phosphorylation, methylation	cytoplasm	cytoplasm	regulation of steroid receptor,heme kinase(HRI)
Hsp110 (c,i)	?	nucleoli	nucleoli cytoplasm	involved in rRNA transcription
Grp75/p75 (c, i)	?	mitochondria	mitochondria	synthesized as a precursor,binds ATP,
Grp78/BIP (c, i)	phosphorylation, ADP-ribosylation	ER	ER	homology to immunoglobulin heavy chain binding protein (BIP)
Grp110 (c, i)	phosphorylation glycosylation	Golgi/ER	Golgi/ER	associated with hsp90

Table 1.5: Biochemical properties of eukaryotic heat shock proteins (revised table of Welch,1990).
(c) constitutive, (i) inducible. This table shows the most important characteristics of hsps, and their distributions before (37°C) or after (42°C) heat shock.

1.2.1.4.2: Hsp90

The proteins collectively referred to as hsp90, have been studied in both eukaryotic and prokaryotic cells. All of them are highly conserved, phosphorylated (Welch *et al*, 1983) and constitutively expressed (Bardwell and Craig, 1987). Two forms of mammalian hsp90 have been identified, hsp89α (constitutive) and hsp89ß (inducible) in humans and hsp86 and hsp84 in mouse (Moore *et al*, 1989);

Hsp90 is located and is associated with other cytoplasmic proteins such as the cellular oncogene *src* pp60 (Oppermann *et al*, 1981). *src* pp60 does not exhibit tyrosine kinase activity or autophosphorylation when it is complexed with hsp90 but after dissociation can recover its activity (Brugge *et al*, 1983).

Furthermore, hsp90 (with hsc73) has been found in association with the haeme-regulated protein kinase that phosphorylates the α subunit of eukaryotic initiation factor 2 (eif-2α) (Rose *et al*, 1989). The phosphorylation of eif-2α results in a reduction in ribosomal initiation complexes and an inhibition of new translational events (Duncan and Hershey, 1984).

1.2.1.4.3: Hsp70 family

Heat shock proteins in this group are mainly expressed either constitutively (p75, hsc70, p72) or are inducible (hsp72/hsp70, hsp68) or both constitutive and stress-inducible (hsp70, grp78) (reviewed by Morimoto and Milarski, 1990) (Figure 1.4). However, a complete classification of the hsp70 family has only been possible in the last few years because specific monoclonal antibodies have been raised against the inducible and constitutive forms of hsps. The nomenclature used for various forms of hsps has, and continues to be different between laboratories: In this work, the constitutive 70kDa protein is called hsc70 and the inducible hsp70 (reviewed by Morimoto and Milarski, 1990).

There is a high degree of homology between hsp70 of different species; for

example, in mouse, a single open reading frame encodes a protein of 642 amino acids, and this has 91% homology to at least one of the human hsp70 genes. Although there is no homology in the 5' (and 3') nontranslated regions, the human and mouse hsp70 gene promoters can be aligned to give 65% homology (Hunt and Calderwood, 1990). A high degree of homology exists between hsc70 and hsp70 with 74% homology at the DNA sequence level, and 81% at the amino acid sequence level. It was demonstrated that while the human hsp70 gene contains no intron, its hsc70 cognate has eight intervening sequences (Dworniczak and Mirault, 1987). The promoter of hsp70 and hsc70 is reviewed at section 1.2.2.

After cellular stress, hsp72 is present in the granular region. As the nucleoli slowly regain their normal morphology, hsp72 disappears from this region and during the later periods of recovery, accumulates again within the cytoplasm. Distinct cytoplasmic localizations are observed; perinuclear, within and underneath the plasma membrane. Subcellular localization of p75 is mitochondrial, p72 is cytoplasmic and nuclear and hsp70 is nucleolar (reviewed by Welch, 1990).

The hsp70 family may play a role in many cellular functions such as thermotolerance (section 1.2.1.2), translocation of proteins across membranes (Deshaies et al, 1988; Shi and Thomas, 1992), reformation of new clathrin coated vesicles (Chappel *et al*, 1986), tissue injuries (Brown *et al*, 1989), protein folding (see section 1.2.3.1), protein degradation (see section 1.2.3.2) and differentiation of haemopoietic cell lines (section 1.2.5).

1.2.1.4.4: Hsp58

Mammalian hsp58 is a mitochondrial protein which is synthesized in the cytoplasm as a precursor protein of 60 kDa and is thought to undergo a proteolytic processing within the mitochondria. Hsp58 is thought to facilitate the proper oligomeric assembly of

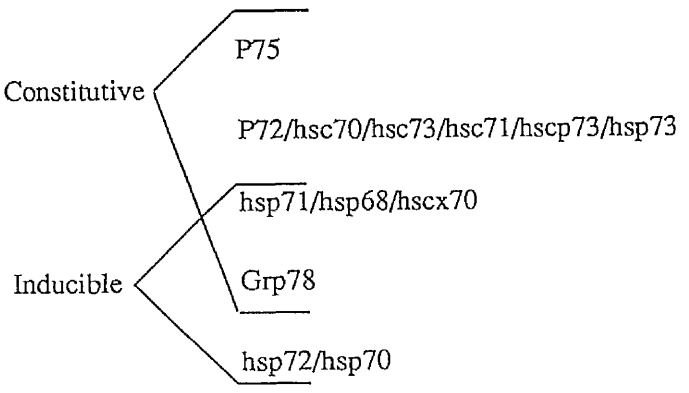


Figure 1.4: HSP70 family. The classification of the hsp70 family varies in each laboratory. P75, P72, hsc70, hsp70, grp78 (Morimoto and Milarski, 1990); hsc73 (Sorger and Pelham, 1987a); hscp73, hsp71 (Hightower and Guidon (1989); hsc71 (Maekawa *et al*, 1989); hsp73 (Palleros *et al*, 1991); hsp68 (Hahnel *et al*, 1986; Lowe and Moran, 1986); hscx70 (Gromov and Celis, 1991).

mitochondrial proteins synthesized in the cytoplasm and translocates them into the mitochondrial matrix (reviewed by Welch, 1990).

1.2.1.4.5: Hsp47

This membrane glycoprotein, from the reticulum endoplasmic, phosphorylates and exhibits an affinity for collagen in a pH dependent manner (Saga *et al*, 1987). Following transformation of chick fibroblasts with Rous sarcoma virus, the synthesis of hsp47 decreases approximately two to threefold and the level of phosphorylation appears to increase 5-7 fold. Although the synthesis of this protein increases after heat shock, the relative levels of hsp47 phosphorylation does not change (Nagata and Yamada, 1986). The hsp47 promoter contains a heat shock element (HSE) similar to that found in the hsp70 gene (see section 1.2.2) (Hosokawa *et al*, 1993).

1.2.1.4.6: Hsp26/27/28

The heat shock proteins of low molecular weights are highly phosphorylated after cells are treated with growth factors or their calcium levels are perturbed (reviewed by Welch, 1985). Hsp28 protein appears to be concentrated around the Golgi cisternae. Following hyperthermia, much of hsp28 relocalizes within the nucleus and to a lesser extent is found dispersed throughout the cytoplasm (Arrigo *et al*, 1988). However these results appears to depend on the experimental conditions, cell type and cell cycle. Rossi and Lindquist (1989) have demonstrated that intracellular distribution of the analogous gene in yeast, hsp26, varies with cellular metabolism and conditions of cell culture. In yeast, in contrast to other heat shock genes, transcription of hsp26 appears to be regulated by a mechanism of basal repression during growth at normal temperatures and derepression during heat shock (Susek and Lindquist, 1989). These workers did not identify any single upstream sequence that is responsible for repressing the basal hsp26 transcription. The

gene appears to contain functionally redundant repression elements. Mouse ehrlich ascites tumour (EAT) cells in the exponential growth stationary phase, a strong accumulation of hsp25 was seen. Constitutive overexpression of hsp25 in EAT cells resulted in a significant decrease in the proliferation rate, when compared to the non transfected cells (Knauf *et al*, 1992). This suggests that hsp25 may downregulate cell growth.

1.2.1.4.7: Grp78/BIP

Grp78 is present within the endoplasmic reticulum (ER) (Carlsson and Lazarides, 1983). It exhibits approximately 50% sequence homology to the hsp 70 proteins and is identical to the immunoglobulin binding protein BIP (Munro and Pelham, 1986). The grp78 gene consists of eight exons (Ting and Lee, 1988). This protein is phosphorylated and/or is ADP-ribosylated. It appears that grp78 functions, in an ATP-dependent manner which may facilitate the stabilization, correct folding and/or oligomeric assembly of proteins which pass through the ER.

1.2.1.4.8: Grp94/100

Grp 94 is a class of hsp which is phosphorylated and glycosylated. It is localized to the ER, Golgi, and possibly the plasma membrane (Welch *et al* 1983). Grp94, like hsp90, is thought to exhibit regulatory interactions with other macromolecules but within its own compartment (reviewed by Welch, 1990).

1.2.1.4.9: Grp75/P75

This protein exhibits high affinity for ATP, is constitutively expressed and located in the mitochondria. It seems that this compartmentalized 75kDa stress protein, like the grp94 protein present in the endoplasmic reticulum, serves a general role in the assembly and/or disassembly of target proteins, but within its own compartment, the mitochondria

(reviewed by Welch 1990).

1.2.2: REGULATION OF HSC70/HSP70 PROMOTER

Most of the studies have been done on the promoter of the inducible gene, hsp70. This promoter is divided into two functional domains, the proximal (1) and the distal (2) domains (Figure 1.5) (reviewed by Morimoto and Milarski, 1990).

(1) The proximal domain (-20 to -70) contains the basal promoter and consists of promoter elements responsive to signals that activate cell growth. This domain is responsive to the adenovirus E1A transcriptional trans-activation (-1 to -74) (reviewed by Morimoto and Milarski, 1990) and also to serum stimulation (SRE) (-58) (Wu *et al*, 1987; Cunniff *et al*, 1991). In this basal promoter, several elements have been identified to play an important role in the regulation of hsp70 gene (Figure 1.5). One of these transcription factors, which is called CBF, binds to a specific element CCAAT (-70) in association with wild-type p53, and blocks the transcription of hsp70 gene (Agoff *et al*, 1993). In response to growth stimuli, wild-type p53 undergoes a conformational change during the G1 stage of cell cycle and adopts a mutant like conformation (Milner and Watson, 1990), and some mutant forms of p53 can oligomerize with wild-type p53 and alter its conformation (Milner and Medcalf, 1991). Agoff *et al* (1993) suggested that mutant forms of p53 may alter the wild-type p53-CBF complex, perhaps by promoting the dissociation of wild-type p53 from CBF, even in the absence of growth stimuli. By using Xenopus laevis oocytes as a model system, Ananthan *et al* (1986) observed that unfolded proteins activate hsp70 promoter in a region between -48 and -62 of the proximal domain.

(2) The distal domain (-70 to -230) contains the heat shock element (HSE), which is necessary for multiple forms of stress induction, and additional basal elements that are redundant relative to the proximal promoter but are still functional (Figure 1.5). In the last few years, the interaction between the heat shock element (HSE) and the heat shock

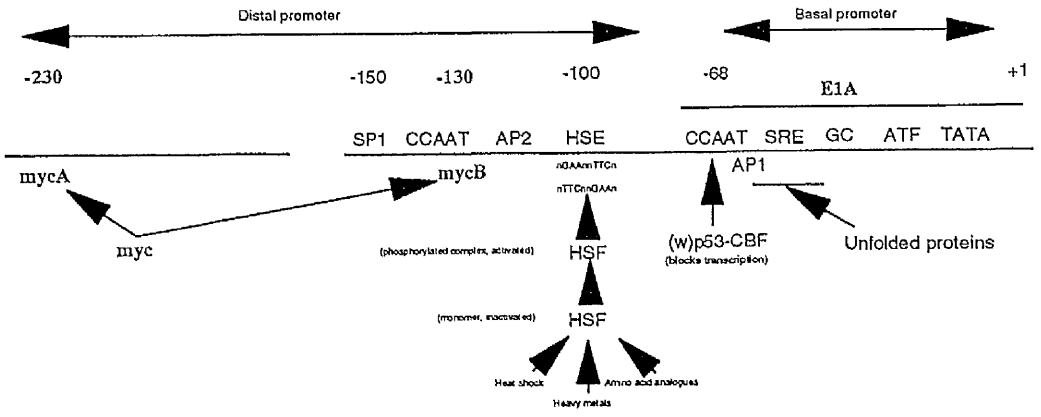


Figure 1.5: Hsp70 promoter (revised from Morimoto and Milarski, 1990). Hsp70 promoter is composed of the basal (-20 to -70) and distal (-70 to -230) promoter. The transcription factor binding sites:SP1, AP2, HSE (heat shock element), SRE (serum stimulation, -58), GC, ATF and as well as the CCAAT and TATA boxes are shown. Following activation of HSF (heat shock factor), HSF binds to HSE (-70 to -120) and increases the transcription rate of hsp70. The wild type p53 in association with CBF binds to CCAAT (-70) to block the transcription of hsp70 (the only known protein complex which blocks the transcription). Denatured or unfolded proteins binds to a region between -48 to -62. The region (-1 to -74) is responsive to adenovirus E1A. *c-myc* binds to two sites, mycA (-230) and mycB (-130) to increase hsp70 expression.

(HSF) has been well investigated (Morimoto *et al*, 1992). HSE seems to be important in regulating the stress response. However a similar sequence has been observed in the promoter of both constitutive gene hsc70 (Sorger and Pelham, 1987a). HSE is described as contiguous arrays of variable numbers of the five base pairs sequence nGAAn arranged in alternating orientation. At least two nGAAn units are needed for high affinity binding of HSF in vitro, and these may be arranged either head-to-head (nGAAnnTTCn) or tail-to-tail (nTTCnnGAAn) (Perisic *et al*, 1989). The binding of HSF to HSE is cooperative binding. When one HSF binds to one of a pair of adjacent trimeric binding sites, it facilitates another HSF to bind to the second trimeric binding site by over 2000 fold (Cunniff *et al*, 1991; Xiao *et al*, 1991). In *Drosophila* and vertebrate cells, it is the binding of HSF to HSE that is induced upon heat shock and following this binding an increase of hsp70 synthesis is observed. It has been suggested that upon a heat shock, pre-existent HSF proteins are activated by a post-translational mechanism because the induction of HSF binding occurs in the absence of protein synthesis (Sorger, 1991). On the contrary, in yeast, HSF is bound constitutively to heat shock promoters and undergoes heat shock-dependent phosphorylation that activates the transcriptional capacity (Gallo *et al*, 1991). It has also been proposed that transcriptionally active complexes (HSF) contain six or more HSF phosphorylated monomers to bound to HSE (Sorger, 1991). The gene encoding HSF was first isolated from *S. cerevisiae* (Sorger and Pelham, 1987a), and subsequently from *Drosophila* melanogaster (Wu, 1984), human (Zimarino *et al*, 1990) and chicken sources (Nakai and Morimoto, 1993). Chicken HSF (-1,-2,-3) exhibit distinct DNA binding properties. HSF1 DNA binding is induced following either heat shock or treatment with non-ionic detergents. HSF2 constitutively binds DNA and HSF3 can not bind DNA unless activated by removal of its carboxyl terminus (Nakai and Morimoto, 1993). In mouse and human cells, two HSF have been identified (Sarge *et al*, 1991). Recently, it has been also suggested that following hsp70 expression, hsp70 could play a role in the conversion of

HSF back to a conformation that no longer binds the heat shock promoter element. The absence of binding of HSF on HSE would decrease the expression of hsp70 (Mosser *et al*, 1993; Liu *et al*, 1993). Such an effect has been shown in the human acute lymphoblastic leukaemia T-cell line, PEER, following transfection with the human hsp70. Overexpression of this gene results in a reduction in the level of HSF activation in response to temperature elevation. The degree of inhibition was proportional to the level of hsp70 expression. These workers suggest that cells sense temperature elevation through a decrease in the level of available hsp70. Activation of the stress response is triggered by a shift in the equilibrium between free and substrate-bound forms of hsp70. A reduction in free hsp70 would lead to the activation of HSF. However Liu *et al* (1993) showed that in rat-1 cells (M21), a constitutive HSE-binding factor (CHBF) appears to be involved in the regulation of hsp70 transcription. The heat-induced decrease of CHBF correlated with the increase of HSF in a dose-dependent manner. Moreover during post-heat shock recovery at 37°C, HSF disappeared within 30 minutes. On the other hand, CHBF recovered with much slower kinetics. The time required for complete recovery of CHBF coincides with the onset of the decline of hsp70 mRNA. In rat-1 cells, constitutively expressing the exogenous human hsp70, a suppression of HSF but not CHBF expression was observed.

c-myc has been shown to bind to synthetic oligonucleotides corresponding to hsp-mycA (-230) and hsp-mycB (-160) in the hsp70 gene (Figure 1.5). The binding of the *c-myc* protein complex to the two sites was sequence specific, and was abolished by a monoclonal antibody against the human *c-myc* protein (Taira *et al*, 1992).

It has been reported that the promoter of the rat constitutive heat shock protein (hsc73) gene contains a similar sequence to the hsp70 promoter in *Drosophila* (Sorger and Pelham, 1987b). Binding sites such as SP1, HSE and CCAAT were present but located at different positions. HSE is much closer to the TATA box than in hsp70 (7bp instead of 15bp). These different locations may result in unfavourable interactions between HSF

and a TATA-binding protein.

However, binding of HSF to HSE is not sufficient for transcriptional activation of the hsp70 gene in MEL cells (Hensold *et al*, 1990). Following heat shock, HSFs are activated and bind to HSE, but this does not lead to the expression of hsp70 transcript.

1.2.3: CHAPERONE FUNCTION AND ROLE IN PROTEIN DEGRADATION OF HEAT SHOCK PROTEINS

1.2.3.1: Heat shock proteins or chaperones

Hsps interact with many cytoplasmic or nuclear proteins. It has been proposed that hsps form part of a larger family of molecular chaperones (Laskey and Earnshaw, 1980). Ellis (1987) suggested that the chaperone function is to ensure correct folding of polypeptides and their assembly into oligomers. The groEL chaperone of *E.coli* (which is similar to the 70kDa hsp of eukaryotic cells) has been shown to bind to various unfolded polypeptides. Bochkareva *et al* (1988) proposed that groEL protein binds the unfolded form of a polypeptide before it is completely synthesized. Thus, the bound polypeptide is prevented from folding prematurely. Bacterial expression of the pre ß-lactamase gene inserted in a plasmid secretion vector demonstrated that when the protein was expressed in the presence of an increasing concentrations of dithiothreitol (DTT) to prevent S-S bond formation (has the effect of keeping pre-ß-lactamase in an unfolded conformation), the unfolded conformation of this protein stimulated its cross-linking with GroEL. The same authors showed that the interaction between the proteins was broken following hydrolysis of ATP.

How do unfolded or denatured proteins stimulate the expression of hsp70? In *Xenopus laevis* oocytes, the expression of hsp70 gene is strictly controlled at only the translation level making them an excellent model to study the expression of hsp70 gene at the transcriptional level (Bienz and Gurdon, 1982). Oocytes were coinjected with a

construct containing the Drosophila hsp70-ß-galactosidase hybrid gene and purified bovine ß-lactoglobulin or bovine serum albumin. This did not result in the activation of the hybrid hsp70-ß-galactosidase gene, whereas when proteins (ß-lactoglobulin or bovine serum albumin), were first denatured by reductive carboxymethylation, each caused the activation of the hsp hybrid gene (Ananthan *et al*, 1986). In order to examine the possibility that a transcription factor or the unfolded protein itself interacted with the hsp70 promoter, different constructs containing various deletions of the hsp70 promoter were made and cotransfected in Xenopus laevis oocytes. Following heat shock or coinjection of denatured proteins, they demonstrated that the region between -48 and -62 on the promoter was necessary for the activation of the hsp70 gene. This region corresponds to the basal region of hsp70 gene (Figure 1.5).

Beckmann *et al* (1990) demonstrated that hsp72/73 in Hela cells is associated with nascent peptides prematurely released from polysomes. This experiment confirmed that the interaction of hsp72/73 with newly synthesized proteins occurs co-translationally. This complex of hsp-proteins is also dissociated by hydrolysis of ATP. It is interesting to note that hsp70 proteins are promiscuous in their ability to bind a variety of synthetic peptides (Flynn *et al*, 1989). The 70kd hsp family has been also shown to be involved in the translocation of proteins across membranes. In prokaryotic cells (Bassford and Collier, 1988) and eukaryotic cells (Munro and Pelham, 1986; Hartl and Neupert, 1990) the 70 kDa heat shock proteins maintain the secondary structure of proteins in order to facilitate their translocation through membranes. In yeast, triple deletion of SSA1, SSA2 and SSA4 proteins, which are hsp70-like proteins, led to the arrest of protein translocation and finally to cell death (Deshaies *et al*, 1988). This suggests that the 70 kd heat shock proteins are necessary for cell survival.

Another heat shock protein, hsp90, is involved in protein folding. Citrate synthase aggregates rapidly in refolding buffer, but in the presence of hsp90, aggregation is

drastically suppressed (Wiech *et al*, 1992). Hsp90 has also been found in association with the steroid receptor (Sanchez *et al*, 1987) and dissociates in presence of the hormone *in vitro*. In contrast, Tuohimaa *et al* (1993) observed that hsp90 is not associated to the progesterone receptor (PR). By immunochemistry, they demonstrated that in chicken oviduct, hsp90 is found only in the cytoplasm, and progesterone receptors in the nucleus. The subcellular distribution of progesterone receptors was not affected by the progesterone administration. This result suggests that the hsp90-PR complex is formed during tissue processing and that hsp90 is unlikely to interfere directly with receptor functions in the nucleus *in vivo*.

1.2.3.2: The role of heat shock proteins in protein degradation

Intracellular protein degradation plays a role in modulating the levels of metabolic enzymes and in removing damaged and abnormal proteins from cells. Although most proteins are relatively stable *in vivo*, a subset of regulated proteins have extremely short half-lives (Goldberg *et al*, 1976). These proteins are required by the cell for limited periods, or under specific metabolic or developmental conditions, but are unnecessary or even detrimental at other times or under other conditions (Gottesman, 1987).

Heat shock proteins, especially hsc70, may play a role in presenting proteins which need to be degraded in lysozomes or by intracellular proteases. The role of hsc70 in protein degradation does not conflict with that "house keeping", in which they chaperon intracellular proteins. Some proteins such as *c-myc* and p53 have very short half-lives, even though they bind to hsc70. It has been suggested that following the interaction of *c-myc* or p53 products with their targets, these proteins would change their conformations and be degraded by proteases or lysozomes. It has been demonstrated that intracellular proteases can recognize specific peptide sequences within most proteins (Gottesman and Maurizi, 1992). It is possible that following the change of *c-myc* or p53 conformation,

these proteins present a degradation signal in order to be transported by hsc70 and degraded in lysozomes or by intracellular proteases (Figure 1.6). It has been demonstrated that hsc70 plays an important role in increasing the up-take of specific proteins into the lysozomes and further stimulates the degradation of these proteins (Chiang and Dice, 1988; Dice and Chiang, 1989; Terlecky *et al*, 1992). In response to serum deprivation, the constitutive heat shock protein hsc70 (identified as prp73) in human fibroblasts is involved in the degradation of a class of cytosolic proteins that contains the peptide sequence motif KFERQ. For example, ribonuclease A (RNase A) is degraded at an increased rate in response to serum withdrawal (the site of degradation is lysozomal). In contrast, lysozyme, ovalbumin, ß-galactosidase, and ubiquitin proteins (that do not contain a KFERQ-motif), do not compete for binding of RNase S-peptide by hsc70. However this peptide sequence KFERQ does not explain all the interactions of hsc70 with other proteins. p53 (Hinds *et al*, 1987), calmodulin (Stevenson and Calderwood, 1990), actin (Margulis and Welsh, 1991) and a preornithine carbamyltransferase-dihydrofolate reductase fusion protein (Baker and Schatz, 1991) interact with hsc70 and yet do not contain KFERQ-like sequences. Flyn *et al* (1989) demonstrated that the abilities of two different peptides to stimulate the ATPase activity of hsc70 (referred to as hsc73) differed by 14-fold. Therefore hsc70 may be able to weakly interact with many polypeptides, but higher affinity binding requires specific peptide characteristics.

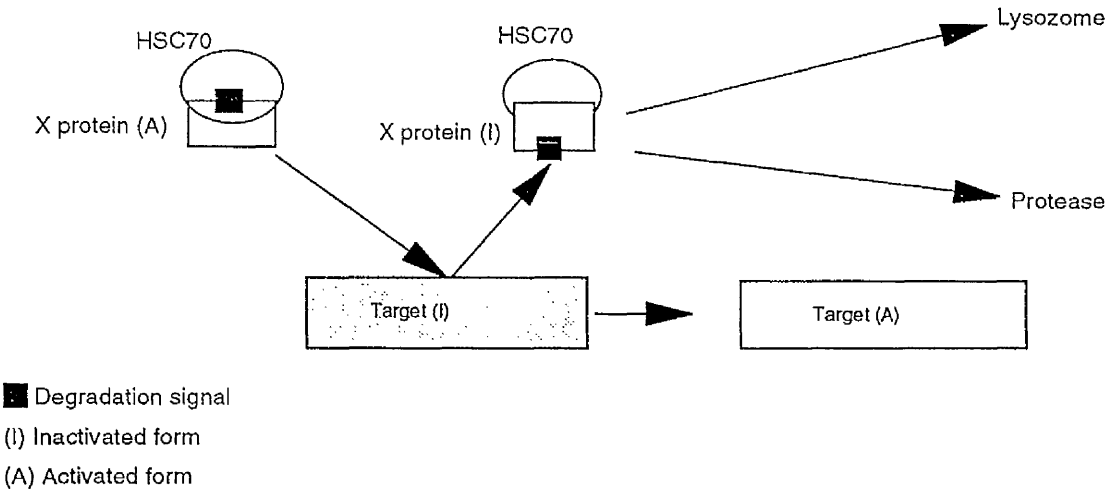


Figure 1.6: The role of hsc70 in degradation of short-lived proteins. A short-lived protein (X protein) which is in an activated form is chaperoned by hsc70. This activated X protein contains a degradation signal (Gottesman and Maurizi, 1992) which is hidden in the complex, X protein-hsc70. Following activation of the target, X protein becomes an inactivated form and changes its conformation. The change of conformation of X protein renders its degradation signal visible. Hsc70 presents X protein and its degradation signal to lysozome or protease to be degraded.

1.2.4: CELL CYCLE - CELL GROWTH AND EXPRESSION OF HEAT SHOCK PROTEINS

It has been reported that the cell cycle regulates the level of expression of hsp70 gene. The subcellular localization of hsp70 protein also varies throughout the cell cycle. In synchronized populations of human cells, hsp70 mRNA levels increase at the G_1/S boundary of the cell cycle in unstressed cells, and then decline rapidly during mid-to late S phase. The rapid increase in hsp70 mRNA in S phase cells corresponds temporally to an increase in hsp70 protein synthesis that is coincident with the localization of hsp70 to the nucleus of S phase cells (Milarski and Morimoto ,1986).

Proliferation of T lymphocytes requires at least two sequential extracellular signals, first an activation signal by antigen which can be mimicked *in situ* by nonspecific polyclonal activators such as phytohemagglutinin (PHA), and secondly, stimulation by IL-2, which allows the activated cells to proceed through the cell cycle. Hsp90 protein synthesis is rapidly and transiently increased in response to PHA, while hsp70 protein synthesis does not rise until later and appears to be part of a generalized increase in protein biosynthesis; hsp70 steady state mRNA was not increased by PHA. After IL-2 stimulation of T cells that had been deprived of IL-2, hsp70 steady state mRNA and protein synthesis is specifically increased (Farrar *et al*, 1988).

These variations in hsp synthesis during the cell cycle may be explained by the interactions that hsp70 has with other proteins expressed in a cell cycle-dependent manner. Monoclonal antibodies to hsp70 do not detect nucleolar-localized hsp70 in heat-shock G2 HeLa cells; this is due neither to the inability of G2 cells to respond to heat shock (as measured by increased hsp70 mRNA and protein synthesis), nor to a lack of accumulation of HSP70 after heat shock in G2 of the cell cycle. Rather that the epitopes recognized by the various antibodies appear to be inaccessible, perhaps due to the association of hsp70 with other proteins (Milarski *et al*, 1989).

1.2.5: ROLE OF HEAT SHOCK PROTEINS IN EMBRYOGENESIS & DIFFERENTIATION

1.2.5.1: Embryogenesis

The first evidence showing the possible link between hsps and development was established by Bensaude *et al* (1983) (Table 1.6). They identified at the late two-cell stage of mouse development, an increase of protein synthesis of hsp70/hsc70, and hsp68. Bensaude and Morange (1983) described an increase in the synthesis of hsp59, hsp70 and hsp89 by day 8 of the mouse embryo development. Moreover Barnier *et al* (1987) also identified in unstressed embryos, an increase in expression of both inducible hsp89ß and constitutive, hsp89α at 8 cell and blastocyst stages.

During early embryogenesis, in *Drosophila melanogaster*, expression of the small hsp28 can be observed in large neuroblast-like cells within the nerve cord after six hours of development. The adult nervous system as well as the ovaries are the tissues that reveals the highest levels of small hsp 28 and hsp83 expression. It is interesting to note that mRNA for hsp28 reaches a plateau by stage 10, while mRNA for hsp83 is present at constant level at all stages of development (Zimmerman *et al*, 1983; Haass *et al*, 1990).

The expression of hsp70 mRNA and proteins in primitive erythroid cells from day 3-8 chicken embryos has been examined and shows a decrease during the late stage of differentiation. The highest levels of hsp70 are detected in polychromatic cells of the day 3-4 primitive erythroid cell population. After an initial burst of hsp70 expression at day 3-4, the level declines. This drop in hsp70 expression is controlled at the transcription level; hsp70 mRNA level decreases following maturation of the red cells. The level of hsp70 synthesis declines in parallel with an overall decrease in total cellular protein synthesis (Banerji *et al*, 1987).

The expression and accumulation of high levels of hsp70 in terminally differentiated erythrocytes is consistent with a suggested role for this hsp complexing with damaged or

Cell	Stage of differentiation	Kinetics of heat shock proteins	Level of analysis	References
Mouse embryos	- 2 and 8 cell stage - blastocyst	constitutive expression hsc70, hsp68, hsp89α, hsp89ß and hsp59	protein	Bensaude et al,1983 Hahnel et al,1986 Barnier et al,1987
Embryos, Drosophila melanogaster	by 6 hours	increases hsp28,hsp83	protein	Haass,1990
Primitive erythroid cells from chicken embryos	From day 0 to adult stage	increases hsc/hsp70 (ND) followed by a decline on the polychromatic stage (days 3-4). In adult reticulocytes, high constitutive expression of hsc70/hsp70 (ND)	protein and RNA	Banerji et al, 1987

Table 1.6: Summary of the kinetics of expression of heat shock proteins during embryogenesis.

ND:not distinguished

denatured proteins (Pelham, 1985). During avian erythroid differentiation, the red cell nucleus condenses, DNA and RNA synthesis is repressed, and many of the intercellular organelles and proteins are degraded as the cell progresses toward its terminal role as a membranous vesicle for haemoglobin (Small and Davis, 1972).

1.2.5.2: Erythrocytic differentiation of haemopoietic cell lines

Using the human leukaemic cell line K562, Singh and Yu (1984) have shown an accumulation of hsp70 or hsc70 (not specified) during erythroid differentiation (Table 1.7). Later, in 1989, Theodorakis *et al* (1989) demonstrated that the haemin-induced differentiation of K562 cells resulted in the expression of the inducible heat shock protein, hsp70 at both protein and RNA levels following 10 hours of induction. The increased levels of hsp70 mRNA gene reflected changes in hsp70 gene transcription rate as measured by the *in vitro* nuclei run-on experiments. It was suggested that the stress response might be provoked by the oxygen-derived free radicals generated by haemin-derived iron within K562 cells. The work of Moore *et al* (1992) does not support this hypothesis. They also reported an increase in hsp70 transcripts following 48 hours of cytosine arabinoside induced differentiation of K562 cells. Following four days of differentiation with this compound, 60% of cells were scored benzidine positive.

Hensold and Housman (1988) differentiated the murine erythroleukaemic cells (MEL) with DMSO along the erythrocytic lineage. They observed a drop of the hsp/hsc70 transcripts (not specified) following 9 and 48 hours of differentiation. They did not report the level of expression of hsp/hsc70 transcript in mature erythrocytes.

The constitutive expression of hscx70 and hsc70 (hscx70 is induced constitutively and after heat shock) has also been identified in mature human erythrocytes (Gromov and Celis, 1991). The presence of 70kDa hsp in erythrocytic cells suggest that they may play a role during differentiation or during the erythrocytic life span (see section 3.7).

Cell	Inducer of differentiation	Kinetics of heat shock proteins	Level of analysis	References
K562, human erythroleukaemic	Haemin	increases of hsp70 after 10 hours, followed by a decrease	protein and RNA	Singh and Yu, 1984; Theodorakis et al,1989
MEL,mouse erythroleukaemic	DMSO	temporally decrease of hsc70/hsp70 (ND) by 9 and 48 hours.	protein and RNA	Hensold and Housman, 1988
Mature human erythrocytes (blood)	N/A	high constitutive expression of hsc70 and hscx70	protein	Gromov and Celis,1991

Table 1.7: Summary of the kinetics of expression of heat shock proteins during differentiation of haemopoietic cells along the erythrocytic lineage. ND:not distinguished

1.2.5.3: Granulocyte/Macrophage differentiation of leukaemic cell lines

These available data contrast with that of erythrocytic differentiation, showing that expression of hsps may be lineage dependent. Differentiation with N-methylformamide of the human myeloid leukaemic cell line, HL-60 along the granulocytic pathway showed a decrease in a 70 kDa protein expression following 12 hours of differentiation (Richards *et al*, 1988) (Table 1.8). Later it was established by Beer *et al* (1993a) that it was the constitutive expression of hsc70 at both protein and RNA levels which decreased during the granulocytic differentiation of HL-60 cells. Analysis of the cell cycle showed that the drop of hsc70 did not reflect accumulating numbers of cells moving out the cell cycle and to growth arrest.

Following 24 hours of differentiation of HL-60 cells along the macrophage lineage with phorbol myristic acid, an increase of the expression and phosphorylation of hsp28 has been reported. The transcripts of hsp28 increased of eight fold following 24 hours of differentiation. Cell cycle analysis showed that by day one and three 69% and 72% of cells are in G_1 cell cycle arrest respectively (Spector *et al*, 1993). These data do not suggest that the increase in the level of hsp28 expression trigger differentiation. As referred to in an earlier section (1.2.1.4.6) the level of expression of the low molecular weight proteins, such as hsp28, increased when cells stop proliferating. Therefore the increase in the level of expression of hsp28, reported during differentiation of HL-60, is probably a consequence of the arrest of the cells in G_1 of the cell cycle.

During the phorbol myristate acetate induced differentiation of the human monoblastic cell line, U937, to a macrophage-like phenotype, the levels of the inducible heat shock proteins hsp90, hsp72 and hsp65 increased to a peak level following 24 hours of treatment, and then declined. This increase was also observed at the RNA level. In contrast, no significant increase was detected for the constitutive hsp73 (equivalent to hsc70) protein (Twomey *et al*, 1993). The increase in the expression of these inducible

Cell	Inducer and lineage of differentiation	Kinetics of heat shock proteins	Level of expression	References
HL-60, promyelocytic cell	granulocytic (N-methylformamide)	decreases hsc70 following 12 hours	protein and RNA	Richards et al,1988; Beer et al,1993a
HL-60	granulocytic (phorbol myristic acid)	increases hsp28 following 24 hours	protein and RNA	Spector et al,1993
U937 human monoblastic cell	macrophage (phorbol myristate acetate)	increases hsp90,hsp72, hsp65 within 24 hours no change hsc73	protein and RNA	Twomey et al,1993

Table 1.8: Summary of the kinetics of expression of heat shock proteins during differentiation of haemopoietic cell lines along the granulocytic and macrophage lineages

hsps may suggest that phorbol myristate acetate has a toxic effect on U937 cells by inducing a stress response.

1.2.5.4: Differentiation of non haemopoietic cell lines along more mature phenotypes

Differentiation of various cell lines along a more mature phenotypes demonstrates that the level of hsps expression is lineage dependent. Bensaude and Morange (1983) reported the retinoic acid-induced differentiation of the F9 cells into parietal endoderm cell type, and the PCC7-S-1009 cells into neuronal cells (Table 1.9). In both pathways, they observed a decrease of a 89 kDa hsp. Giebel *et al* (1988) differentiated F9 cells with retinoic acid and observed a fall of hsc70 at both RNA and protein levels. Nuclear-run on suggested that the decrease of expression of hsc70 protein was due to a decrease of the transcription rate of hsc70.

In contrast, an increase in the expression of hsp47, a major collagen-binding stress protein residing in the endoplasmic reticulum, has been observed during differentiation of F9 cells with retinoic acid (Takechi *et al*, 1992). These authors suggested that this increase in hsp47 expression is closely related to the increase of collagen synthesis during F9 cell differentiation. The expression of the small hsp25 has been reported to increase constitutively during the first three days of the differentiation of F9 cells with retinoic acid and then to decrease by day 7 (Stahl *et al*, 1992). This data show that hsc70, hsp47 and hsp25 may play different roles during F9 differentiation. An increase in the constitutive hsp25 expression following 7 days of P19 embryonal carcinoma cell differentiation with retinoic acid has been also reported by Stahl *et al* (1992).

The human neuroblastoma (GOTO) cells were differentiated by dibutyryl cyclic AMP and retinoic acid to yield a network of neurites after 3-4 days (Murakami *et al*, 1991). These workers observed a down regulation of hsp70 transcripts by day three when the growth of these cells decrease sharply. The expression of the oncogene N-myc, a

marker of growing cells, decreases dramatically from day 0 to day 3. They did not link the fall of the expression of hsp70 to the differentiation of GOTO cells.

1.2.5.5: Conclusion

The kinetics of the expression of hsps during differentiation may vary with the cell line, the inducing reagents and the lineage of differentiation. During differentiation of some leukaemic cell lines such as K562, it has been shown that the expression of the inducible hsps increased (Table 1.7). The overexpression of hsps during chemically-induced differentiation of some leukaemic cell lines may be a consequence of the toxic effect of the drugs used. However into more mature phenotypes: This may partially explain the triggering effect of anti-cancer drugs in the differentiation of leukaemic cells.

Cell	Lineage and inducer of differentiation	Kinetics of heat shock proteins	References
F9 embryonal carcinoma cell	parietal endoderm (retinoic acid)	-decreases hsp89 (1) (a); hsc70 (2); -Increases hsp47 (3) (c); -increases hsp25 (4) (3 days) followed by a decrease (7 days) (a)	(1)Bensaude and Morange,1983; (2)Giebel et al, 1988; (3)Takechi et al,1992 (4)Stahl et al,1992
(a) P19 embryonal carcinoma cell	(retinoic acid)	increases hsp25 within 24 hours	Stahl et al, 1992
(b) GOTO neuroblastoma cell	dendrites (dibutyryl cyclic AMP)	decreases hsp70 following 3 days	Murakami et al, 1991

Table 1.9: Kinetics of expression of heat shock proteins during differentiation of non haemopoietic cell lines.
(a) Analysis at the protein level; (b) Analysis at the RNA level; (c) Analysis at both protein and RNA levels

2.1: SOURCES

Purchased from Sigma Chemical Company Ltd., Poole, Dorset: Ammonium persulphate, bovine serum albumin (powder), bromophenol blue, carbenicillin, coomassie brilliant blue R250, deoxycholic acid, diethyl pyrocarbonate, dimethyl sulfoxide, formaldehyde, glycerol, MOPS, Nonidet P-40, N,N,N',N'-tetramethylethylenediamine, phenylmethylsulphonyl-fluoride, silver nitrate, sodium azide, sodium deoxycholate, sodium nitrate, streptomycin sulphate.

Purchased from BDH Chemicals Ltd., Poole Dorset: Glacial acetic acid, acetone, EDTA, ethanol magnesium chloride, ß-mercaptoethanol, Giemsa stain, glycine, lithium chloride, May-Grunwald, methanol, sodium carbonate, sodium chloride, trichloroacetic acid, Triton X-100, Tween 20.

Purchased from Becton Dickinson Labware, New Jersey, U.S.A.:Conical tubes:50, 15 and 6ml; universal tubes:30ml; culture flasks:12.5, 25, 75 and 175 cm^2; bottle top filters: 22 and 45 micron pore size membrane filters.

Purchased from Gibco Ltd, Cowley Mill, Uxbridge, Middlesex, U.K.: Iscove Mofified Dulbecco Medium (17.67g), Fisher's Medium (10X), horse serum, methionine free medium (MEM, with earle's salts, without L-methionine and L-glutamine.

Purchased from Amersham, Buckinghamshire, U.K.:ECL kit, Hybond-C-extra, Hybond-N, [α^{32}P]-dCTP and protein rainbow marker.

Other purchases

Acrylamide Mix (Protogel), National Diagnostics, Atlanta, Georgia, U.S.A.

Agarose, Flowgen Instruments Ltd, Sittingbourne, Kent, U.K.

Antibodies: Rabit antiserum to mouse haemoglobin, Organon Teknika Corporation, Durham, U.S.A; rabit antiserum to human lysozyme, Dako, Glostrup, Denmark; mouse monoclonal to hsc70 and hsp70 (3A3) and to hsp70 (4G4), Affinity BioReagents, distributed by Cambridge Research Biochemicals, Cheshire, U.K.; mouse monoclonal to actin, Amersham International plc, Buckinghamshire, U.K.; FITC-conjugated rabbit to mouse immunoglobulins, Dako, Glostrup, Denmark.

Bacto-Agar, Difco Laboratories, Detroit, Michigan, U.S.A.

Bacto yeast extract, Difco Laboratories, Detroit, Michigan, U.S.A.

Benzyl penicillin sodium BP (300mg/ml), Britannia Pharmaceuticals Ltd, Redhill Surrey, U.K. BSA (stock solution:100mg/ml), Boehringer Mannheim, Germany.

Erythropoietin (recombinant), Cilag, High Wycombe, U.K.

Fetal calf serum, Sera Laboratories, Crawley Down, Sussex, U.K.

Glass fiber filters (2.5cm), Whatman International Ltd, Maidstone, U.K.

Glutamine, Flow Laboratories S.A., Woodcock Hill, Rickmansworth, Herts, U.K.

Haemin, Calbiochem, La Jolla, U.S.A.

Kodak X-OMAT film, Eastman Kodak, Rochester, NY, U.S.A.

[^{35}S]-methionine, NEN, Du Pont Ltd, Stevenage, Hertfordshire, U.K.

Phosphate buffered saline, OXOID, Unipath Ltd, Basingstoke, Hampshire, U.K.

Random primed DNA labeling kit, Mannheim Boehringer, Germany

RNAzol B, Biotecx Laboratories Inc, Houston, Texas, U.S.A., (supplier, Biogenesis Ltd., Bournemouth, U.K).

Sephadex G50 spin columns, Pharmacia Biotech, Herts, U.K.

Syringe (1ml), Terumo Europe N.V., Leuven, Belgium

Sodium bicarbonate, Northumbria Biologicals, U.K.

Vial A/S Nunc (1.8ml), Kamstrup, Roskilde, Denmark

2.2: MEDIA

2.2.1: Fisher medium

This medium was prepared by mixing 870ml of double distilled water, 100ml Fisher's medium (stock solution:10X), 17.6ml of a 7.5% (w/v) solution sodium bicarbonate, 1ml of benzyl penicillin (stock solution:300mg/ml) and 0.5ml of streptomycin sulphate (stock solution:5mg/ml) and stored at 4°C.

2.2.2: Iscove's Modified Dulbecco's Medium (IMDM)

The IMDM powder (17.67g) was dissolved in 870ml of double distilled water. The solution was sonicated for 5 minutes to completely dissolve the powder. This solution was supplemented with 40ml sodium bicarbonate (stock solution:7.5% w/v), 1ml of benzyl penicillin and 0.5ml of streptomycin. The osmolarity was determined using a cryoscopic osmometer and adjusted by adding H_20 to reach a final osmolarity between 320 and 325 mOsm/kg. This culture medium was filtered with a 0.22 μm pore size membrane filter and stored at 4°C.

2.2.3: L-Broth

L-Broth was prepared with 950 ml of deionized water, 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl. The pH was adjusted to 7.0 with NaOH (stock solution:5N). The solution was sterilized by autoclaving for 20 minutes at 15 lb/sq.in.on liquid cycle. The medium was supplemented with antibiotics after cooling the media to 60°C.

2.2.4: Agar

Cold sterile water (2l) and agar (66g) were mixed, heated at 100°C for 1 hour, aliquoted and stored at room temperature.

2.3: GROWTH FACTORS

2.3.1: Mouse IL-3 (MIL-3)

MIL-3 was obtained from a mouse cell line NIH 3T3 fibroblasts which constitutively secretes large quantities of IL-3 using modified cDNA expression vector (Karasuyama and Melchers, 1988; Heyworth and Spooncer, 1993). MIL-3 was used as conditioned medium to culture FDCP-mix cells (2% (v/v)).

2.3.2: Recombinant IL-3 and EPO

Recombinant IL-3 was provided by Biogen at a concentration of 0.13 mg/ml ($2.1x10^7$U/ml). Recombinant EPO was provided by Cilag ($3x10^7$U/ml).

2.3.3: Lung conditioned medium

Lung conditioned medium (LCM) was used as source of GM-CSF and G-CSF. For the preparations, an intravenous injection of 0.2ml of lipopolysaccharide (LPS) in PBS (stock solution LPS:1μg/0.2ml) was given to each mouse (1 mouse yields 5ml of LCM). Three hours after injection of LPS, mice were killed by cervical dislocation and the lungs were removed and incubated for two days at 37°C with 5% CO_2 in a McCartney bottle containing 25ml Fisher's medium (one pair of lungs for 5ml of medium). The solution was sterilised by filtration through 0.45μm and then 0.22 μm pore size membrane filters. The activity of the lung conditioned medium was evaluated by performing the GM-CFC assay (Burgess and Metcalf, 1980; Heyworth and Spooncer, 1993).

2.3.4: L929-cell conditioned medium

The L929 mouse cell line constitutively produces M-CSF (or CSF-1) and secretes this growth factor into the culture medium (Stanley and Heard, 1977; Heyworth and Spooncer, 1993). Following 1 week of culture (see section "growth conditions for L929 cells"), the medium was harvested and centrifuged at room temperature at 800g for 7 minutes. The culture medium was sterilised through 0.45μm and then 0.22μm pore size membrane filters.

2.4: CULTURE CONDITIONS

2.4.1: Growth conditions of culture medium for FDCP-Mix (clones A4, 1B and 2J) cells

The cells were suspended in IMDM supplemented with 20% (v/v) horse serum (HS), 2% (v/v) M-IL3 and subcultured twice a week at a concentration of $4x10^4$, 10^5 or $1.5x10^5$ cells per ml. The flask was gassed with 5% CO_2 and incubated at 37°C.

2.4.2: Growth conditions for the mouse eythroleukaemic MEL cells

The cells were suspended in IMDM supplemented with 15% (v/v) fetal calf serum (FCS) for MEL cells or 20% (v/v) HS for HL-60 and K562 cells and subcultured twice a week as described in the above section.

2.4.3: Growth conditions for L929 cells

The cells were suspended in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS. The culture flask was gassed with 5% CO_2 and incubated for 1 week at 37°C.

2.4.4: Granulocytic differentiation of FDCP-mix (clones A4 and 1B) cells

The cells were harvested by centrifuging at 800g (MSE) for 7 minutes, washed

twice with 50ml IMDM and resuspended in IMDM supplemented with 20% (v/v) fetal calf serum, 1.5 units per ml of recombinant IL-3 and 10% (v/v) lung conditioned medium. The cells were seeded at a concentration varying between $4x10^4$ and $3x10^5$ cells per ml. The flask was gassed with 5% CO_2 and incubated for 8 (clone 1B) and 9 (clone A4) days at 37°C.

2.4.5: Erythrocytic differentiation of FDCP-mix (clone A4) cells

The culture conditions were the same as section 2.4.5 but the medium was supplemented with 3 units/ml erythropoietin (EPO) and 0.2mM haemin (instead of lung conditioned medium).

2.4.6: Macrophage differentiation of FDCP-mix (clone A4) cells

The cells were harvested, centrifuged at 800g for 7 minutes, washed twice with 50ml IMDM, and resuspended at a concentration of $5x10^5$ cells/ml in IMDM supplemented with 20% (v/v) FCS, 15% (v/v) of conditioned medium from L-cells, 5U/ml of recombinant IL-3. The flask was gassed with 5% CO_2 and incubated for 8 days at 37°C.

2.4.7: Erythrocytic differentiation of the mouse eythroleukaemic MEL cells

The cells were seeded at a concentration varying between $4x10^4$ and $3x10^5$ in growth culture medium (section D2) supplemented with 1.5% (v/v) DMSO. The flask was gassed with 5% CO_2 and incubated for three days at 37°C. By day three, an equal volume of fresh medium supplemented with 1.5% (v/v) DMSO was added to the same flask. It was incubated under the same conditions for a further 4 days.

2.4.8: Colony-forming cell-mix [Mix-CFC] assay

IMDM was supplemented with 20% (v/v) fetal calf serum, 10% (v/v) BSA, 10%

(v/v) agar, 10% (v/v) of cell suspension (500-5000 cells) to give a final volume of 3.3ml. In to each 35mm petri dishes, 1ml was decanted quickly. Petri dishes were placed in a incubator with 5% CO_2 and 5% O_2 and incubated at 37°C for a period of time varying between 7 and 12 days. A colony is defined as a minimum of 50 cells (Heyworth and Spooncer, 1993).

2.5: STORAGE OF CELLS IN LIQUID NITROGEN

2.5.1: Cell freezing

The cells were harvested by centrifugation in a conical tube for 7 minutes at 800g, at 4°C. The pellet was resuspended in cold freezing mixture composing 90% (v/v) horse serum and 10% (v/v) DMSO. This mixed solution (1ml solution:10^6 cells) was aliquoted in each vial (1.8ml), stored for 45 minutes in the position up (not immersed) and 15 minutes in the position down (immersed) in a tank with liquid nitrogen.

2.5.2: Cell thawing

The vial of frozen cells was thawed rapidly in a water bath at 37°C, the cells were then transferred to a sterile tube on ice and 5ml of complete medium were added slowly with gentle shaking. The suspension was left at room temperature for 10 minutes, followed by centrifugation at 800g for 10 minutes. The pellet was resuspended in growth medium and transferred to a culture flask. The flask was gassed with 5% CO_2 and incubated at 37°C for 5 days.

2.6: ASSESSMENT OF CELL DIFFERENTIATION

2.6.1: Cytocentrifugation

The cells ($5x10^4$) were resuspended in 200µl of IMDM, added to the plastic carrier and centrifuged against a slide at 1000rpm for 5 minutes in a cytospin centrifuge

(Shandon).

2.6.2: Cell staining with May-Grunwald-Giemsa

The slides (section 2.6.1) were stained for 2 minutes with May-Grunwald staining solutions, rinsed with water, stained for a further 15 minutes with Giemsa stain solution (1:10 dilution with distilled water), rinsed with water and dried at room temperature.

2.6.3: Erythroid cell staining with O-Dianidisine

The slides were fixed in methanol for 10 minutes to suppress granulocyte peroxidase activity. They were kept in the dark for 10 minutes in a solution containing 35ml of O-Dianidisine [stock solution:0.2% (w/v) diluted in methanol], 7ml of 3% (v/v) hydrogen peroxide and 7ml of of sodium nitroferricyanide [stock solution:1% (w/v) diluted in double distilled water]. The slides were rinsed with water and counterstained with May-Grunwald and Giemsa staining solutions as described at the previous section (2.6.2).

2.7: PROTEIN PREPARATION AND ANALYSIS

2.7.1: [^{35}S]-methionine *in vitro* incorporation (FDCP-Mix and MEL cells)

Cells (10^6) were centrifuged at 800g for 5 minutes and washed twice with 1ml methionine free medium (MFM) in a conical tube. The pellet was resuspended in final volume of 200ul MFM per 10^6 cells, supplemented with 2% (v/v) MIL-3 (exclusively for FDCP-mix cells), 20% (v/v) of dialysed horse serum or fetal calf serum [+10% (v/v) LCM, when FDCP-mix cells were differentiated along the granulocyte lineage] and 50uCi of [^{35}S]-methionine (specific activity:1176.4 Ci/mmol). The medium was gassed with 5% CO_2 and the tube was incubated for 1 hour at 37°C in a water bath. Total protein was extracted as described below (section 2.7.3) and the level of protein incorporation of [^{35}S]-methionine was determined as followed: 5μl of protein extract was transferred in a microcentrifuge tube

with 100µl of PBS containing 1mg/ml of BSA mixing with 1ml of 16% (w/v) TCA. Protein incorporation was assessed in triplicate as described below (section 2.7.2).

2.7.2: TCA precipitation-filtration

TCA precipitates were put on ice for 30 minutes. Glass fiber filters were washed twice with 2ml of 16% (v/v) TCA before TCA precipitates were filtered through the filter. Following filtration, the glass fiber filter was washed twice with 2ml of 95% (v/v) ethanol. The filters were allowed to dry and the level of radioactive incorporation was assessed by scintillation countering (Beckman, LS5000CE).

2.7.3: Extraction of proteins in SDS polyacrylamide electrophoresis buffer

Cells were washed three times in ice-cold PBS in a microcentrifuge tube and centrifuged at 12,000g for a few seconds. The pellet (10^6 cells) was resuspended in 30µl ice cold PBS, 30µl SDS polyacrylamide gel electrophoresis buffer [stock solution: 100mM Tris HCl, 200mM dithiothreitol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue and 20% (v/v) glycerol] and heated at 100°C for 5 minutes (Laemmli, 1970). The mixture was centrifuged at 12,000g for 10 minutes, the supernatant was recovered, transferred to another microcentrifuge tube and frozen at - 20°C.

2.7.4: SDS-polyacrylamide gel electrophoresis

The resolving SDS polyacrylamide was composed of 7.5% (v/v) (to resolve proteins with a Mw of 40kDa and above) or 12.5% (v/v) (to separate proteins with a Mw below 40kD) acrylamide mix (Protogel), 0.375M Tris (pH: 8.8), 0.1% (w/v) SDS and the stacking gel was composed of 5% (v/v) acrylamide mix, 0.25M Tris HCl (pH: 6.8) and 0.1% (w/v) SDS. Polymerisation was performed using 200µl of ammonium persulfate (stock solution:10% w/v) and 40 ul of TEMED in 40ml of acrylamide mixture. Before the

electrophoresis, aggregation of proteins was prevented by heating samples for 2 minutes at 100°C. The same radioactive count (for autoradiography) or the protein extract from the same number of cells (between 10^5 and $2x10^5$ cells, for Western blot analysis) was loaded and the proteins resolved by electrophoresis at 220 volts for 3 hours in the Tris-glycine electrophoresis running buffer [25mM Tris, 250mM glycine:pH 8.3 and 0.1% (w/v) SDS] (Laemmli, 1970).

2.7.5: Staining of SDS Polyacrylamide gel with Coomassie blue R250

Coomassie Brilliant Blue R250 is a triphenylmethane textile dye which is also known as Acid Blue 83. The stock solution was prepared by dissolving 0.25 g of Coomassie Blue R250 in 90ml of methanol:distilled water 1:1 (v/v) and 10ml of glacial acetic. The gel was immersed in at least 5 volumes of staining solution and rocked on a slowly rotating platform for 2 hours at room temperature. Gels were destained by soaking it in 20% (v/v) methanol/10% (v/v) glacial acetic acid for 5 hours. The destaining solution was changed four times.

2.7.6: Staining of SDS polyacrylamide gel with silver nitrate

Silver staining has a sensitivity of between 1 and 10ng of proteins per band. Following electrophoresis, the SDS polyacrylamide gel was incubated at room temperature on a slowing rotating platform for two hours in 5 gel volumes of 20% (v/v) methanol and 10% (v/v) acetic acid in order to fix the proteins. The solution was discarded and the gel was incubated in five gel volumes of 30% (v/v) ethanol for 30 minutes. This step was repeated twice. The ethanol was discarded and the gel was rehydrated in distilled water for 10 minutes. This was repeated four times. The distilled water was removed and the gel stained for 30 minutes at room temperature with five gel volumes of 0.3% (w/v) of silver nitrate diluted in distilled water. The solution was discarded, the gel was washed

twice with distilled water and incubated with 5 gel volumes of an aqueous solution of 2.5% (w/v) sodium carbonate and 0.02% (v/v) formaldehyde at room temperature. Stained bands of protein appeared within 5 to 10 minutes. The reaction was quenched in 1% (v/v) acetic acid (2 minutes).

2.7.7: Western blot analysis

After separating the total protein extract on SDS polyacrylamide gel electrophoresis (section 2.7.4), the gel was incubated in transfer buffer [48mM Tris base, 39mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol] for 20 minutes and placed on Hybond-C-extra filter in between three sheets of Whatman 3MM filter paper. Proteins were transferred to Hybond-C-extra using transblotter apparatus (Biometra, 130mA for 60 minutes). The efficiency of protein transfer was checked with two controls. First coloured markers (Rainbow marker:myosin, 200kDa; phosphorylase, 97.4kDa; BSA, 69kDa; ovalbumin, 46kDa; carbonic anhydrase, 30kDa; trypsin inhibitor, 21.5kDa; lysozyme, 14.3kDa). Second, the filter was stained with Ponceau S (section 2.7.8).

The filter was blocked for 1 hour at room temperature in PBS containing 5mg/ml BSA. It was followed by three washings of 10 minutes at room temperature with 0.1% (v/v) Tween-20 in PBS.

The Hybond-C-extra filter was incubated in a sealed bag with PBS supplemented with 5mg/ml BSA and the primary antibody (section 2.7.9) for 1 hour at room temperature. The filter was washed three times for 20 minutes with 0.1% (v/v) Tween-20 in PBS. Then it was incubated in a sealed bag for 1 hour with PBS, 5mg/ml BSA and the secondary antibody (mouse immunoglobulin, peroxidase conjugated, dilution 1/3000). The filter was washed 6 times for 10 minutes with PBS and 0.1% (v/v) Tween. The detection was performed by cheminoluminescence following the manufacturer's instructions (Amersham, ECL kit).

The Western blot filter was stripped of antibodies as followed: It was washed twice with 0.1% (v/v) Tween in PBS, incubated at 50°C with 2% (w/v) SDS, 100mM ß-mercaptoethanol, 63mM tris-HCl (pH:6.8), and rinsed three times with 0.1% (v/v) Tween in PBS.

2.7.8: Staining of a Western blot filter with Ponceau S

The stock solution is composed of 3-hydroxy-4-(2-sulfo-4-(sulfo-phenylazo)-2,7-naphtalene disulfonic acid in 30% (v/v) trichloroacetic acid and 30% (v/v) sulfosalicylic acid. The blot was incubated for 10 minutes in the dye (diluted 1 in 10 with double distilled water). The filter was rinsed with 5% (v/v) acetic acid for 2 minutes. All proteins were coloured pink/red. The blot was washed twice in PBS for 5 minutes and was then ready for blocking.

2.7.9: Monoclonal and polyclonal antibodies used for Western blotting

Monoclonal to both human hsp70 and hsc70, dilution 1/10,000 (3A3); monoclonal to human hsp70, dilution 1/10,000 (4G4). Both monoclonal antibodies detect hsc70/hsp70 (3A3) or hsp70 (4G4) proteins from different species as human, mouse, chicken, frog and yeast. Monoclonal to mouse actin molecules, dilution 1/10,000; rabbit polyclonal to human lysozyme, dilution 1/1,000; rabbit polyclonal to mouse haemoglobin, dilution 1/1000; mouse to human *bcl-2*, dilution 1/500 (Pezzella *et al*, 1990); FITC-conjugated (peroxidase) rabbit to mouse immunoglobulins, dilution 1/3,000.

2.8: LARGE SCALE PREPARATION OF PLASMID DNA FROM E.coli

In a 30ml universal tube, 5ml of L Broth plus 10mg/ml carbenicillin were added and a loop of bacteria was inoculated. The solution was incubated with shaking (model G24, Environmental incubator shaker) overnight at 37°C. This culture medium was

transferred to a 1 liter flask with 500ml of L Broth plus the selective antibiotic and incubated overnight at 37°C (model G25, Environmental incubator shaker). The cells were lysed by alkali (Maniatis, 1989). The cultured medium was poured in a 500ml bottle and centrifuged for 15 minutes at 2000g (Sorvall GS3), the supernatant was discarded and the pellet was resuspended and lysed in 10ml of solution 1 [stock solution: 50mM glucose, 25mM Tris-Cl (pH 8.0), 10mM EDTA (pH 8.0)] supplemented with 10mg of lysozyme diluted in 1ml 10mM Tris-Cl (pH 8.0). It was mixed with 20ml of freshly prepared solution 2 [stock solution: 0.2 N NaOH and 1% (v/v) SDS] and stored at room temperature for 10 minutes. Following this incubation, 15ml of ice-cold solution 3 [stock solution: 3M KAc, 11.5 % (v/v) glacial acetic acid] was added and transferred to ice for 10 minutes. After centrifugation at 4000g for 15 minutes at 4°C, the supernatant was filtered through four layers of cheesecloth into a 250ml centrifuge bottle, 0.6 volume of isopropanol was added and the bottle was stored at room temperature for 10 minutes. The nucleic acids were recovered by centrifugation at 5000g for 15 minutes at room temperature. The supernatant was discarded, the precipitate was washed with 70% (v/v) ethanol and the pellet was resuspended in 3ml of TE [stock solution:10mM tris.HCl, pH7.6 and 1mM EDTA,].

Plasmid DNA was purified by precipitation with PEG according to the method of Maniatis (1989). The nucleic acid solution was transferred to a 15ml Corex tube, an equal volume of an ice cold solution of LiCl (stock solution:5M) was added and the mixture centrifuged at 10,000g (Sorvall SS34) for 10 minutes at 4°C. The supernatant was transferred to a 30ml Corex tube, an equal volume of isopropanol was added and the nucleic acids were precipitated by centrifugation at 10,000g for 10 minutes at room temperature. The pellet was rinsed with 70% (v/v) ethanol and allowed to air dried at room temperature. The precipitate was dissolved in 500µl of TE (pH 8.0) supplemented with 20µg/ml of DNAase-free pancreatic RNAase, transferred to a microcentrifuge tube and

incubated at 37°C for 30 minutes. It was mixed with 500µl of solution composed [stock solution:1.6M NaCl and 13% (w/v) PEG] and the plasmid DNA were recovered by centrifugation at 12,000g for 5 minutes at 4°C. The supernatant was removed and the precipitate was resuspended in 400µl of TE (pH 8.0). The solution was extracted twice with phenol:chloroform and once with chloroform. The aqueous phase was transferred to a microfuge tube, a final concentration of 0.3M ammonium acetate was mixed with two volumes of ethanol. The solution was kept at room temperature for 10 minutes and the plasmid DNA was recovered by centrifugation at 12,000g for 5 minutes at 4°C. The pellet was rinsed with 70% (v/v) ethanol at 4°C and resuspended in 500µl of TE (pH 8.0).

2.9: DNA PROBES

pHA7.6 is a vector derived from pUC18 in which a 600bp fragment encoding the 5'sequence of human hsc70 has been inserted (C. Hunt, unpublished). A fragment of 2.3kb cDNA from the inducible gene, hsp70 was isolated from the human DNA library and inserted between the restriction sites, HindIII-BamHI of the bacterial vector pAT153 to give pH2.3 (Wu *et al*, 1985). Human *bcl-2* is 598kb PstI-PstI insert of pFL1 which detects both human and mouse *bcl-2* (Cleary and Sklar, 1985); mouse *c-myc*, 0.9kb SstI-XbaI exon 2 fragment of pSV*c-myc*1 (Land *et al*, 1983); mouse ß-globin, pSP64MßI34 (Baron and Maniatis, 1986); mouse α-actin (Minty *et al*, 1981); mouse m-lysozyme (Cross *et al*, 1988).

2.10: RNA PREPARATION AND ANALYSIS

2.10.1: RNA extraction using RNAzol

Cells were harvested by centrifugation in a conical tube at 2000g for 5 minutes at 4°C. The supernatant was removed and the cells were resuspended in RNAzol (2ml/10^7cells) plus 200µl of chloroform per 2ml of RNAzol and transferred to a 15ml Corex tube. The Corex tube was covered tightly, shaken vigorously for 15 seconds and put

on ice for 5 minutes. The suspension was centrifuged at 12,000g for 15 minutes at 4°C. Following the centrifugation the homogenate forms two phases of equal volumes: the lower blue phenol-chloroform phase and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are at the interface and on the organic phase. The upper phase was transferred to another 15ml Corex tube and an equal volume of isopropanol was added. Samples were stored at -70 overnight and centrifuged at 9,000g for 20 minutes at 4°C to precipitate RNA. The supernatant was removed and the pellet was resuspended with 400µl of 0.5% SDS (w/v) dissolved in distilled water treated with diethylpyrocarbonate [DPEC, stock solution:0.1% (w/v)].

2.10.2: Preparation of labelled probes with [α^{32}P]-dCTP

Random-primed probes of isolated cDNA inserts were prepared by boiling 50ng of DNA in 9µl of double distilled water for 2 minutes and then put on ice for 2 minutes to keep as single strand DNA. This solution was supplemented with 3µl of nucleotide cocktail consisting of 0.16mM each of dATP, dGTP and dTTP, 2µl of reaction mix (Random priming kit), 5µl of [α^{32}P]-dCTP (50µCi, 3000Ci/mmol, Amersham) and 1µl of Klenow enzyme (1 unit/µl). The mixture was incubated in a water bath at 37°C for 30 minutes. Following the random priming reaction, the reaction was stopped with 80µl of SDS column buffer [stock solution:10mM Tris-HCl pH 7.5, 1mM EDTA and 7% (w/v) SDS], heated at 100°C for 2 minutes and put on ice for 2 minutes.

All probes were purified by the use of Sephadex G50 spin columns. In order to prepare this column, the plunger of 1ml disposable syringe was removed, a small plug of siliconised autoclaved glass wool was placed at the bottom, the syringe was filled with a 75% of Sephadex G50 and placed in a 15ml Corex tube. Following a centrifugation at 2000g for 2 minutes using a Sorval HB4 rotor the syringe was loaded with 200µl of SDS column buffer and centrifuged as described previously. The cap from a microcentrifuge

tube was removed and dropped into a Corex tube and the dry Sephadex column was replaced and ready for used. After centrifugation, the radiolabelled probe was recovered in a microfuge tube ready for subsequent hybridization.

2.10.3: RNA preparation for electrophoresis on agarose gel

Total RNA (5μg) was precipitated in ethanol in a microcentrifuge tube, the supernatant was discarded and 15μl of denaturing buffer was added [stock solution: 7% (v/v) formaldehyde, 50% (v/v) deionised formamide, 20mmol/l morpholinopropanesulfonic acid (MOPS) (pH 7.0)] plus 2μl of loading buffer [50% (w/v) sucrose and 0.05% (w/v) bromophenol blue]. The solution was incubated at 65°C for 10 minutes and put on ice for 2 minutes.

2.10.4: Electrophoresis on agarose gel and transfer of RNA on Hybond-N

A 1% (w/v) agarose gel was prepared with 7.5% (v/v) formaldehyde and 20mM pH 7.0. The formaldehyde was poured when the agarose cooled to 60°C. Following the loading of samples, the electrophoresis was carried on at a voltage of 10 V/cm^2 of agarose gel until the bromophenol blue reaches the end of the gel. The electrophoresis buffer contained also 7.5% (v/v) formaldehyde and 20mM MOPS. The RNA was transferred directly onto a nylon membrane, Hybond-N (Amersham) by capillary blotting overnight with 20X SSC [stock solution:20x (3M NaCl and 0.33M tris-sodium citrate, pH 7.0)]. The filter membrane was UV cross-linked for 2 minutes and baked for 30 minutes at 85°C (Church and Gilbert, 1984).

2.10.5: Hybridization

The membrane filter was prehybridized for 1 hour at 68°C in a prehybridization buffer [0.1M NaCl, 0.05M Na_2PO_4, 1% (w/v) bovine serum albumin (BSA) and 7% (w/v)

SDS] (Church and Gilbert, 1984). BSA is used to reduce non-specific binding. Hybridization was performed at 68°C overnight in 1ml or 2ml of prehybridization buffer with the labelled probes as described above.

The filter was washed in two steps. First, it was incubated at 68°C in a buffer containing 2x SSC (0.3M NaCl and 0.03M tris-sodium citrate, pH 7.0), 1% w/v SDS for 30 minutes and second in a buffer with 0.2x SSC, 0.1% (w/v) SDS for 1 hour at the same temperature. The filter was sealed in a plastic bag and exposed to kodak film for 1 or 2 hours at -70°C. In order to reprobe, the filter was stripped of probe by boiling buffer [10 mM Tris/HCl, pH 7.5, 1% (w/v) SDS]. Thereafter, it was incubated for 30 minutes at 68°C and left at room temperature for the same time in the same stripped buffer. This step was repeated two or three times.

3.1: HSP70/HSC70 GENE AND PROTEIN EXPRESSION IN FDCP-Mix (clone A4) CELLS DURING IL-3 DEPENDENT SELF-RENEWAL

3.1.1: Introduction

Following heat shock or chemical stress, most eukaryotic and prokaryotic cells increase their level of hsp expression. The most extensively studied heat shock protein belongs to the 70kD family. Only the constitutive (hsc70) and the inducible (hsp70) forms are investigated in this study. The hsp70 gene is not usually expressed in non-stressed cells, whereas the constitutive expression of hsc70 is found in many cell types (Welch, 1987; Lindquist and Craig, 1988). Theodorakis and Morimoto (1987) reported that hsp70 mRNA from the human cell line HeLa, becomes stable only following heat shock, and therefore it is not possible to detect any hsp70 transcripts in non-stressed cells. Likewise in *Drosophila*, it has been shown that the rapid degradation of hsp70 messages at normal temperatures operates through recognition of the hsp70 3' UTR (Petersen and Lindquist, 1989). It is thought that hsps somehow provide a measure of protection against heat shock or chemically-induced death, whereas cells unable to express hsps show less tolerance when challenged by heat or chemical stress (Mattei *et al*, 1988). In many early published experiments, no distinction was made between the constitutive hsc70 and the inducible hsp70 products. Only recently have monoclonal antibodies capable of recognizing these different forms of hsps been available. For this reason, the initial experiments identified which forms of 70kDa hsp at both the RNA and protein levels were present in stressed and normal FDCP-mix (clone A4) cells.

3.1.2: Identification of hsc70 and hsp70 in FDCP-mix (clone A4) cells

In order to distinguish which members of the hsp70 gene family are being expressed in FDCP-mix (clone A4) cells, cells grown in culture medium supplemented with IL-3 were heat shocked at 42°C for 1 hour and then allowed to recover for 2 hours at 37°C under normal culture conditions. FDCP-mix (clone A4) cells were also treated in the same fashion but were not heat shocked at 42°C. MEL cells were treated in exactly the same way. Total proteins were extracted from each of the cell lines under the conditions outlined above. Western blot analysis using monoclonal antibody 4G4 (Figure 3.1) which recognises only the heat-inducible form of hsp70 from different species (section 2.7.9), and antibody 3G3, recognising both the heat-inducible and constitutive forms (hsp70 and hsc70 respectively) from different species (section 2.7.9), showed that in FDCP-Mix (clone A4) cells only hsc70 was expressed in both treated (2-3 fold increase) and non-treated cells (Figure 3.1). Heat shocked FDCP-mix cells (clone A4) also expressed the heat-inducible hsp70 protein which, in the case of the murine line examined here, migrates with an apparently smaller molecular mass than hsc70 (Hahnel *et al*, 1986). The MEL cell line also expressed the constitutive (hsc70) protein in both treated (2-3 fold increase) and non treated cells but, following heat shock, no inducible hsp70 was detected, in agreement with Hensold *et al* (1990) (Figure 3.1) who also showed the absence of hsp70 expression in MEL cells.

Total RNA was isolated from FDCP-Mix (clone A4) and MEL cells which were treated under the same conditions as described above. Northern analysis using specific probes for either hsc70 or hsp70 RNA transcripts is shown in figure 3.2. In FDCP-mix (clone A4) cells, hsc70 gene expression is increased only 2-3 fold following heat shock, whereas the hsp70 gene is expressed at barely detectable levels in non-heat shocked cells, but was highly induced (more than 100 fold) following heat shock (Figure 3.2). Analysis of the total RNA extracted from the MEL cells showed that this cell line constitutively

expressed an hsp70 RNA but Western blot analysis demonstrated that no inducible hsp70 was produced. Perhaps it should be noted that the hsp70 probe appears to hybridize weakly with hsc70 RNA in MEL cells (Figure 3.2).

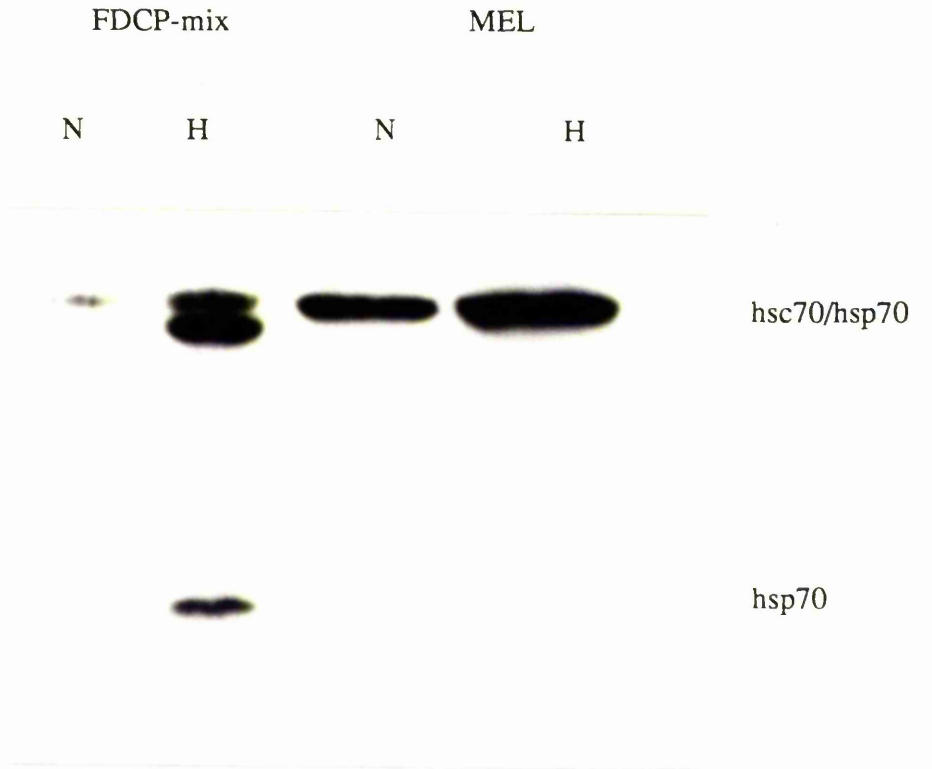


Figure 3.1: Western blot analysis of total proteins isolated from FDCP-mix (clone A4) and MEL cells following 42.5°C heat shock. The cells were treated at 42.5°C for 1 hour and allowed to recover for two hours. Total proteins were resolved by 7.5% PAGE. The Western blot was probed with monoclonal antibody 4G4 (hsp70) (shown at bottom). The same blot was stripped and probed with monoclonal antibody 3A3 (hsc70/hsp70) (shown at top). Lanes N are from normal cells and lanes H from heat-shocked cells. Each lane was loaded with 40µg of total proteins.

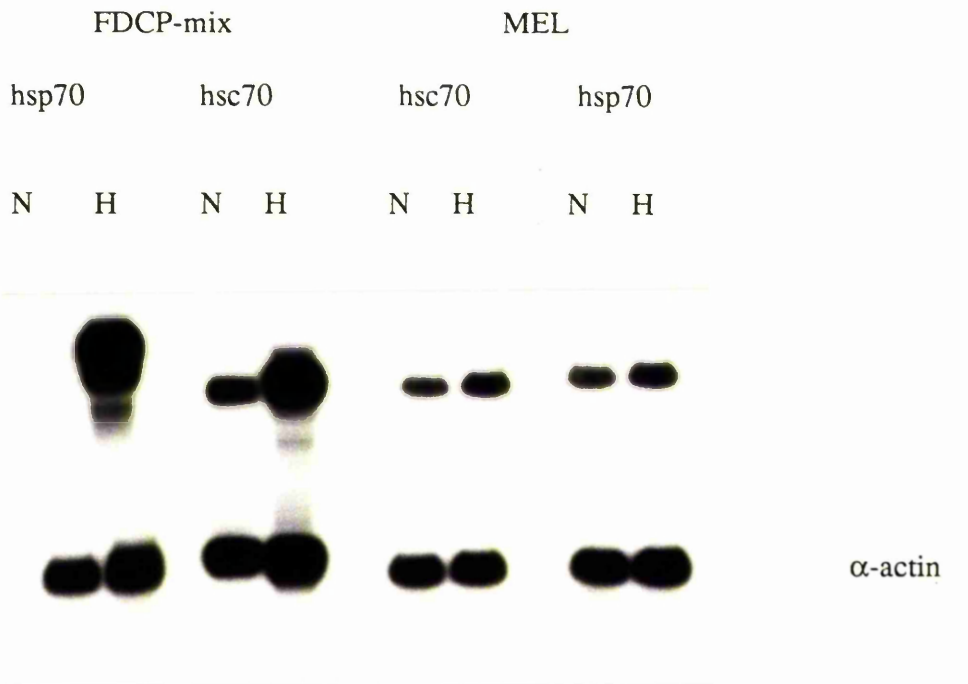


Figure 3.2: Northern blot analysis of total RNA isolated from FDCP-mix (clone A4) and MEL cells following 42°C heat shock. The cells were treated at 42°C for 1 hour and allowed to recover for 2 hours. Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The Northern blot was hybridized consecutively with [^{32}P]-labelled probes for hsp70, hsc70 (shown at top) and α-actin (shown at bottom). Lanes N are from normal FDCP-mix and MEL cells and lanes H from heat shocked cells. Each lane was loaded with 5μg of total RNA.

3.2: EXPRESSION OF HSC70 DURING THE GRANULOCYTIC DIFFERENTIATION OF FDCP-mix (clone A4) CELLS

3.2.1: Introduction

It has been suggested that stress inducible hsps may trigger the differentiation of haemopoietic cells. For example, during the chemically-induced differentiation of the human leukaemic cell line, U937, to macrophages (Twomey *et al*, 1993) and the human erythroleukaemic cell line K562 (Singh and Yu, 1984) to reticulocytes, an increase in the inducible heat shock protein hsp70 expression has been reported. However, during the chemically-induced differentiation of the human promyelocytic cell line, HL-60, to granulocytes induced by N-methylformamide (NMF), no inducible hsp70 was detected (Beere *et al*, 1993a). In addition these workers also showed that the constitutive expression of hsc70 in HL-60 cells decreases following 4 hours (RNA) and 12 hours (protein) of differentiation prior to commitment (36-48 hours). It is therefore not clear whether the increase in the level of expression of hsp70 during differentiation of various haemopoietic cell lines with chemical inducers (see introduction) triggers differentiation but merely arises as a consequence of the toxic effect of chemical inducers.

In this study the kinetics of hsc70 expression were analyzed during granulocytic differentiation in the cell line, FDCP-mix (clone A4). Cells are switched from a 100U/ml of IL-3 (self renewal) to 1U/ml of IL-3 and GM-CSF/G-CSF (in LCM). Although the FDCP-mix cell line model allows the study of heat shock protein expression in the absence of non-physiological chemical agents, it was important to establish whether the change of culture conditions could induce stress in the cells.

3.2.2: Measurement of cell density, primitive cell numbers and morphology of FDCP-mix (clone A4) during the granulocytic differentiation

FDCP-mix (clone A4) cells were differentiated along the granulocytic lineage in the presence of GM-CSF, G-CSF: The presence of a low concentration of IL-3 was required for cell survival. During the first 6 days of granulocytic differentiation, the cell number increased 5 fold (Figure 3.3). As a measure of the maturation and development, the number of clonogenic cells was monitored (Figure 3.3). 75% cells on day 3 were capable of forming colonies whereas by day 6 there was only 20% primitive cells, which disappeared by day 9. Morphological analysis also showed that during differentiation, primitive or blast cells gave rise to the more mature phenotypes, early granulocytes and late granulocytes (Figures 3.4a and 3.4b).

3.2.3: Western blot analysis of hsc70 protein

Total protein was extracted from cells at various times during the granulocytic differentiation of FDCP-mix (clone A4), separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and analyzed by immuno probing of the Western blot. Hsp70 expression was detected on the blot using the monoclonal antibody (4G4) (Figure 3.5). During the time course of FDCP-mix (clone A4) differentiation, no inducible heat shock protein hsp70 was detected. In the absence of a specific monoclonal antibody for hsc70, the same blot was then stripped and probed with monoclonal antibody 3A3 which recognises both hsc70 and hsp70. Between day 0-5, no change of the expression of hsc70 protein was detected, but by day 6 a dramatic decrease was observed (Figure 3.5). These results show that the change of growth conditions required for differentiation did not trigger the expression of the inducible hsp70 gene product and this suggests that the cells did not produce a stress response. The observed decrease in the level of hsc70 suggested that, at least at the translational level, hsc70

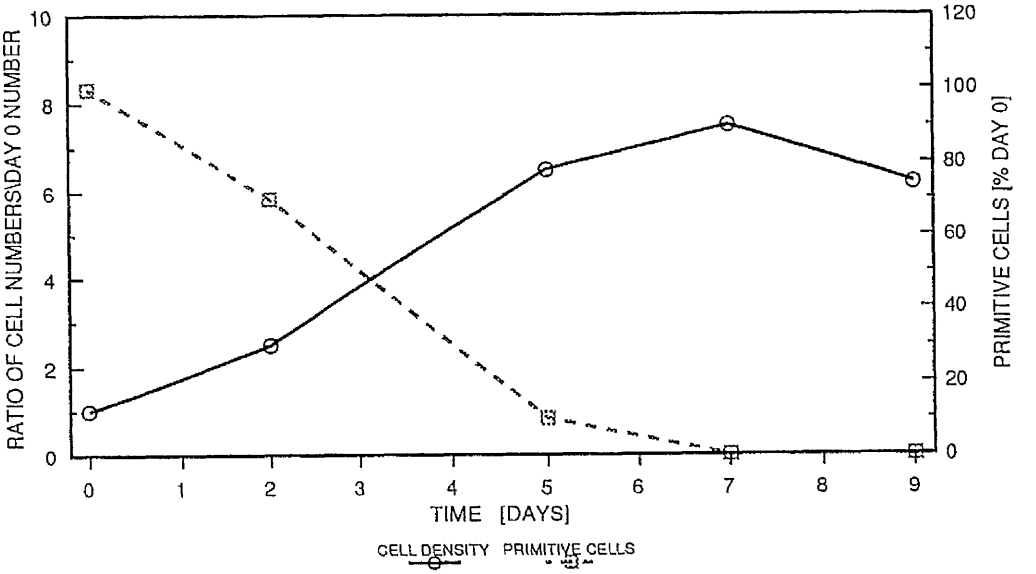


Figure 3.3: Measurement of cell density and primitive cell numbers during the granulocytic differentiation of FDCP-mix (clone A4) cells in the presence of LCM and IL-3 (1.5U/ml). Day 0 value for the cell density was $4x10^4$ cells/ml, and for the clonogenic assays, 200 colonies. Cell density was expressed as fold increase of day 0 value. Numbers of primitive blasts cells were expressed as a percentage of the day 0 number. The cell density was assessed by trypan blue exclusion test. Each point represents the average of three experiments.

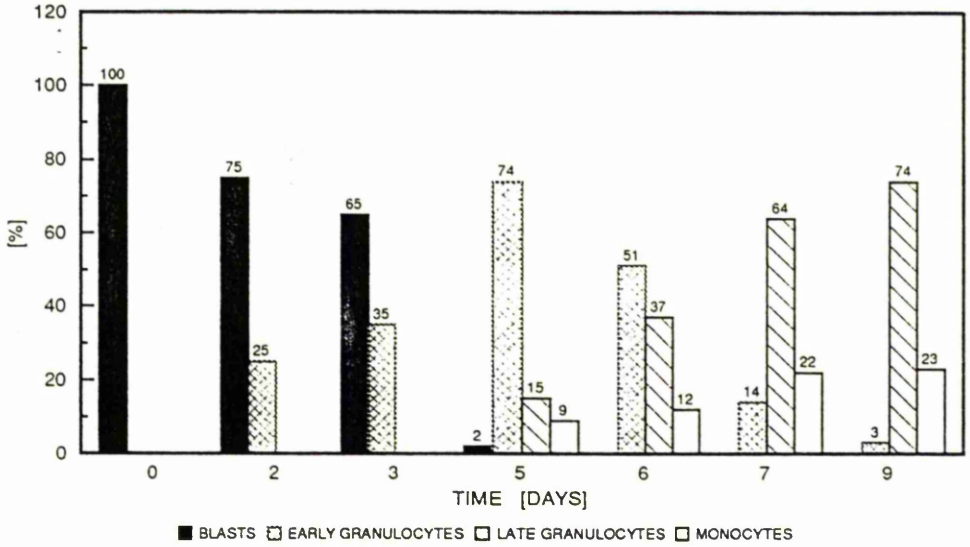
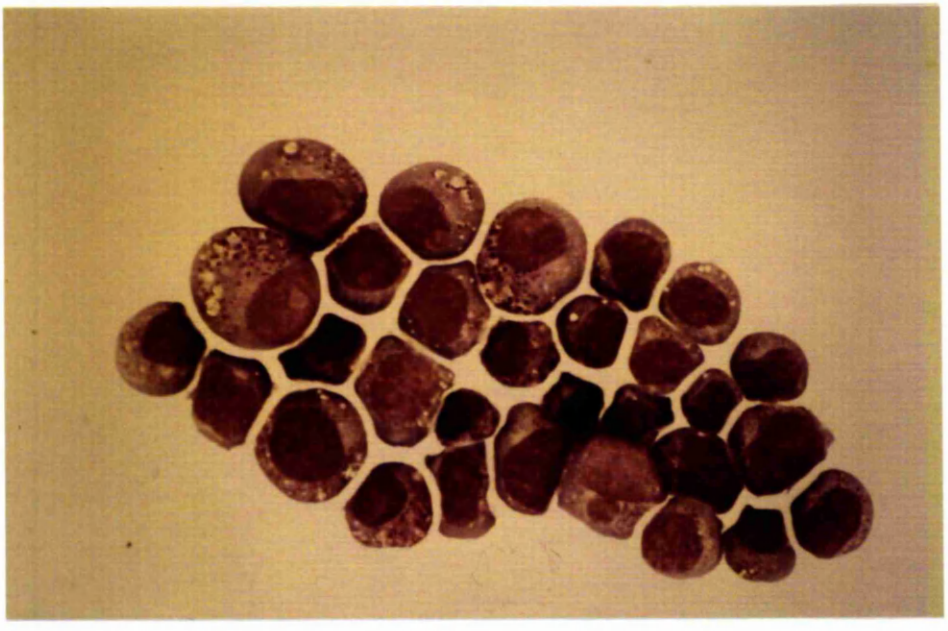


Figure 3.4a: The morphology of FDCP-mix (clone A4) cells during the granulocytic differentiation in the presence of LCM and IL-3 (1.5U/ml). The cells were stained with May-Grunwald and Giemsa stains. Each point represents the average of three experiments.

Day 0 (blasts: Undifferentiated FDCP-mix cells)



Day 9 (Differentiated FDCP-mix cells)

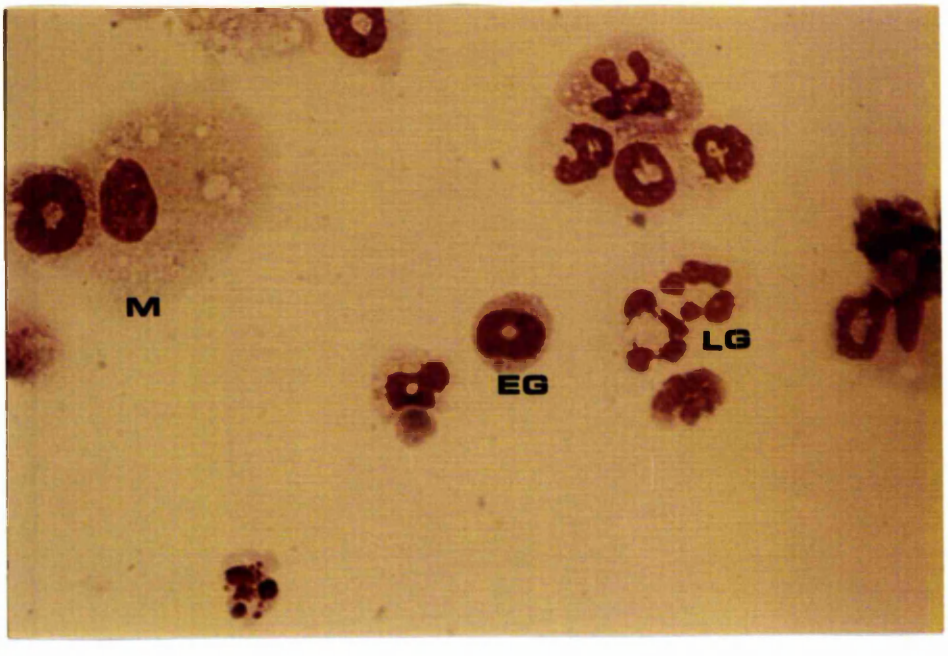


Figure 3.4b: Changes of the nuclear morphology of FDCP-mix (clone A4) cells, during the granulocytic differentiation in the presence of LCM and IL-3 (1.5U/ml). The cells were stained at days 0 and 9 with May-Grunwald and Giemsa stains. B:blasts, EG:early granulocytes, LG:late granulocytes, M:monocytes/macrophages. Magnification (600x).

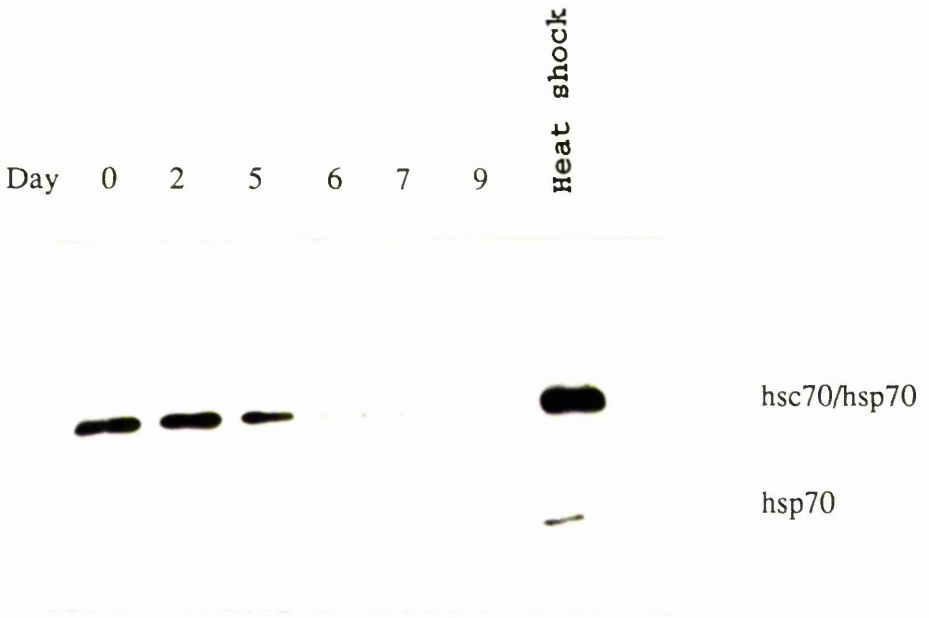


Figure 3.5: Western blot analysis for hsc70 and hsp70 expression during the granulocytic differentiation of FDCP-mix (clone A4) cells in the presence of LCM and low IL-3 (1.5U/ml). Total proteins were resolved by 7.5% PAGE. The Western blot was probed successively with monoclonal antibodies 4G4 (hsp70) (shown at bottom) and 3A3 (hsc70/hsp70) (shown at top). Lane heat shock is the total protein from FDCP-mix cells grown in culture medium, treated for 1 hour at 42°C and allowed to recover for 2 hours. Each lane was loaded with 40µg of total proteins.

expression was down regulated between the early and late granulocyte (Figure 3.4a). To investigate whether this change was significant and occurred as a consequence of reduced cell growth rate further experiments were performed.

3.2.4: Is the down-regulation of hsc70 expression regulated at the transcriptional or translational level?

Northern blot analysis was performed in order to determine if the drop of hsc70 protein observed during granulocytic differentiation was also seen at the transcriptional level. Total RNA was extracted from cells at different times during the differentiation, separated by formaldehyde agarose gel electrophoresis, transferred on Hybond-N nylon membrane and hybridised with [^{32}P]-labelled hsc70 probe. Hsc70 RNA started to decrease gradually after day 3 of the granulocytic differentiation, but still remained detectable up to day 8 (Figure 3.6). These results show that the level of expression of hsc70 is regulated at the transcriptional level during the granulocytic differentiation of FDCP-mix (clone A4) cells. However, in order to measure the hsc70 trancriptional activity, nuclear run-on analysis should be done.

As a marker of cell proliferation, the expression of *c-myc* was shown to decrease dramatically by day 3 and became undetectable by day 8 (Figure 3.6). The m-lysozyme RNA was used as a marker of granulocytic differentiation. It was detectable between day 2 and day 6 and the level of expression increased dramatically (more than 10 fold) between day 7 and 8 (Figure 3.6). Equal loadings of RNA for each time point were confirmed by using a specific probe for α-actin (Figure 3.6).

3.2.5: Does decreasing the IL-3 concentration during the IL-3-dependent growth of FDCP-mix (clone A4) cells affect the expression of hsc70?

The results of the previous experiments (sections 3.2.3 and 3.2.4) suggested that the level of expression of hsc70 might be linked to cell growth, as it decreases dramatically

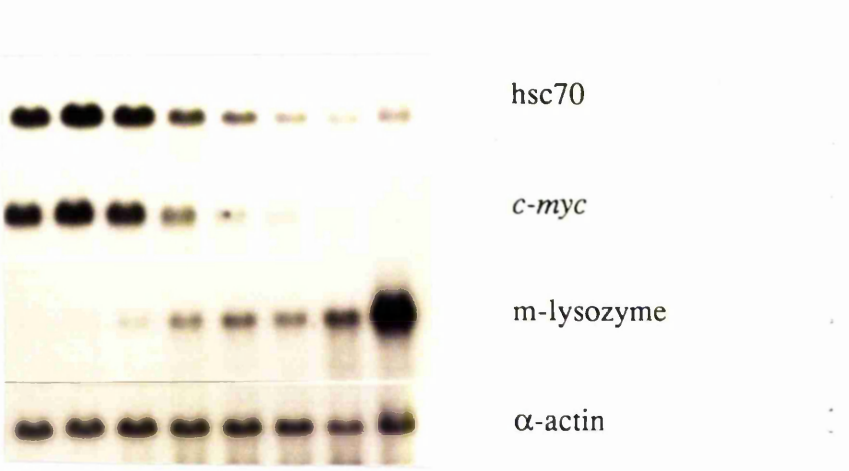


Figure 3.6: Northern blot analysis of total RNA from FDCP-mix (clone A4) cells during the granulocytic differentiation in the presence of LCM and IL-3 (1.5U/ml). Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The same blot was hybridized successively with [^{32}P]-labelled probes for hsc70, *c-myc*, m-lysozyme, and α-actin. RNA was a gift from Dr M. Cross.
Each lane was loaded with 5μg of total RNA.

when the cells stop proliferating (measured by clonogenic assay and cell density and correlated with *c-myc* RNA expression). Therefore it was of interest to determine if the level of expression of hsc70 would also vary with the cell growth rate of FDCP-mix (clone A4) cells at different concentrations of IL-3. In previous studies it has been reported that the level of hsp70 and/or hsc70 expression (not distinguished) changes with the cell cycle in eukaryotic cells. For example, the level of hsp70/hsc70 expression has been found to increase during the S phase of the cell cycle of HeLa cells (Milarski and Morimoto, 1986) and of human IL-2-dependent T-lymphocytes (Farrar *et al*, 1988). Farrar *et al* (1988) reported that after IL-2 stimulation of IL-2-dependent T lymphocytes cells (previously deprived of IL-2), hsp70/hsc70 steady state mRNA and protein synthesis are specifically increased, suggesting that the synthesis of hsp70 increases when T-cells start to enter S phase. Likewise, in arrested rat fibroblast cells which were starved of FCS, and consequently were not growing, the level of hsc73 expression (rat equivalent of hsc70) transcript was lower than that in non-starved cells (Sorger and Pelham, 1987b). Cairo *et al* (1989) observed that the level of the constitutive expression of hsc73 transcript in rapidly growing hepatoma rat cells was higher than in either slow-growing ones or and normal rat hepatocytes.

In order to analyze the level of hsc70 expression, FDCP-Mix (clone A4) cells were grown for either 24 or 48 hours in different concentrations of IL-3 [2, 1, 0.75, 0.5, 0.25 or 0.1% (v/v) of IL-3 conditioned medium (CM)]. The rate of cell growth decreased as the concentration of IL-3 was lowered (Figure 3.7). With 0.1% (v/v) of IL-3 CM, the viable cell number did not change significantly between 24 and 48 hours. After 48 hours, 25% of cells were dead (as determined by trypan blue exclusion test), suggesting that 75% of the cells survive and that some of these divide. Second, the effect of complete withdrawal of IL-3 from the FDCP-mix (clone A4) was determined by reproducing the experiment of Williams *et al* (1990). Following 24 hours of incubation of FDCP-mix (clone A4) cells

in culture medium devoid of IL-3, approximately 75% of the cell population were dead as assessed by trypan blue exclusion test (results not shown).

Total proteins were extracted from FDCP-mix (clone A4) cells incubated for 24 or 48 hours in various concentrations of IL-3 [2, 1, 0.75, 0.5, 0.25 or 0.1% (v/v)]. Western blot analysis demonstrated that the level of hsc70 expression remained constant in both experiments (Figure 3.8), suggesting that it is not associated with growth rate of FDCP-mix cells. In addition, no change in hsc70 expression was seen in total protein extracted at each hour during the first 15 hours of incubation of FDCP-mix cells in culture medium deprived of IL-3 (data not shown). Hsp70 protein could not be detected in either of these experiments (data not shown).

To confirm this data, Northern blot analysis of total RNA from cells incubated for 48 hours in growth culture medium supplemented with either 2% (v/v) or 0.1% (v/v) of IL-3 (CM) also showed that hsc70 expression did not change (Figure 3.9).

The results presented suggest that the level of hsc70 expression is not linked to the rate of cell growth of FDCP-mix (clone A4) cells. Therefore the decrease in the level of expression of hsc70 observed during granulocytic differentiation of FDCP-mix (clone A4) cells (when most of the cells stop proliferating) is associated with differentiation of these cells.

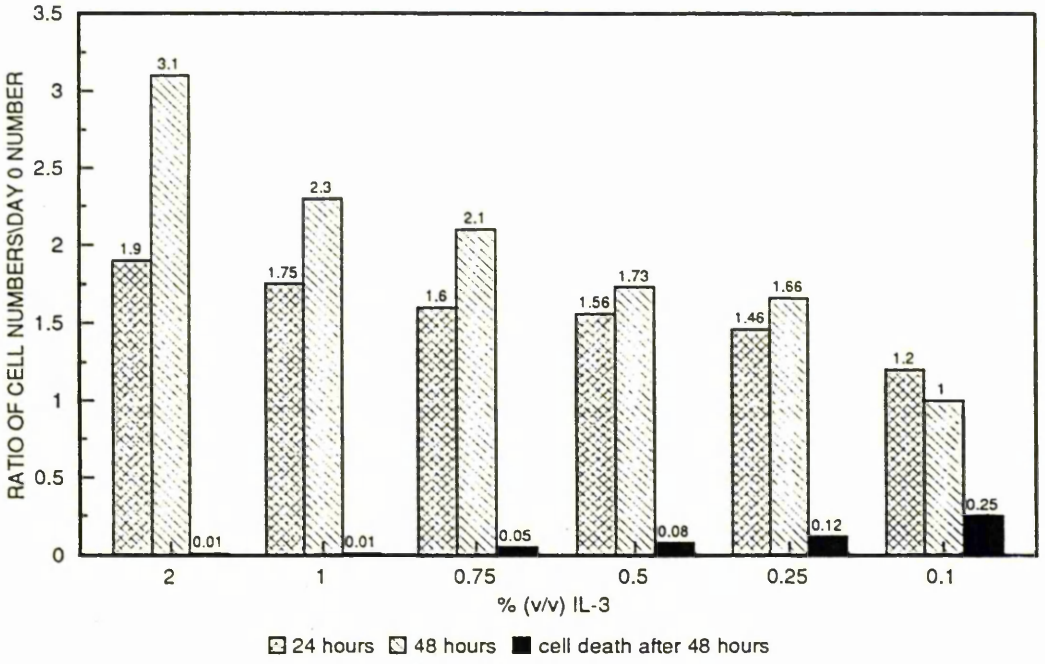


Figure 3.7: The effect of lowering the IL-3 concentration on the cell density of FDCP-mix (clone A4) cells after 24 or 48 hours. The cells were incubated for 24 or 48 hours in a culture medium supplemented with 2, 1, 0.75, 0.5, 0.25 or 0.1% (v/v) IL-3-conditioned medium. The cell density was compared to day 0. The cell density at day 0 was $3x10^5$/ml as measured by trypan blue exclusion test. Each point represents the average of three experiments.

Figure 3.8: Western blot analysis of hsc70/hsp70 expression of FDCP-mix (clone A4) cells grown in different concentration of IL-3. FDCP-mix cells were incubated with 2 (A), 1 (B), 0.75 (C), 0.5 (D), 0.25 (E) or 0.1 (F) % (v/v) IL-3 CM for 24 or 48 hours. Total proteins were resolved by 7.5% PAGE. The Western blot was probed with 3A3 (hsc70/hsp70). Lane heat shock is the total protein from FDCP-mix cells heat shocked at 42.5°C for 1 hour and allowed to recover for 2 hours.
Each lane was loaded with 40μg of total proteins.

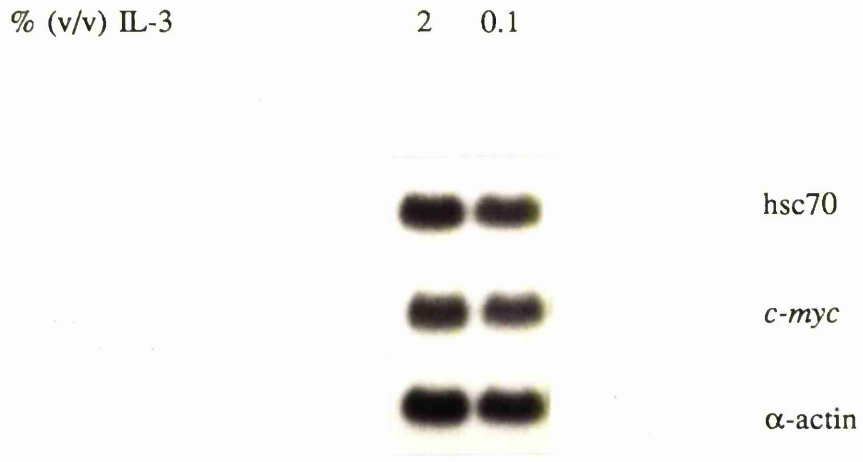


Figure 3.9: Northern blot analysis of total RNA from FDCP-mix (clone A4) cells following 48 hours of incubation in a culture medium supplemented with 2% or 0.1% (v/v) IL-3 CM. Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The same Northern blot was hybridized consecutively with [^{32}P]-labelled probes for hsc70, *c-myc* and α-actin. Each lane was loaded with 5μg of total RNA.

3.3: THE EFFECT OF OVER-EXPRESSING THE HUMAN *bcl-2* GENE IN FDCP-mix (*bcl-2*, clone 1B) CELLS ON HSC70 EXPRESSION

3.3.1: Introduction

In this work and elsewhere, it has been shown that growth factors or viability factors, which suppress apoptosis, are required throughout the differentiation process from immature cells to mature granulocytes and macrophages. The *bcl-2* gene can suppress apoptosis without inducing growth in growth factor-dependent cell lines (Wyllie *et al*, 1980; Vaux, 1993; Sachs and Lotem, 1993; section 1.1.3). Different haemopoietic growth factor-dependent cell lines have been transfected with human *bcl-2* gene. In the absence of growth factors, these cell lines survive and the process of apoptosis is delayed (Vaux, 1988; Nunez *et al*, 1990). The cell line FDCP-mix (clone A4) was transfected with the human *bcl-2* gene and clones which could survive for several days in the absence of IL-3 were selected (Fairbairn *et al*, 1993). Two different clones of FDCP-mix cells transfected with *bcl-2* were examined: (I) FDCP-mix (*bcl-2*, clone 1B) which differentiates along the granulocytic lineage in both the presence and absence of growth factors (GM-CSF, G-CSF, IL-3); (II) FDCP-mix (*bcl-2*, clone 2J) which differentiates along the erythrocytic lineage in both the presence and absence of growth factors (EPO/IL-3). Fairbairn *et al* (1993) suggested that differentiation is intrinsically determined, and that the primary role of the growth factors may be enabling rather than inductive. Within the bone marrow, therefore, these workers suggested that regional variations in the production and/or accessibility of certain growth factors determine the fate (survival or death) and amplification (by proliferation) of the progenitor cells that are produced as a consequence of stem cell differentiation. Haemopoietic cell growth factors may be able to influence stem cell differentiation, resulting in the preferential production of progeny that are committed to one

or more cell lineages.

3.3.2: Does the absence of IL-3 in FDCP-mix (*bcl-2*, clone 1B) cells accelerate the granulocytic differentiation in the presence of GM-CSF and G-CSF?

It is thought that the low concentration of IL-3 used in granulocytic differentiation of the FDCP-mix (clone A4) cells is required for survival but not differentiation (Heyworth *et al*, 1990). In FDCP-mix (*bcl-2*) cells, survival is governed by the presence of over-expressed human *bcl-2* but it was not known whether the presence of IL-3 was required for differentiation.

To establish whether a low concentration of IL-3 was an optional component for this process, FDCP-mix (*bcl-2*, clone 1B) were differentiated in LCM in the presence (A) or absence (B) IL-3. The cells grew continuously in both experiments but at day 7 of the differentiation time course, the total cell number was two fold higher in cells containing IL-3 (Figure 3.10). As a result of the absence of IL-3 in experiment B (LCM, no IL-3), the cells differentiated earlier along the granulocytic lineage (Figure 3.10). For example, the number of clonogenic cells decreased to 62% (LCM, no IL-3) and 77% (LCM, low IL-3) by day 2. More pronounced decreases, to 23% (LCM, low IL-3) and 5% (LCM, no IL-3), were noticed by day 5. No obvious differences in cell morphology were observed between both experiments A and B (Figures 3.11a and 3.11b).

3.3.3: Western blot analysis of hsc70, lysozyme, human *bcl-2* and actin proteins

Total proteins were extracted from cells at various time points during the 8 days of granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells grown in conditions A or B (A:LCM, low IL-3; B:LCM, no IL-3). The proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and analyzed by immuno probing of the Western blot. In both experiments, A (LCM, IL-3) and B (LCM, no IL-3),

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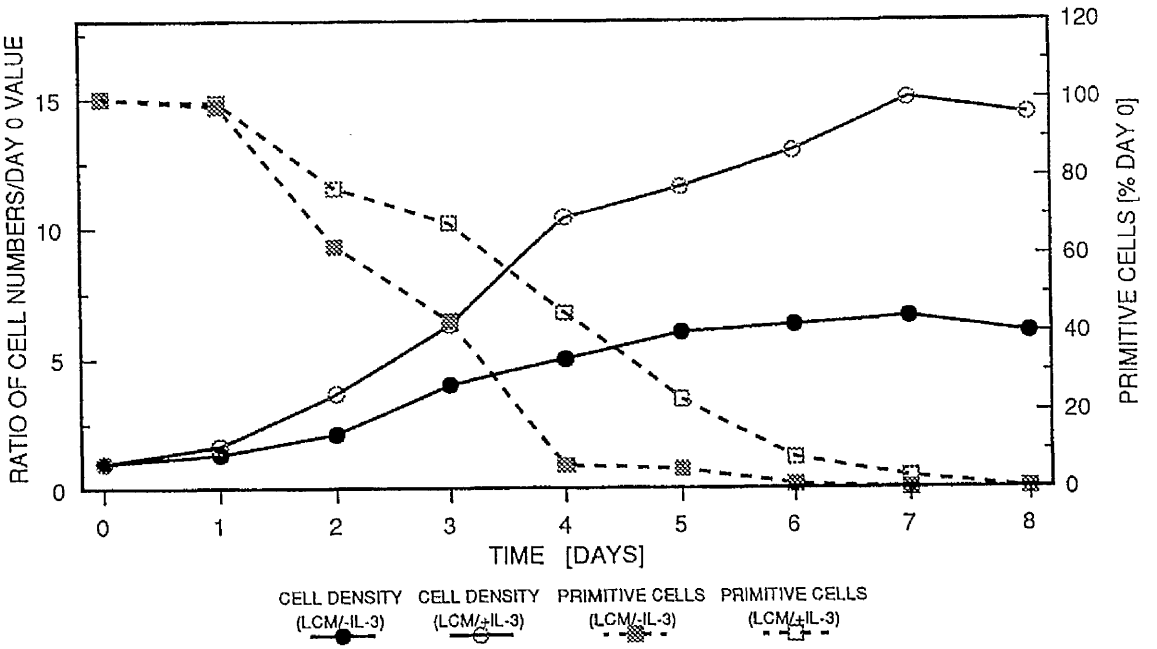


Figure 3.10: Measurement of cell densities and primitive cell numbers during the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells. The cells were differentiated under different conditions of growth factor supplementation: experiment A (LCM supplemented with low concentration of IL-3:1.5U/ml) and B (LCM without IL-3). For both experiments, day 0 value for the cell densities was 10^5 cells/ml, and for the clonogenic assays, 210 colonies. Cell density was expressed as fold increase of the day 0 value. Numbers of primitive blasts cells was expressed as a percentage of the day 0 number. The cell density was assessed by trypan blue exclusion test. Each point represents the average of three experiments.

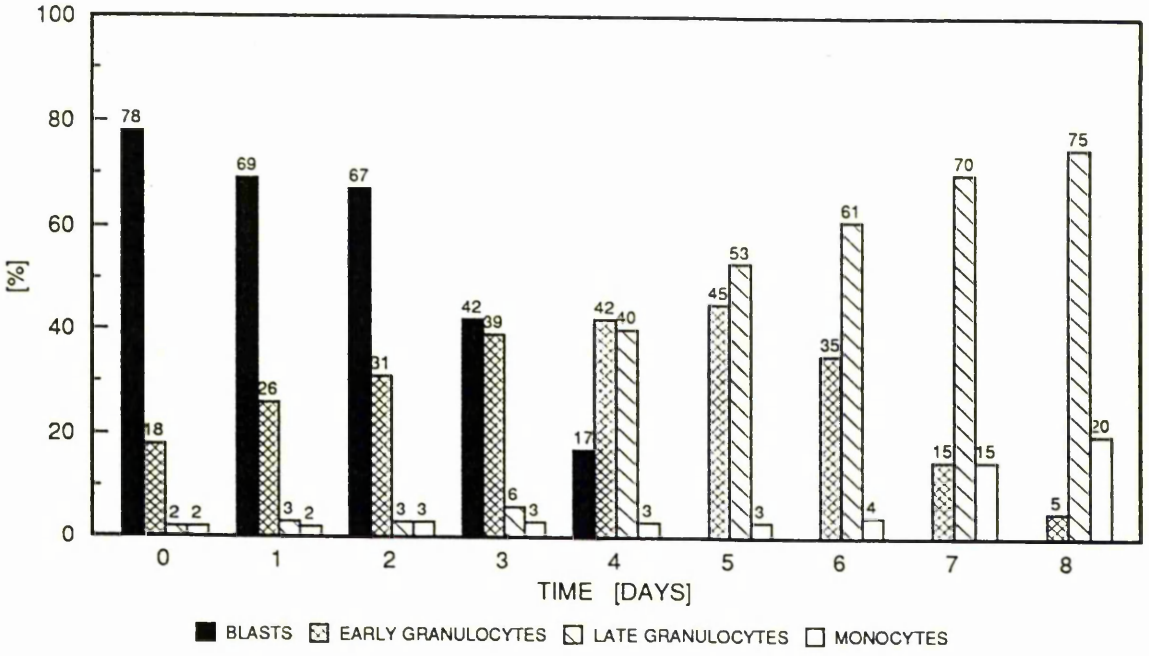


Figure 3.11a: The morphology of FDCP-mix (*bcl*-2, clone 1B) cells during the granulocytic differentiation in the presence of LCM and IL-3 (1.5U/ml). The cells were stained with May-Grunwald and Giemsa stains. Each point represents the average of three experiments.

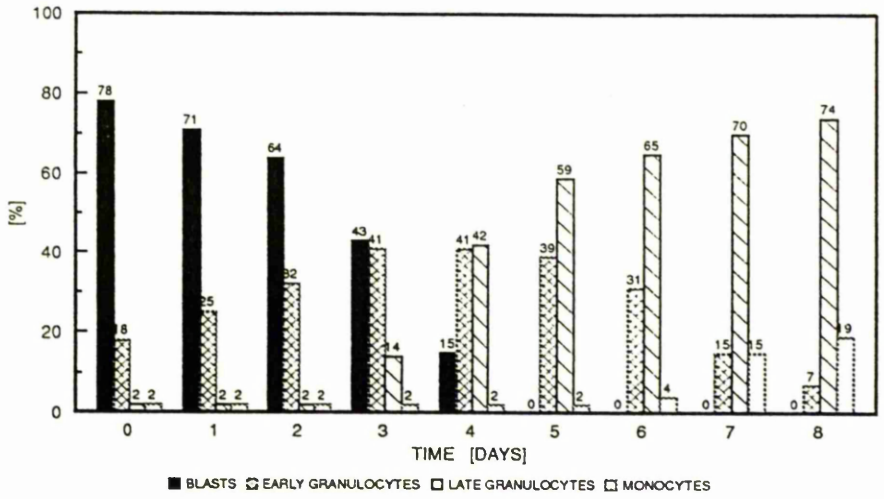


Figure 3.11b: The morphology of FDCP-mix (*bcl-2*, clone 1B) cells during the granulocytic differentiation in the presence of LCM. The cells were stained with May-Grunwald and Giemsa stains. Each point represents the average of three experiments.

the level of the constitutive expression of hsc70 protein did not change until day 5 (Figure 3.12). By day 6, it decreased sharply and remained at a very low level until the end of the differentiation on day 8. No inducible heat shock protein, hsp70 was detected at any time during the course of differentiation (Figure 3.12). In FDCP-mix (*bcl-2*, clone 1B) cells, the decrease in hsc70 appeared at the same time as that found in FDCP-mix (clone A4) cells and yet as measured by cell density and primitive cell number, FDCP-mix (*bcl-2*, clone 1B) cells grown in the absence of IL-3 clearly had an accelerated differentiation programme.

Expression of lysozyme protein, a marker of the granulocyte differentiation, increased sharply at day 4 for the experiment B (LCM, no IL-3) and at day 6, in the experiment A (LCM, IL-3), and remained high until the end of differentiation, at day 8 (Figure 3.12). These differences in the level of expression of the lysozyme protein reflect an earlier on set of differentiation in experiment B (LCM, no IL-3) than in experiment A (LCM, low IL-3). Equal loading of proteins was confirmed by probing with specific monoclonal antibody for actin (Figure 3.12). The level of expression of the recombinant human *bcl-2* protein, detected by a specific anti-human *bcl-2* antibody, decreased dramatically by day 6 (Figure 3.12).

3.3.4: Northern blot analysis of hsc70, *c-myc*, m-lysozyme, human *bcl-2* and α-actin RNA

Total RNA was extracted from cells at various times during the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells grown in conditions A (LCM, low IL-3) or B (LCM, no IL-3). It was established that RNA hsc70 expression decreased 2-3 fold between days 6 and day 8 (Figure 3.13a), coinciding with the observed decreased of hsc70 protein (Figure 3.12). No significant difference was found between differentiation conditions.

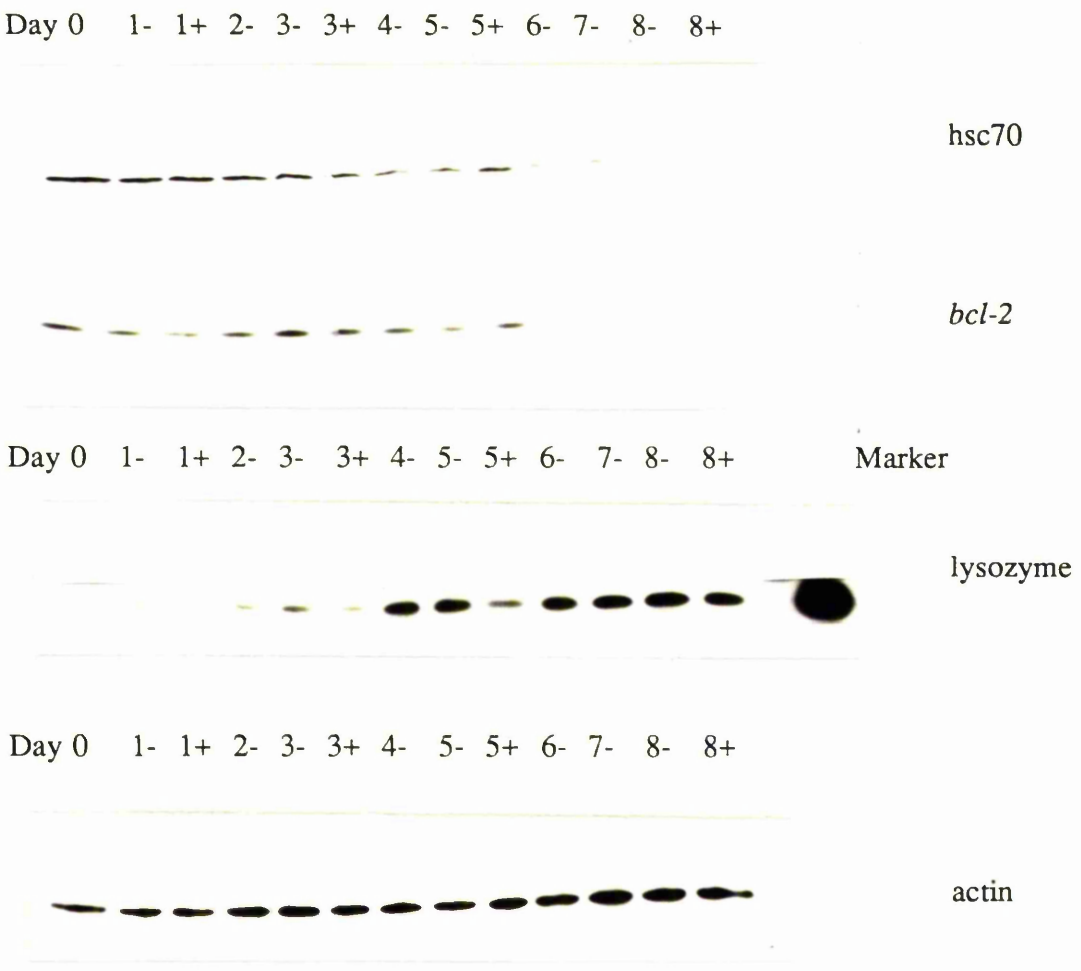


Figure 3.12: Western blot analysis of total proteins from FDCP-mix (*bcl-2*, clone 1B) cells during the granulocytic differentiations (A, B). These cells were differentiated with LCM, no IL-3 (-) or with added IL-3 (+:1.5U/ml). Total proteins were resolved by 7.5% or 12.5% PAGE. One Western blot (7.5%) was probed successively with monoclonal antibodies 3A3 (hsc70) and anti-actin and another Western blot (12.5%) with polyclonal anti-lysozyme and monoclonal anti-human *bcl-2* antibodies. Lane marker consists of lysozyme from the Rainbow marker (section 2.7.7).
Each lane was loaded with 40μg of total proteins.

The level of expression *c-myc* decreased by more than 10-fold by day 3 in experiment B (LCM, no IL-3) but a decrease of similar magnitude was observed only after day 6 (data not shown) in experiment A (LCM, IL-3). This result suggests that hsc70 expression is not co-ordinated to the level of expression *c-myc* during the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells unlike FDCP-mix (clone A4) (Figure 3.6). The m-lysozyme RNA which was constitutively expressed at a very low level on day 0, increased steadily to reach a maximum (more than 10 fold) of expression by day 4 (B:LCM, no IL-3) or day 6 (A:LCM, low IL-3) (Figure 3.13a). During this period, the level of expression of the endogenous *bcl-2* RNA (7.5kb) had decreased 10-fold by day 5 in experiment B, and the same decrease was observed in the experiment A (Figure 3.13b) after day 6. The level of expression of human *bcl-2* RNA (2.3kb), whose expression was under the SV40 promoter (Vaux *et al*, 1988), also decreased faster in the experiment B (LCM, no IL-3) (Figure 3.13b). Equal loading of RNA was confirmed with a probe for α-actin (Figure 3.13a).

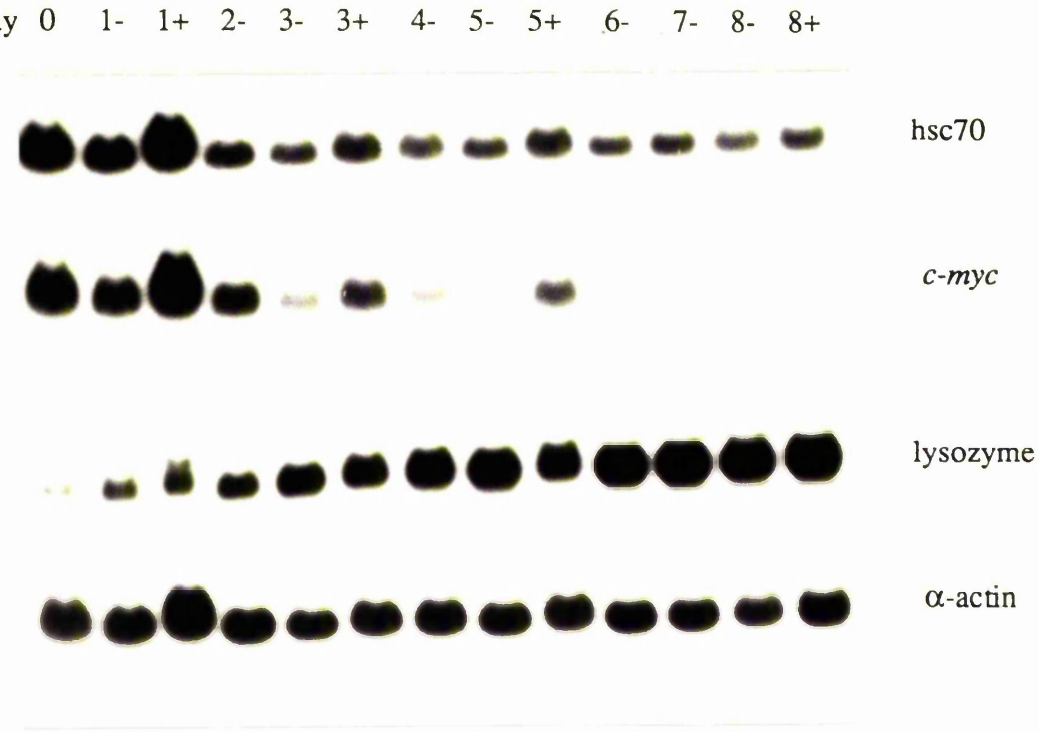


Figure 3.13a: Northern blot analysis of total RNA from FDCP-mix (*bcl-2*, clone 1B) cells during the granulocytic differentiation. The cells were differentiated with LCM, without IL-3 (-) or with added IL-3 (+:1.5U/ml). Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The Northern blot was hybridized successively with [^{32}P]-labelled probes for hsc70, m-lysozyme, *c-myc* and α-actin. Each lane was loaded with 5μg of total RNA.

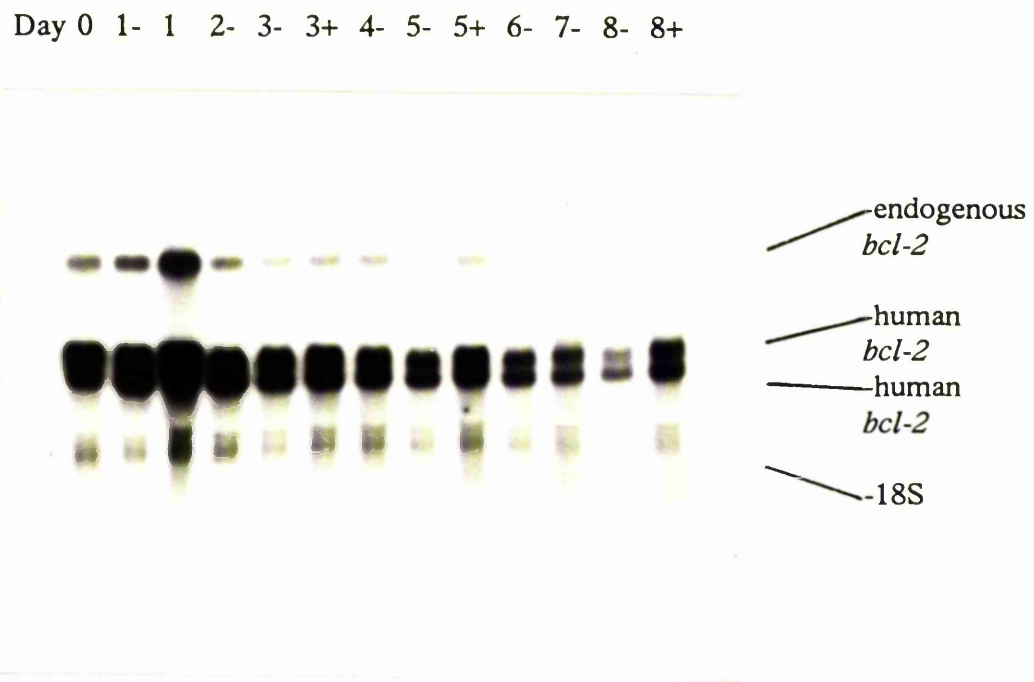


Figure 3.13b: Northern blot analysis of the viral and endogenous expression of *bcl-2* in FDCP-mix (*bcl-2*, clone 1B) cells during the granulocytic differentiation. The cells were differentiated along the granulocytic lineage with LCM no IL-3 (-) or with added IL-3 (+:1.5U/ml) (see figure 3.13a). Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The Northern blot (see figure 3.13a) was hybridized successively with the [^{32}P]-labelled probes for the human *bcl-2* (see figure 2 from Negrini *et al*, 1987). Each lane was loaded with 5µg of total RNA.

3.4: THE EXPRESSION OF HSC70 DURING THE GRANULOCYTIC DIFFERENTIATION OF FDCP-mix (*bcl-2*, clone 1B) CELLS IN THE ABSENCE OF GROWTH FACTORS

3.4.1: Introduction

During the granulocytic differentiation of FDCP-mix cells (clone A4) and FDCP-mix (*bcl-2*, clone 1B) cells, using GM-CSF and G-CSF, there was a decrease in the expression of hsc70 following the maturation of more than 80% of the cell population, as showed by clonogenic assays (Figures 3.3 and 3.10). In order to determine if hsc70 expression was associated with the presence or not of growth factors (GM-CSF, G-CSF, IL-3), it was of interest to use a cell line which could be differentiated along the granulocytic lineage without using growth factors. Because of the over-expression of the human recombinant *bcl-2*, the cell line FDCP-mix (*bcl-2*, clone 1B), survived in the absence of IL-3, both in serum containing and serum-deprived conditions, and this survival was accompanied by granulocytic differentiation (Fairbairn *et al*, 1993). However a small population (27%) of erythroblast cells was also observed (Fairbairn *et al*, 1993).

3.4.2: Measurements of cell density, primitive cell numbers and morphology

When IL-3 was removed from the culture medium of FDCP-mix (*bcl-2*, clone 1B), a 40% increase in cell number was observed at day 1 (Figure 3.14). At day 1, Fairbairn *et al* (1993) showed by flow cytometry that following 20 hours of withdrawal of IL-3, 10-15% of viable cells were in S or G_2/M phase while 80-85% were in G_0/G_1 phase of the cell cycle. The cell number remained constant between days 1 and 3 and decreased by 50% until day 6. The number of clonogenic cells decreased slightly by day 1 (95%), 35% by day 3, and no clonogenic cells were detected by day 6 (Figure 3.14). The morphology of

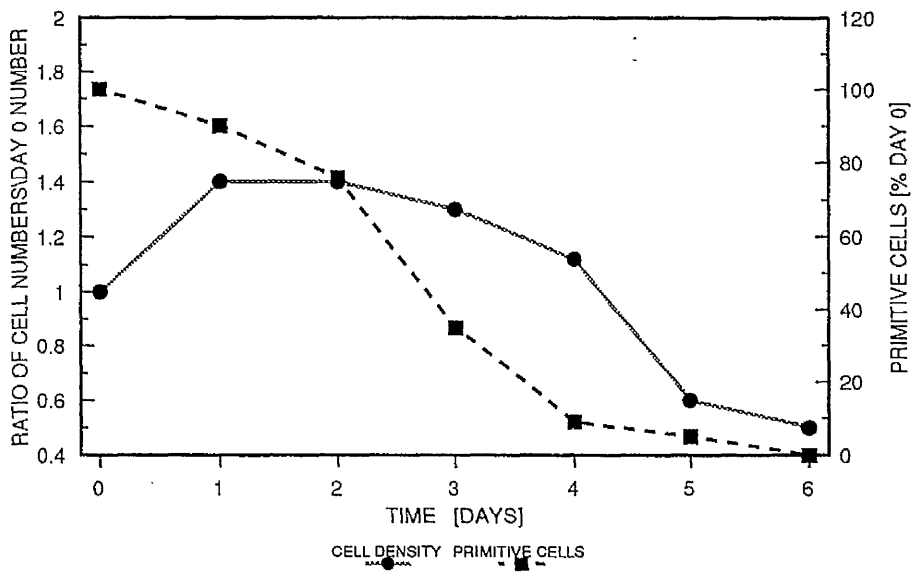


Figure 3.14: Measurement of the cell density and primitive cell numbers of FDCP-mix (*bcl-2*, clone 1B) during the granulocytic differentiation in the absence of growth factors. Day 0 value for the cell density was $5x10^5$ cells/ml, and for the clonogenic assays, 210 colonies. Cell density was expressed as fold increase of the day 0 value. Numbers of primitive blast cells was expressed as a percentage of the day 0 number. The cell density was assessed by trypan blue exclusion test. Each point represents the average of three experiments.

the cell population is shown in figure 3.15. It produced a mixed population composed of erythroblasts and granulocytes/macrophages.

3.4.3: Western blot analysis of hsc70, lysozyme, human *bcl-2* and actin proteins

Total proteins were extracted from cells at each day during the differentiation. Protein analysis by Western blot showed that the level of expression of hsc70 decreased rapidly at day 1 to remain just detectable by day 6 (Figure 3.16). Unlike the granulocytic differentiation of FDCP-mix clones (A4 and 1B) in the presence of growth factors, the decrease of the level of hsc70 expression occurred before the maturation of the cell population. No expression of the inducible, hsp70 was detected during the time course (Figure 3.16).

The lysozyme protein showed a detectable expression at day 1 and 2, to increase sharply (more than 5 fold) by day 3 (Figure 3.16). This confirmed that by day 3 more than 65% of cells were differentiating along the granulocytic lineage. The level of expression of the recombinant *bcl-2* protein remained unchanged (Figure 3.16).

Equal loading of proteins between samples was confirmed by the detection of α-actin (Figure 3.16).

3.4.4: Northern blot analysis of hsc70, *c-myc*, lysozyme and α-actin RNA

Total RNA was isolated during the differentiation. The expression of hsc70 RNA remained unchanged during the 6 days of differentiation (Figure 3.17), and yet a fall in hsc70 protein was observed by day 1 (Figure 3.16). This result also showed that hsc70 expression does not appear to be regulated at the transcriptional level during the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B).

Loading of RNA between samples was confirmed with the probe to α-actin (Figure 3.17). The level of expression of *c-myc* RNA fell more than 10 fold by day 1 (Figure

3.17). As it has been already shown during other granulocytic differentiations of the same cell line with growth factors, *c-myc* expression is not associated with that of hsc70. The expression of the m-lysozyme RNA was detected at day 0 to reach a maximum of expression by day 3 (more than 10 fold increase) and remained constant until day 6.

In regard to the above data it was interesting to determine if the fall of hsc70 expression was linked to a fall of total protein synthesis occurring during the granulocytic differentiation of *bcl-2* transfected FDCP-mix cells in the presence or absence of growth factors.

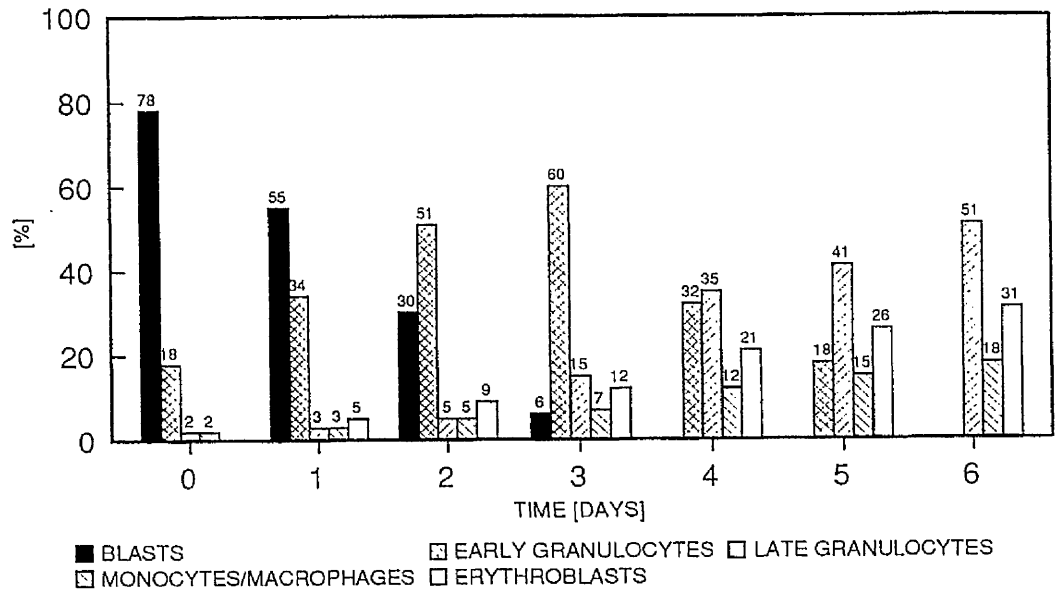


Figure 3.15: The morphology of FDCP-mix cells (*bcl-2*, clone 1B) during the granulocytic differentiation in the absence of growth factors. Cells were stained with May-Grunwald and Giemsa stains. Each point represents the average of three experiments.

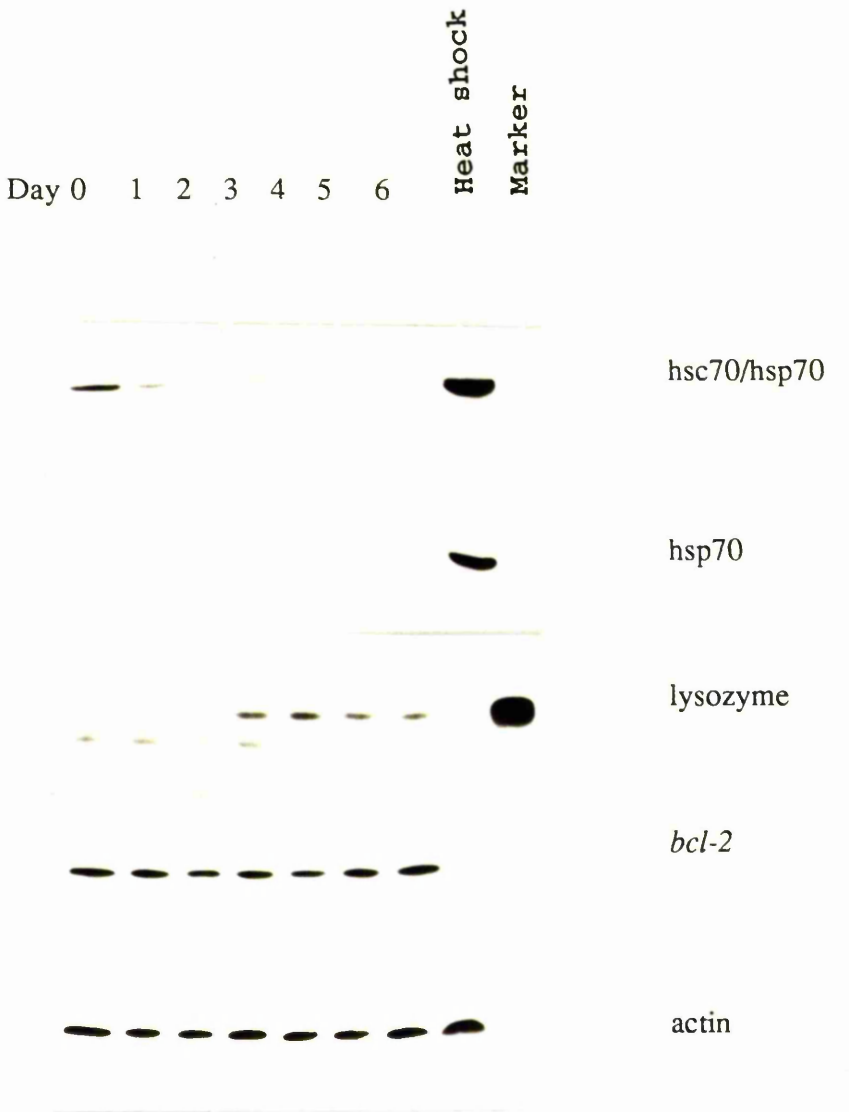


Figure 3.16: Western blot analysis of total proteins from FDCP-mix (*bcl-2*, clone 1B) during the granulocytic differentiation in the absence of growth factors. The cells were differentiated in the absence of growth factors (no LCM, no IL-3). Total proteins were resolved by 7.5% or 12.5% PAGE. The same blot (7.5%) was probed successively with monoclonal antibodies 4G4 (hsp70), 3A3 (hsc70/hsp70) and anti-actin. Another blot (12.5% gel) was probed successively with monoclonal antibody anti-human *bcl-2* and polyclonal antibody anti-lysozyme. Lane heat shock consists of total protein extracted from FDCP-mix (clone A4) cells heat shocked for 1 hour at 42°C and allowed to recover for 2 hours. Lane marker consists of lysozyme protein from the Rainbow marker (section 2.7.7). Each lane was loaded with 40µg of total proteins.

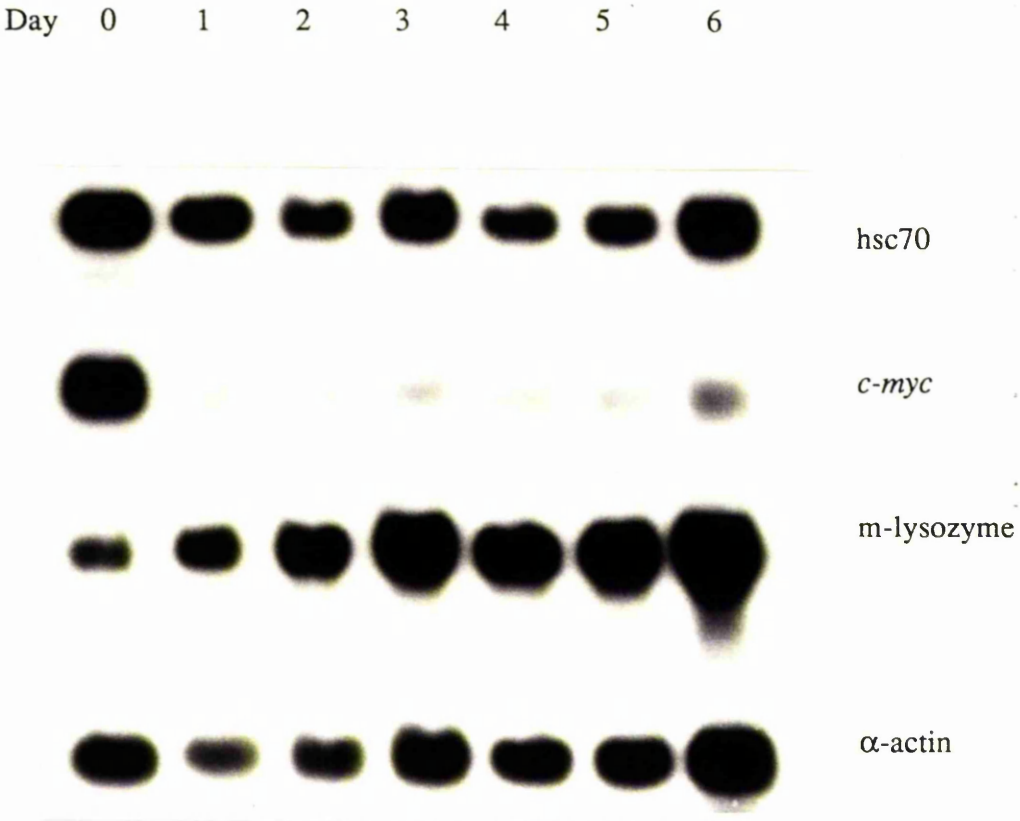


Figure 3.17: Northern blot analysis of total RNA from FDCP-mix (*bcl-2*, clone 1B) during the granulocytic differentiation in the absence of growth factors. Total RNA was resolved on formaldehyde-agarose (0.1%) gel and transferred to Hybond-N. The same blot was hybridized successively with [^{32}P]-labelled probes for hsc70, *c-myc*, m-lysozyme and α-actin. Each lane was loaded with 5μg of total RNA.

3.4.5: Is the decrease of the level of hsc70 expression during the granulocytic differentiation of FDCP-mix cells (*bcl-2*, clone 1B) associated with a fall in total protein synthesis?

It has been reported that the level of hsc70/hsp70 expression is linked to the rate of protein synthesis. For example, following stimulation of T lymphocyte cells with phytohemagglutinin and IL-2, the level of hsp70 expression increases as part of a generalized increase in protein biosynthesis (Farrar *et al*, 1988). During the DMSO-induced granulocytic differentiation of HL-60 cells, a decrease in total protein synthesis was observed (Fibach *et al*, 1985). Therefore the decrease in hsc70 expression during the granulocytic differentiation of FDCP-mix clones with or without growth factors, may be the consequence of a decrease in the total protein synthesis.

In the following experiments, the *in vitro* incorporation of [^{35}S]-methionine into total protein at various times during the granulocytic differentiation of FDCP-mix (*bcl-2*, 1B clone) cells in the presence of growth factors (LCM/IL-3) was measured. This showed a 70% decrease of incorporated radiolabelled methionine in total proteins at day 1 (Figure 3.18). Following this, protein incorporation decreased gradually to reach less than 5% at day 8 (Figure 3.18).

In vitro incorporation of [^{35}S]-methionine into total protein in FDCP-mix (*bcl-2*, clone 1B) cells during differentiation (no LCM, no IL-3), showed a 77% decrease of incorporation at day 1 (Figure 3.18) and following this, it decreased gradually to reach 11% at day 6.

These results showed that in FDCP-mix (*bcl-2*, clone 1B) cells grown in the presence or absence of growth factors, the decrease in hsc70 expression did not appear to be associated with the decline in total protein synthesis seen during granulocytic differentiation.

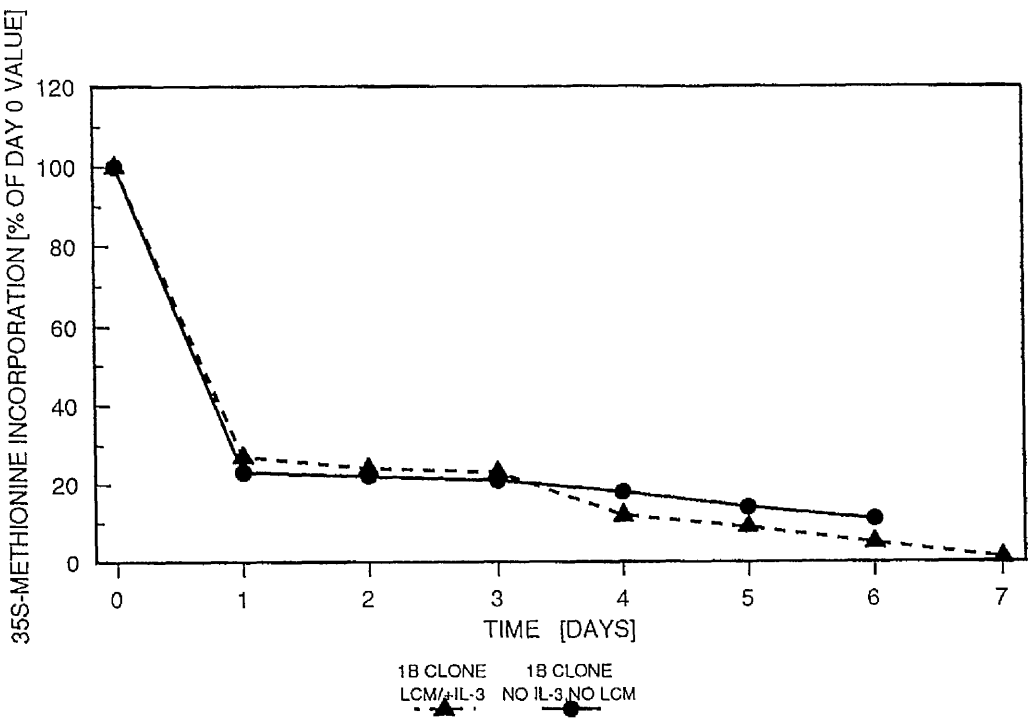


Figure 3.18: Decrease of total protein incorporation of [^{35}S]-methionine during the granulocytic differentiations of FDCP-mix (*bcl-2*, clone 1B). Measurement of *in vitro* incorporation of ^{35}S methionine was performed every day:
During the 6 days of the granulocytic differentiation in the absence of growth factors (no LCM, no IL-3).
During 7 days of granulocytic differentiation in the presence of LCM and low IL-3 (1.5U/ml).
Each point represents the average of three experiments.

3.5: DISCUSSION: GRANULOCYTIC DIFFERENTIATION OF FDCP-mix CLONES

3.5.1: Stress and granulocytic differentiation of haemopoietic cells

The granulocytic differentiation of FDCP-mix (A4) cells and FDCP-mix (*bcl-2*, clone 1B) was performed by transferring cells from a culture medium containing 100U/ml IL-3 to a low concentration of IL-3 (1.5U/ml) or in the absence of IL-3, and LCM (G-CSF, GM-CSF). To establish whether or not the drastic decrease in IL-3 concentration might be stressful for FDCP-mix cells, this stress was measured by determining hsp70 protein expression since this protein has been shown to be induced exclusively in stressed cells (section 1.2.1). By Western blot analysis, no hsp70 was detected during the granulocytic differentiation of FDCP-mix clones, suggesting that differentiation was not induced by stress (Figure 3.5).

These results confirmed the work on NMF-induced granulocytic differentiation of HL-60 which demonstrated that differentiation was not triggered by stress (Beere *et al*, 1993b).

To my knowledge, they are the only studies to have investigated hsp70 expression during the granulocytic differentiation of haemopoietic cells.

3.5.2: Markers of differentiation

In the work presented here, the number of colony forming cells during differentiation was determined to assess the maturation of FDCP-mix cells. The number of colony forming cells decreased with differentiation of haemopoietic cells because differentiating cells lose the capacity to proliferate, and therefore to form colonies (reviewed by Metcalf, 1984).

At day 6, 8% primitive cells were observed, as measured by the number of colony-forming cells, during the granulocytic differentiation of FDCP-mix (*bcl-2*, 1B clone), in the presence of growth factors (Tables 3.1,2,3). In the same differentiating conditions, 21%

primitive cells were found by day 6 in FDCP-mix (clone A4) cells. In each case, the cell population was composed of both early and late granulocytes, but did not contain blast cells, as measured by morphology. The significant number of cells capable of forming colonies in the presence of IL-3 by day 6, shows that early granulocytes proliferate. Therefore, the number of primitive cells is a marker which seems to represent both blasts and early granulocytic cells.

The early granulocyte stage consists of a broad range of cells, from myeloblasts (first differentiated stage) to metamyelocytes (last stage of early granulocytes), which is recognized by the progression of the retracted shape in the nucleus. It has been reported that myeloblasts, promyelocytes, and myelocytes from human bone marrow form colonies in the presence of IL-3 (Lopez *et al*, 1988; Aglietta *et al*, 1993), suggesting that FDCP-mix cells, which formed colonies by day 6 of the granulocytic differentiation, consisted at least partially of these cells (Figure 3.19). In the presence of IL-3, promyelocyte-myelocyte cells (from $0.5x10^5$ to 10^5 cells) purified from human bone marrow, formed approximately 10 clusters on semi-solid media Lopez *et al*, 1988). This result shows that from 0.0002% to 0.0001% of cells formed colonies in the presence of IL-3. During the granulocytic differentiation of FDCP-mix (clone A4) cells, 42 clusters were observed, when day 6 cells ($5x10^3$ early granulocytes) were incubated on semi-solid media in the presence of IL-3. This result suggests that 0.0084% of FDCP-mix cells formed colonies, in the presence of IL-3, suggesting that early granulocytes (FDCP-mix, clone A4) are more responsive (42-84 fold) to IL-3 than promyelocyte-myelocyte purified from human bone marrow.

The second molecular marker which was used in this work to assess the granulocytic differentiation of FDCP-mix cells was the detection of m-lysozyme RNA. In the presence of growth factors (LCM, low IL-3) maximum expression of m-lysozyme RNA was reached by day 6 (FDCP-mix, *bcl-2*, clone 1B) or day 8 (FDCP-mix, clone A4), when more than 80% of the cell population was composed of both early and late granulocytes

(Tables 3.2,3). In FDCP-mix (*bcl-2*, clone 1B) cells, in the absence of growth factors, maximum of m-lysozyme RNA expression was reached by day 3, when 60% of the cell population was composed of early granulocytes (Table 3.4). This result suggests that this cell line may differentiate faster in the absence rather than in the presence of growth factors (section 3.5.3). However it remains unclear whether or not expression of m-lysozyme RNA increases at the early or late granulocyte stage in FDCP-mix cells. Measurement of m-lysozyme RNA expression from a single cell by PCR would resolve this question. A previous study on the granulocytic differentiation of the mouse cell line 32D C13 (Valtieri *et al*, 1987) in the presence of G-CSF, has showed that there is strong evidence that m-lysozyme RNA expression increased at the early granulocytic stage of FDCP-mix cells. Valtiri *et al*, 1987 have shown that molecular markers which characterise the granulocytic lineage are expressed at early stage of the differentiation. For example, when 55% of 32D C13 cells were at the metamyelocyte stage, which corresponds to early granulocytic stage in FDCP-mix cells, 88% of these cells synthesized myeloperoxidase RNA (Valtieri *et al*, 1987).

3.5.3: Cell survival leading to differentiation

3.5.3.1: Role of growth factors in survival and differentiation

Growth factors have been demonstrated to promote proliferation and/or differentiation of haemopoietic cells (section 1.1.1). However Fairbairn *et al* (1993) showed that growth factors were necessary for cell survival but not for differentiation. FDCP-mix (clone A4) cells with the transfected human *bcl-2* gene, differentiated to granulocytes in the absence of growth factors, suggesting that *bcl-2* was the survival signal to allow FDCP-mix cells to differentiate.

In this work, the measurement of primitive cells shows that the granulocytic differentiation of FDCP-mix clones is accelerated in the absence of growth factors,

Day	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	Lysozyme RNA	Morphology (%)	Primitive cells (% day 0)	Cell growth Ratio of cell numbers/day 0 number
0	BE	BE	BE	-	B:100	100	1.0
2	BE	BE	BE	-	B:75,EG:25	89	1.7
3	BE	↓↓	↓↓	↑	B:65,EG:35	75	3.7
5	BE	↓↓	↓↓↓↓	↑	B:2,EG:74 LG:15,M:9	26	15.0
6	↓↓↓	↓↓↓↓	↓↓↓↓	↑↑	EG:51 LG:37,M:12	21	27.5
7	↓↓↓	↓↓↓↓	↓↓↓↓	↑↑↑	EG:14 LG:64,M:22	15	27.0
9	↓↓↓	↓↓↓↓	↓↓↓↓	↑↑↑↑	EG:3 LG:74,M:23	0	2O.0

Table 3.1:Summary of granulocytic differentiation of FDCP-mix (clone A4) cells in the presence of LCM and IL-3(1.5U/ml).
B:blasts, EG:early granulocytes, LG:late granulocytes, M:monocytes/macrophages, BE:basal expression.
Increase of expression:↑ (<2 fold), ↑↑ (2-5 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold), - (not detected).
Decrease of expression:↓↓ (2-5 fold), ↓↓↓ (5-10 fold), ↓↓↓↓ (>10 fold).

Day	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	Lysozyme protein	Lysozyme RNA	Morphology (%)	Primitive cells (% Day 0)	Cell growth Ratio of cell numbers/day 0 number
0	BE	BE	BE	BE	BE	B:78,EG:18 LG:2,M:2	100	1.0
1	BE	BE	BE	BE	↑↑	B:69,EG:26 LG:3,M:2	99	1.6
2	BE	BE	BE	BE	↑↑↑	B:67,EG:31 LG:3,M:3	77	3.5
3	BE	BE	↓↓	BE	↑↑↑↑	B:42,EG:39 LG:16,M:3	68	6.3
4	BE	BE	↓↓	BE	↑↑↑↑	B:17,EG:42 LG:40,M:3	45	10.4
5	BE	BE	↓↓	↑↑	↑↑↑↑	EG:45 LG:53,M:3	23	11.6
6	↓↓↓	↓↓	↓↓↓	↑↑↑	↑↑↑↑	EG:35 LG:61,M:4	8	13.0
7	↓↓↓	↓↓	↓↓↓↓	↑↑↑	↑↑↑↑	EG:15, LG:70,M:15	3	15.0
8	↓↓↓	↓↓	↓↓↓↓	↑↑↑	↑↑↑↑	EG:5,LG:75 M:20	0	14.4

Table 3.2: Summary of granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells in the presence of LCM and IL-3 (1.5U/ml).
B:blasts, EG:early granulocytes, LG:late granulocytes, M:monocytes/macrophages. Increase of expression:↑↑ (2-5 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold).
Decrease of expression:↓↓ (2-5 fold), ↓↓↓ (5-10 fold), ↓↓↓↓ (>10 fold)

Day	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	Lysozyme protein	Lysozyme RNA	Morphology (%)	Primitive cells (% day 0)	Cell growth Ratio of cell numbers/ day 0 number
0	BE	BE	BE	BE	BE	B:78,EG:18 LG:2,M:2	100	1.0
1	BE	BE	BE	BE	↑↑	B:71,EG:25 LG:2,M:2	98	1.3
2	BE	BE	BE	BE	↑↑↑	B:64,EG:32 LG:2,M:2	62	2.1
3	BE	↓	↓↓	↑↑	↑↑↑↑	B:43,EG:41 LG:14,M:2	43	4.0
4	BE	↓↓	↓↓	↑↑↑↑	↑↑↑↑	B:15,EG:41 LG:42,M:2	6	5.0
5	BE	↓↓	↓↓↓↓	↑↑↑↑	↑↑↑↑	EG:39 LG:59,M:2	5	6.0
6	↓↓↓	↓↓	↓↓↓↓	↑↑↑↑	↑↑↑↑	EG:31 LG:65,M:4	1	6.3
7	↓↓↓	↓↓	↓↓↓↓	↑↑↑↑	↑↑↑↑	EG:15 LG:70,M:15	0	6.6
8	↓↓↓	↓↓	↓↓↓↓	↑↑↑↑	↑↑↑↑	EG:7,LG:74 M:19	0	6.0

Table 3.3: Summary of granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells in the presence of LCM.
B:blasts, EG:early granulocytes, LG:late granulocytes, M:monocytes/macrophages, BE:basal expression. Increase of expression:↑↑ (2-5 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold). Decrease of expression:↓ (<2 fold), ↓↓ (2-5 fold), ↓↓↓↓ (>10 fold)

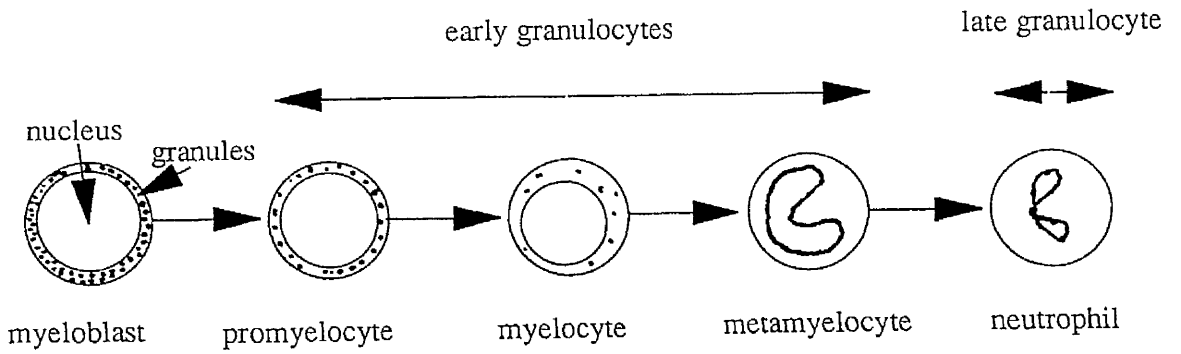


Figure 3.19: The morphology of normal human cells during the granulocytic differentiation (Hann *et al*, 1983). Each primitive cell or myeloblast differentiates to give rise successively to promyelocyte, myelocyte, metamyelocyte, and finally neutrophil, which is the final mature phenotype. During the differentiation, the nucleus is progressively retracted and twisted. The cytoplasm is intensely basophilic [granules due to its high RNA content] in myeloblast, which progressively loses its granules during differentiation.

Day	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	Lysozyme protein	Lysozyme RNA	Morphology (%)	Primitive cells (% Day 0)	Cell growth Ratio of cell numbers/ day 0 number
0	BE	BE	BE	BE	BE	B:78,EG:18 LG:2,M:2	100	1.0
1	↓↓↓	BE	↓↓↓↓	BE	↑↑	B:55,EG:34 LG:3,EB:5 M:3	90	1.4
2	↓↓↓	BE	↓↓↓↓	↑↑	↑↑↑	B:30,EG:51 LG:5,EB:9 M:5	76	1.4
3	↓↓↓	BE	↓↓↓↓	↑↑↑↑	↑↑↑↑	B:6,EG:60 LG:15,EB:12 M:7	35	1.3
4	↓↓↓	BE	↓↓↓↓	↑↑↑↑	↑↑↑↑	LG:32,EG:35 EB:21,M:12	9	1.1
5	↓↓↓	BE	↓↓↓↓	↑↑↑↑	↑↑↑↑	LG:41,EG:18 EB:26,M:15	5	0.6
6	↓↓↓	BE	↓↓↓↓	↑↑↑↑	↑↑↑↑	LG:51,M:18 EB:31	0	0.5

Table 3.4: Summary of granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) in the absence of growth factors. B:blasts, EG:early granulocytes, LG:late granulocytes, M:monocytes/macrophages, EB:erythroblasts, BE:basal expression. Increase of expression:↑↑ (2-5 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold). Decrease of expression:↓↓↓ (5-10 fold), ↓↓↓↓ (>10 fold).

suggesting that growth factors, by promoting both cell proliferation and survival, slow down cell differentiation (Tables 3.1,2,3,4). Therefore proliferation is not required for the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells. In the presence of growth factors, differentiation of FDCP-mix clones is coupled to proliferation for 5-7 days and the decrease in hsc70 expression is delayed, compared to the differentiation in the absence of growth factors (Tables 3.1,2,3,4). This result suggests that hsc70 may be a marker for cell survival because when hsc70 expression decreases, cells start to differentiate and die, as measured by the decrease in the total cell numbers.

Other workers have shown for human T-lymphocytes, that M-CSF, G-CSF, GM-CSF and IL-2 upregulated genes known to be involved in cell proliferation such as hsp70/hsc70, *c-fos* and *c-myc* (Farrar *et al*, 1988). These results demonstrate, that growth factors by stimulating the expression of these genes may delay the granulocytic differentiation.

During the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells, in the presence of growth factors, the cell population was composed of only granulocytes and macrophages. In contrast to the differentiation in the presence of growth factors, in the absence of growth factors, the cell population contained erythroblasts and granulocytes (Table 3.4). These results suggest that in the absence of growth factors, the cells differentiated upon a stochastic model (Fairbairn *et al*, 1993), giving rise to a mixed population composed of granulocytes and erythroblasts. Therefore, the presence of growth factors restricted the differentiation to only one lineage of FDCP-mix cells.

3.5.3.2: Bcl-2 and hsc70 as markers of cell survival

This work shows that growth factors delays the decrease of hsc70 expression and the programmed cell death during the granulocytic differentiation of FDCP-mix clones (A4 or 1B). In the presence of growth factors, hsc70 expression remained high until day 5 of

the differentiation, to decrease dramatically by day 6. On the contrary, in the absence of growth factors, hsc70 expression decreases sharply by day 1 (Tables 3.1,2,3,4). These results suggest that hsc70 may be a good marker to measure cell survival during the granulocytic differentiation of haemopoietic cells.

The association between cell survival and hsps expression has been thoroughly investigated only in the yeast *S. cerevisiae*. In *S. cerevisiae*, there are four groups of hsp70 genes (SSA,B,C,D), and the SSA subgroup contains four members SSA1,2,3,4. SSA1 and SSA2 are expressed constitutively but SSA3 and SSA4, only following heat shock (Werner-Washburne *et al*, 1987). SSA gene products appear to function both in mitochondrial and endoplasmic reticulum import pathways, though each may have distinct primary responsibilities while retaining the capacity to act interchangeably (Deshaies *et al*, 1988). The yeast strain MW141, harbours chromosomal disruptions of the SSA1, SSA2 and SSA4 genes. This strain is not viable without transfection of a plasmid vector containing the coding region of SSA1 (Deshaies *et al*, 1988), and in the absence of SSA1, translocation of proteins through the endoplasmic reticulum does not occur. In other eukaryotic cells, hsc70 and hsp70 (SSA-D related genes) have also been shown to have a role of "chaperon" (Laskey and Earnshaw, 1980; Ellis, 1987), in the translocation of proteins through membranes (Beckmann *et al*, 1990) and protein degradation (Chiang and Dice, 1988; Chiang *et al*, 1989; Terlecky *et al*, 1992).

Therefore hsc70/hsp70 gene products have a crucial role in the survival of all eukaryotic cells, suggesting that the low hsc70 expression in granulocytes may reflect the short life span of these cells (Dresh *et al*, 1969; Rubinson *et al*, 1976). However, as observed previously, in the absence of growth factors, a decrease in hsc70 expression was observed by day 1 during the differentiation of FDCP-mix (*bcl-2*, clone 1B) cells. Therefore, *bcl-2* expression does not delay the decrease of hsc70 to day 6, as is observed during granulocytic differentiation in the presence of growth factors. These results suggest

that *bcl-2*, a potent survival signal, acts via a mechanism different to the survival signal promoted by growth factors. Evidence to support this idea. For example, from the demonstration that IL-2, M-CSF, GM-CSF and G-CSF increased *c-myc* and *c-fos* expression in T-lymphocytes (Farrar *et al*, 1988) whilst no change in *c-myc* or *c-fos* expression was observed following transfection of *bcl-2* in FDCP-mix (clone A4) cells (data not shown), incubated in non-differentiating culture medium (100U/ml IL-3).

In FDCP-mix (*bcl-2*, clone 1B) cells, human *bcl-2* expression is under the control of the LTRenhancer (Vaux *et al*, 1988, Fairbairn *et al*, 1993). Therefore human *bcl-2* expression was expected to remain constant during granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells. Granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) cells in the presence of growth factors (experiments A:LCM, IL-3; B:LCM, no IL-3) is accompanied by a decrease in human *bcl-2* protein expression by day 6. The expression of the human *bcl-2* RNA decreased 2 or 3 fold during granulocytic differentiation A (LCM, IL-3) and between 5 and 10 fold in experiment B (LCM, no IL-3). The reasons of the decrease in human *bcl-2* expression during differentiation is unknown. The endogenous expression of *bcl-2* RNA decreased severely by day 5, to be undetectable by day 8 in experiment B (LCM), whilst in experiment A (LCM, IL-3), the endogenous *bcl-2* RNA expression decreased gradually and was still detectable by day 8 (Figure 3.13b). These data suggest that IL-3, a known survival factor (Spooncer *et al*, 1986; Shearman *et al*, 1993), delayed the decrease in *bcl-2* RNA expression.

It has been shown that with only LCM and without IL-3 in the culture medium, FDCP-mix (*bcl-2*, clone 1B) differentiated faster along the granulocytic lineage but it could be suggested that the cells die faster, due to a more rapid decrease in *bcl-2* expression. Sachs and Lotem (1993) reported that endogenous *bcl-2* down regulation which occurs during normal differentiation is involved in determining the limited life span of mature granulocytes. The activation of the LTR enhancer during differentiation has been

investigated on haemopoietic and non haemopoietic cell lines. For example LTR-ORF mRNA expression (ORF from the minor lymphocyte stimulating superantigens of mice) has been found to be activated in immature thymocytes (CD4-), and down-regulated following differentiation to mature phenotype (CD4+) (Jarvis *et al*, 1994). Other workers observed a down-regulation of HIV-LTR-CAT activity during the macrophage differentiation of U937 cells in the presence of PMA (Bernstein *et al*, 1991). These results suggest that during granulocyte differentiation of FDCP-mix (bcl-2, clone 1B) cells, in the presence of growth factors, and during erythrocyte differentiation of FDCP-mix (*bcl-2*, clone 2J), in the absence of growth factors, the decrease in *bcl-2* expression may be regulated transcriptionally. During differentiation of FDCP-mix (*bcl-2*) clones, new transcription factors may be synthesized and they down-regulate the LTR enhancer from the retroviral construct (Vaux *et al*, 1988) transfected in FDCP-mix cells, resulting in a decrease in bcl-2 gene expression, which is under the control of this retroviral LTR enhancer. In contrast, no change in the human *bcl-2* protein (Figure 3.16) and RNA (data not shown) expression was observed over the 6 days of the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B), in the absence of growth factors (no LCM, no IL-3) since these experiments were done at different times, the biology of FDCP-mix clones may change from batch to batch, as has been noted by others (Dr. C. Heyworth, personal communication). The significance of the constant human *bcl-2* RNA expression during differentiation, in the absence of growth factors is therefore unknown.

3.5.4: Cell survival versus apoptosis

3.5.4.1: Role of hsc70

In contrast to the programmed cell death, which is used to describe the mode of cell death that is a normal part of the life of a multicellular organism, apoptosis can be induced by a wide variety of cytotoxic drugs, physical stimuli or following withdrawal of growth factors from growth factor-dependent cell lines (reviewed by Martin *et al*, 1994). However,

scientists working on cell death and/or apoptosis do not seem to agree about the definitions of these two concepts. Buttyan *et al* (1988) reported a possible involvement of hsc70/hsp70 (not distinguished) in cell death of the ventral prostate gland after castration of adult rats. These workers demonstrated an increase in expression of the hsc70 and/or hsp70 transcript (not distinguished) on the fourth day after castration, when the cells were beginning to die.

In the present study, following the incubation of FDCP-mix (clone A4) cells in a culture medium supplemented (1) for 24 hours with 0.5 μg/ml of cytosine arabinoside (this chemical compound blocks DNA replication) and a high concentration of IL-3 [2% (v/v) CM] (data not shown) or (2) for 48 hours with a low concentration of IL-3 [0.1% (v/v) CM] (Figures 3.8 and 3.9), no change in hsc70 protein and RNA expressions was observed. These culture conditions stopped or decreased the rate of cell proliferation and killed 10% (1) or 25% (2) of the cell population as assessed by trypan blue exclusion. As described elsewhere, a similar result was obtained when HL-60 cells were treated with a toxic concentration of 300mM NMF (Beere *et al*, 1993b). However, from these results, obtained using heterogenous cell populations in which some cells died whilst other survived, it is not clear whether hsc70 expression is required for apoptosis or survival.

3.5.4.2: Role of c-myc

No change in *c-myc* RNA expression was detected in dying FDCP-mix (clone A4) cells when they were cultured for 48 hours in a low concentration of IL-3 [0.1% (v/v) CM] (Figure 3.9).

In *c-myc* transfected rat-1 fibroblasts, which over-express *c-myc* constitutively, Evan *et al* (1992) demonstrated that upon serum starvation, transfected cells were more prone to death than cells which expressed low levels of *c-myc* protein, or an inactive *c-myc* mutant. Following castration in adult rats, the level of expression of *c-myc* transcript was transiently

increased (6 fold) in the ventral prostate gland during the period of cell death (Buttyan *et al*, 1988). However, Askew *et al* (1991) showed that the level of expression of *c-myc* transcript decreased dramatically following 2 hours of deprivation of IL-3 in the IL-3-dependent haemopoietic cell line 32D. These workers reported that, following deprivation of IL-3, there was an accumulation of 32D cells in the G_1 phase of the cell cycle associated with a rapid induction of apoptosis (Askew *et al*, 1991). They showed that, after 15 hours of IL-3 deprivation, 70% of these cells were viable, but this had fallen to 15-20% after 24 hours as assessed by trypan blue exclusion. Apoptosis must therefore have occurred some time before death as assessed by trypan blue exclusion.

The results presented here seem to preclude a general role for *c-myc* in cell death for several but not all cell types.

3.5.5: Hsc70 regulation

3.5.5.1: Translational or transcriptional regulation of hsc70

During the granulocytic differentiation of FDCP-mix (*bcl-2*, 1B clone) cells, in the absence of growth factors (no LCM, no IL-3), no change in the level of hsc70 RNA was observed, and yet hsc70 protein expression fell dramatically (Table 3.4). Granulocytic differentiation of FDCP-mix (clone A4) and FDCP-mix (*bcl-2*, clone 1B) cells, in the presence growth factors, resulted in a dramatic (more than 10 fold) and a slight (2-3 fold) decrease of hsc70 RNA respectively (Tables 3.2,3) and in both differentiations, hsc70 protein expression had decreased by day 6. In both cell lines these results suggest that hsc70 is not regulated at the transcriptional level, but at the translational or post-translational level. In contrast, other workers have demonstrated that hsc70 and hsp70 were regulated transcriptionally during the granulocytic (Beere *et al*, 1993a) or erythrocytic (Theodorakis *et al*, 1989) differentiation of HL-60 or K562 cells respectively.

The results presented here and elsewhere (Beere *et al*, 1993b), do not confirm that

c-myc regulated hsc70 RNA expression as has previously been suggested by Taira *et al* (1992) and Koskinen *et al* (1991). Taira *et al* (1992) have reported that the *c-myc* protein complex binds to two sites in the human hsp70 promoter region (section 1.2.2). Following over-expression of *c-myc* protein in *c-myc* transfected COS cells, hsp70 was observed to co-localise with *c-myc* in the nucleus (Koskinen *et al*, 1991). Because of the high homology between hsp70 and hsc70 (81%) at the amino-acid sequence level (section 1.2.1.4.3), these results suggest that the level of hsc70 expression may be regulated by *c-myc*. During the granulocytic differentiation of FDCP-mix clones (A4 and 1B), the decrease in *c-myc* RNA expression did not correlate with a decrease in hsc70 RNA, irrespective of granulocytic differentiation conditions. For example, during the differentiation of FDCP-mix (clone A4) in the presence of growth factors (LCM, IL-3), the expression of *c-myc* and hsc70 RNA decreased profoundly after day 3 (5-10 fold) (Table 3.1). In contrast, during the granulocytic differentiation of FDCP-mix (*bcl-2*, 1B clone) under the same culture conditions (LCM, IL-3), *c-myc* RNA expression decreased dramatically only after day 6 (5-10 fold) (Table 3.2) and hsc70 RNA decreased only 2-3 fold over the same period. Moreover, the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) in the absence of growth factors (no IL-3, no LCM), showed a drastic decrease of *c-myc* RNA by day 1, and hsc70 RNA expression did not change during differentiation (Table 3.4). Therefore, the regulation of the hsc70 gene may vary with the cell lines which are differentiated and the growth factor conditions of differentiation.

Thus *c-myc* expression during the granulocytic differentiation of FDCP-mix clones (A4 and 1B), seems to be dependent on (1) the cell line being studied, (2) and the inducers of differentiation. For example Vass *et al* (1990) reported that following the DMSO-induced granulocytic differentiation of HL-60, the steady-state level of *c-myc* transcript in total RNA fractions decreased rapidly to reach one-tenth of the value found in non-induced HL-60 cells by 45-60 minutes. In contrast, during the TPA-induced differentiation of the

same cell line along the monocyte/macrophage lineage, an initial twofold increase occurred, after which the steady-state level of *c-myc* transcript decreased more slowly; only reaching one-tenth of its control value by 16-24 hours. During the retinoic-induced granulocytic differentiation of HL-60 cells, an increase of *c-myc* transcript was observed following 24 hours of incubation. This increase persisted until 96 hours and then declined to below the level of untreated cells at 120 hours (Yen and Guernsey, 1986). These authors proposed that *c-myc* may play different roles in different processes of terminal differentiation. These results suggest that hsc70 and *c-myc* RNA expressions are not co-ordinated, and that the cell line and the differentiating inducers, and the lineage of differentiation may affect their expression in different ways.

3.5.5.2: Is hsc70 protein expression associated with the general protein synthesis?

Analysis of the incorporation of [^{35}S]-methionine in total cellular protein, showed that there was a sharp decrease in protein synthesis by day 1 during differentiation of FDCP-mix (*bcl-2*, clone 1B) cells which were performed with or without growth factors (Figure 3.18). These similar observations suggest that the expression of hsc70 protein is not related to the level of synthesis of total protein because hsc70 protein expression decreased by days 6 or 1 during granulocytic differentiations of FDCP-mix (*bcl-2*, clone 1B) cells with or without growth factors respectively (Tables 3.2,3).

My results do not confirm the work of Farrar *et al* (1988). By using T-lymphocytes, they observed that the increase of expression of hsp70/hsc70 (not distinguished) protein synthesis observed during the S phase was part of a generalized increase in protein biosynthesis, suggesting that hsc70/hsp70 proteins chaperoned newly synthesized proteins and unfolded proteins (Bienz and Gurdon, 1982; Ananthan *et al* 1986; Beckmann *et al*, 1990). No other work has investigated protein synthesis during granulocytic differentiation.

3.5.5.3: Hsc70 expression and proliferation of haemopoietic cells

In human fibroblasts (Milarski and Morimoto, 1986) and T lymphocytes (Farrar *et al*, 1988), it has been reported that the level of hsp70 increased during the S phase of the cell cycle.

During the granulocytic differentiation of FDCP-mix clones, a decrease in hsc70 protein expression occurred, when most of the cells stopped proliferating, as demonstrated by the analysis of cell density, in the presence (Tables 3.1,2,3) or not (Table 3.4) of growth factors. Fairbairn *et al* (1993) showed that 20 hours after withdrawal of IL-3, the proportion of FDCP-mix (*bcl-2*, clone 1B) cells in S or G_2/M phase was only 10%-15% of viable cells and 80%-85% were in G_0/G_1. In contrast 40% of the cells were in S or G_2/M phase and 60% in G_0/G_1 phase when incubated in the presence of IL-3. In non differentiating conditions, following a decrease in the rate of proliferation of FDCP-mix (clone A4) cells, after treatment with cytosine arabinoside (data not shown) or after transferring cells to a low concentration of IL-3 (Figure 3.8), no change in hsc70 expression was observed, suggesting that hsc70 expression is not associated with cell proliferation in FDCP-mix cells. However cell cycle analyses should be done on FDCP-mix (clone A4) cells for both experiments, to compare these results to those obtained for human fibroblasts and T-lymphocytes (Milarski and Morimoto, 1986; Farrar *et al*, 1988).

3.5.5.4: Effect of growth factors on hsc70 expression

During the granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) in the presence of LCM, supplemented with IL-3 (1.5U/ml) (experiment A) or no IL-3 (experiment B), hsc70 protein expression decreased dramatically by day 6 for both experiments A and B (Tables 3.2,3). Following the lowering of IL-3 concentration from FDCP-mix (clone A4) cells, no change of hsc70 protein expression was observed (Figure 3.8), supporting the hypothesis that IL-3 does not regulate hsc70 expression. During the

granulocytic differentiation of FDCP-mix (*bcl-2*, clone 1B) in the absence of growth factors (no LCM, no IL-3), hsc70 expression decreased rapidly by day 1 (Table 3.1). The late fall (day 6) in hsc70 protein expression observed during differentiation, in the presence of LCM may suggest that LCM (GM-CSF and G-CSF) delay the decrease of hsc70 expression.

Other laboratories have demonstrated an activation of the hsp70 gene by growth factors. For example, Farrar *et al* (1988) demonstrated that after IL-2 stimulation of IL-2-dependent T-lymphocyte cells that had been deprived of the growth factor, hsc70/hsp70 (not distinguished) protein expression was specifically increased. The human hsp70 promoter, from PC12 cell line (nerve type) was found to be transcriptionally activated by nerve growth factor (Visvader *et al*, 1988). Because hsp70 and hsc70 may have similar promoter (section 1.2.2), these results suggest that hsc70 expression may also be activated by G-CSF/GM-CSF during the granulocytic differentiation of FDCP-mix cells.

3.6: THE EXPRESSION OF HSC70 DURING THE MACROPHAGE DIFFERENTIATION OF FDCP-mix (clone A4) CELLS

3.6.1: Introduction

The human macrophage leukaemic cell line, U937, induced to differentiate to macrophages with phorbol myristate acetate, showed an increase in the level of expression of hsp65, hsp72 and hsp90 at both protein and RNA levels, (Twomey *et al*, 1993). No change in the level of expression of the constitutive, hsc73 (similar characteristics to hsc70). They suggested that the increase in the level of expression of the inducible hsp may the trigger of the differentiation of U937 along the macrophage lineage but were not able to distinguish between this process and the consequences of chemical stress on the cells. To address this problem, hsp expression was analyzed during the differentiation of FDCP-mix (clone A4) to macrophages.

3.6.2: Analysis of hsc70 protein expression

FDCP-mix cells were differentiated in the presence of L-cell conditioned medium (containing M-CSF) and IL-3 (1.5U/ml) along the macrophage lineage over a period of 8 days. Complete differentiation to macrophages was observed by day 8 (Figures 3.20a and 3.20b) but unlike the granulocytic differentiation time course, FDCP-mix (clone A4) cells did not proliferate under these culture conditions and by day 8 only 29% of cells were viable and identified as macrophages (Figure 3.20a and 3.20b).

Total proteins were extracted from viable cells at day 0, 1, 2, 4, 6 and 8 of the macrophage differentiation and analyzed by Western blot. No inducible heat shock protein, hsp70 was expressed (Figure 3.21), and no change of the expression of hsc70 was detected over the 8 days of the differentiation (Figure 3.21). To establish equal loadings of protein, the same blot was stripped of antibody and probed with an anti-actin antibody.

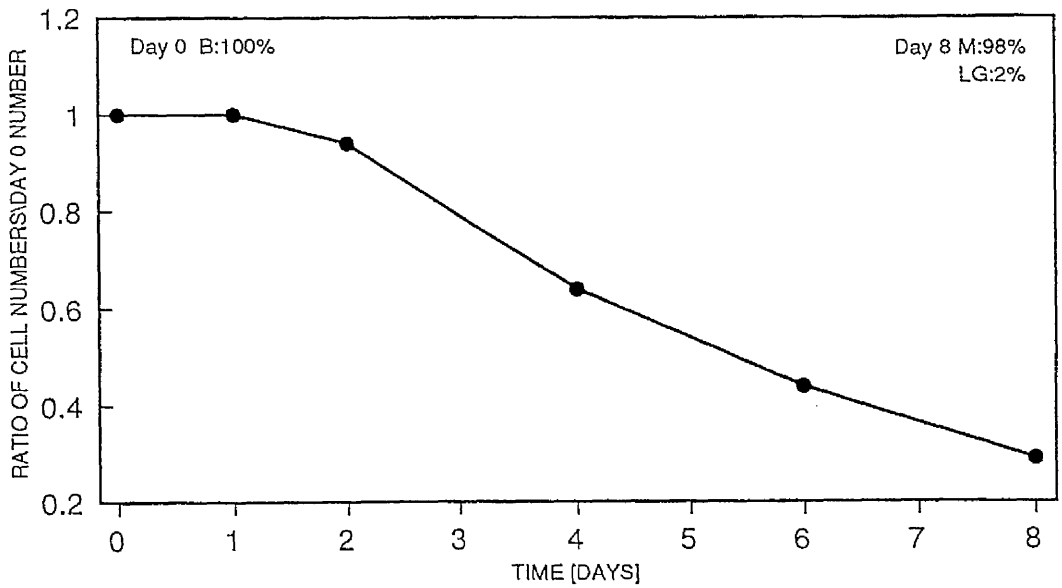


Figure 3.20a: Measurement of cell density of FDCP-mix (clone A4) cells during the macrophage differentiation in the presence of L-cells conditioned medium (M-CSF) and IL-3 (1.5U/ml). Cell density was compared to the day 0 value ($3x10^5$ cells/ml) and expressed as the number of fold. The cell density was assessed by trypan blue exclusion test. Each point represents the average of three experiments. The morphology at days 0 and 8 is shown (at top). B:blasts, M:macrophages, LG:late granulocytes.

Day 8 (Macrophages)

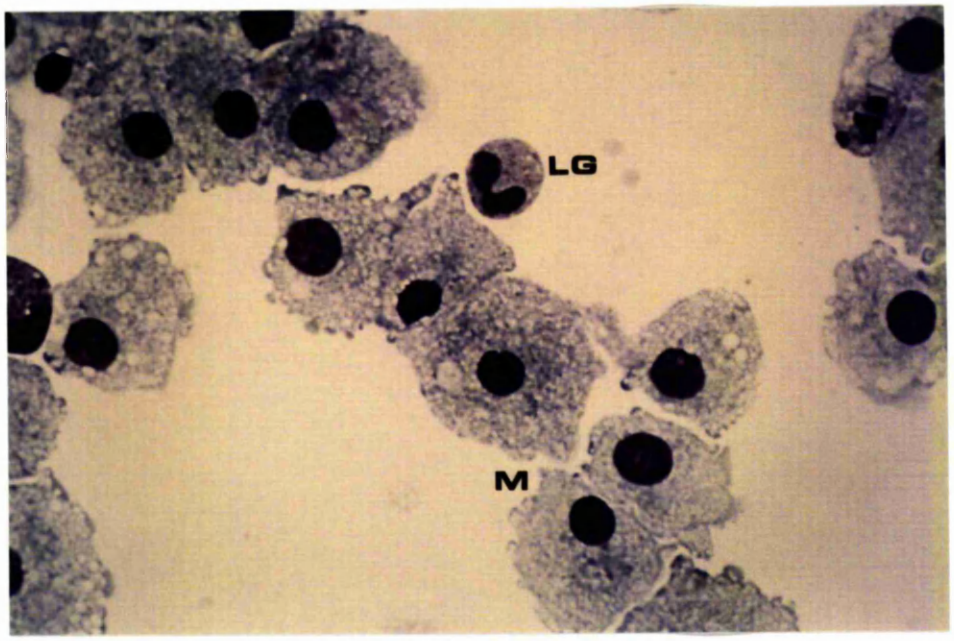


Figure 3.20b: The morphology of FDCP-mix (clone A4) cells differentiated into macrophages in the presence of L-cells conditioned medium (M-CSF) and IL-3 (1.5U/ml). At day 8 of the differentiation, the cells were stained with May-Grunwald and Giemsa solutions. Magnification (600x).

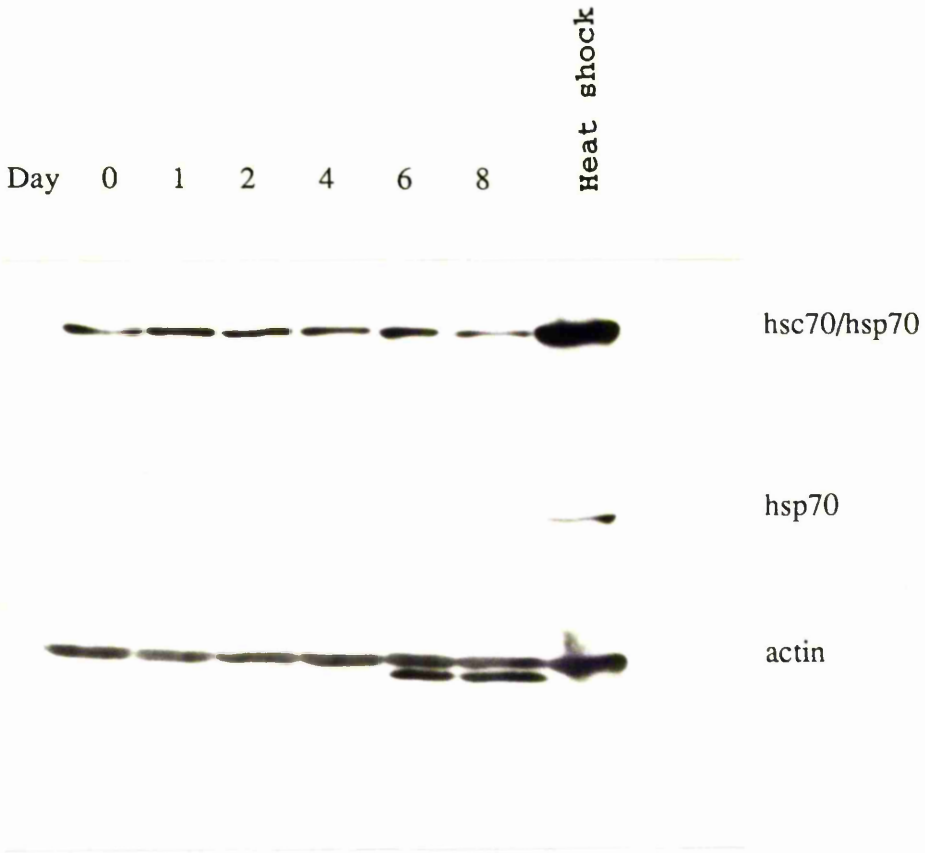


Figure 3.21: Western blot analysis of total proteins from FDCP-mix (clone A4) cells during the differentiation along the macrophage lineage in the presence of L-cells conditioned medium (M-CSF) and IL-3 (1.5U/ml). Total proteins were resolved by 7.5% PAGE. The Western blot was probed successively with monoclonal antibodies 4G4 (hsp70), 3A3 (hsc70/hsp70) and actin. Lane heat shock consists of total protein from FDCP-mix cells heat shocked at 42°C for 1 hour and allowed to recover for 2 hours.
Each lane was loaded with 40µg of total proteins.

After day 6, it was of interest that the expression of two actin proteins were observed (Figure 3.21).

3.6.3: Discussion: Macrophage differentiation of FDCP-mix (clone A4)

3.6.3.1: Stress and macrophage differentiation of haemopoietic cells

In contrast to the expression of hsp70 observed in retinoic acid-induced macrophage differentiation of U937 (Twomey *et al*, 1993), no hsp70 expression was reported during macrophage differentiation, with growth factors, of FDCP-mix (clone A4) cells, suggesting that the macrophage differentiation of normal haemopoietic cells does not require an increase in hsp70 expression (Figure 3.21).

The increase of hsp70 expression in retinoic acid-induced differentiation of U937 may be the consequence of DNA damage caused by the drug, but the association between DNA damage and hsp70 expression is very controversial in the literature. For example, Schaefer *et al* (1988) reported that the anti-cancer drugs, BCNU and CCNU, at concentrations varying between 100 and 300μM, increased hsp70 expression and damaged DNA in the human colon adenocarcinoma cell line HT-29. However the same workers did not observe an increase in hsp70 expression following treatment at low concentrations of BCNU (5-15μM) of another adenocarcinoma cell line (BE), deficient in alkylation repair of DNA damage. Only at high doses ranging from 100 to 300μM, was hsp70 expression increased in BE cells. However BCNU and CCNU have been shown to also damage proteins (Ali-Osman *et al*, 1990; Tomlinson *et al*, 1991), suggesting that the increase in hsp70 expression, which was observed in BE cells following treatment with these drugs, may be induced by the increased number of damaged or unfolded proteins in the cells (section 1.2.3.1) rather than an increase in DNA damage. Moreover, other drugs known to induce only DNA damage, such as CHLZ and BHCNU, did not increase hsp70 expression at toxic doses, suggesting that hsp70 expression and DNA damage are not

associated. During the parietal endoderm retinoic acid-differentiation of F9 cells, an increase in hsp90 expression was shown (Kohda *et al*, 1991). These workers incubated retinoic acid with a mutant of this cell line which does not differentiate, and did not observe a change in hsp90 expression, suggesting that the increase in hsp90 observed in the wild type during differentiation was associated with F9 maturation.

U937 and HL-60 cell lines are commonly used as models for haemopoietic differentiation; However studies of hsc70/hsp70 expression during differentiation show that they appear to differ from normal non-leukaemic FDCP-mix cells, which are more representative of physiological situation. In contrast to chemically-induced differentiation, cell differentiation with growth factors may not be induced by the same biochemical pathway. These data seem to show that the increase in hsps expression may trigger differentiation of chemically-induced cells, but that they do not seem to play a role in "normal" haemopoietic cells, that are stimulated by growth factors.

3.6.3.2: M-CSF and survival of haemopoietic cells

In the previous section (3.5.3.2), it was suggested that hsc70 expression was associated with cell survival. The macrophage differentiation of FDCP-mix (clone A4) cells suggest that M-CSF is a potent candidate for promoting macrophage survival (Hamilton *et al*, 1988; Bennet *et al*, 1992). For example, following stimulation of murine bone marrow-derived macrophages (BMM) with M-CSF, Hamilton *et al* (1988) observed an increase in [^{3}H]-2-deoxyglucose uptake and DNA synthesis in BMM, suggesting that M-CSF may promote cell survival of BMM. Other workers have shown that M-CSF is required for survival of differentiated mononuclear phagocytes selected from adherent bone marrow-derived macrophages (Tushinski *et al*, 1982). They observed that the complete removal of M-CSF resulted in a decrease in the cell concentration and a loss of DNA.

3.6.3.3: Lineage-dependence of hsc70 expression

During both macrophage differentiation, U937 and FDCP-mix (clone A4) cells, hsc70/hsc73 expression did not change, suggesting that hsc70/hsc73 expression is lineage restricted. What is the role of hsc70/hsc73 in macrophages? Erythrophagocytosis has been shown to induce several heat shock proteins such as hsp70 and hsp90 (Clerget and Polla, 1990). These workers suggested that the generation of hydroxy radical ($OH^{\cdot}$) released from oxidized haemoglobin may participate in the induction of stress proteins. The extremely reactive $OH^{\cdot}$ group can induce DNA damage as well as protein degradation, both of which may signal for induction of hsp synthesis (Ananthan *et al*, 1986; Davis and Goldberg, 1987). Then hsps expression may have a protective role during the life span of macrophage.

3.6.3.4: Actin expression and macrophage differentiation

After day 4 of the macrophage differentiation, an increase in the total content of actin, and the appearance of a lower molecular weight (not determined) actin protein has been detected by Western blot analysis, using a monoclonal antibody to all actin molecules (Figure 3.21). The detection of two actin proteins may reflect a reorganization of filaments in macrophages. Induced-TPA monocytic differentiation of HL-60, also increased actin protein expression after 12 hours of treatment (Uemura *et al*, 1989). These workers observed an increase in globular (G) and polymerized actin (F). Moreover, other workers have observed an increase in actin protein expression (distinguished as p42) following PMA-treated HL-60 cells (Miyamoto and Wu, 1990) and M1 differentiation (Ichikawa, 1983). Normal human monocytes stimulated in the presence of a differentiating concentration of M-CSF, were found to increase ß-actin RNA expression after 30 minutes of induction (Mufson, 1990).

These data support the observation that actin protein increased during the

macrophage differentiation of FDCP-mix (clone A4), but the significance of the appearance of two actin proteins remains unclear.

3.7: THE EXPRESSION OF HSC70 DURING THE ERYTHROCYTIC DIFFERENTIATION OF FDCP-mix (clone A4) CELLS

3.7.1: Introduction

It has been shown that the expression of stress inducible heat shock protein, hsp70 increased at both protein and RNA levels within the first 24 hours of the haemin-induced (30μM) maturation of the erythroleukaemic cell line, K562, along the erythrocytic lineage (Singh and Yu, 1984; Theodorakis *et al*, 1989). After 24 hours hsp70 expression returned to the basal level. These data suggest that the increase in hsp70 expression was a consequence of a cellular stress, possibly due to the toxic effect of haemin.

In this study, the expression of hsp70 and hsc70 was determined during the erythrocytic differentiation of FDCP-mix (clone A4) cell line. FDCP-mix (clone A4) require both EPO and haemin (0.2mM) for erythrocyte differentiation. It was therefore important to establish whether this higher concentration of haemin would stress the cells and thus increase the levels of inducible hsp70.

3.7.2: Measurement of cell density, primitive cell numbers and morphology

FDCP-mix cells (clone A4) were differentiated along the erythrocytic lineage in the presence of growth factors [EPO (3U/ml, IL-3 (1.5U/ml)] and haemin (0.2mM). The cell population increased continuously until day 7 (Figure 3.22). Primitive cell number decreased with time and by day 7, no clonogenic cells were present (Figure 3.22). Morphology showed that the cell population on day 7 consisted of 63% erythroblasts, 33% benzidine positive cells (cells containing the haemoglobin protein) and 3% monocytes. Further maturation was observed by day 9 (Figures 3.23a and 3.23b).

It should be noted that although the differentiation of leukaemic cell lines such as MEL (Hensold and Housman, 1988) and K562 (Singh and Yu, 1984) leads to the formation

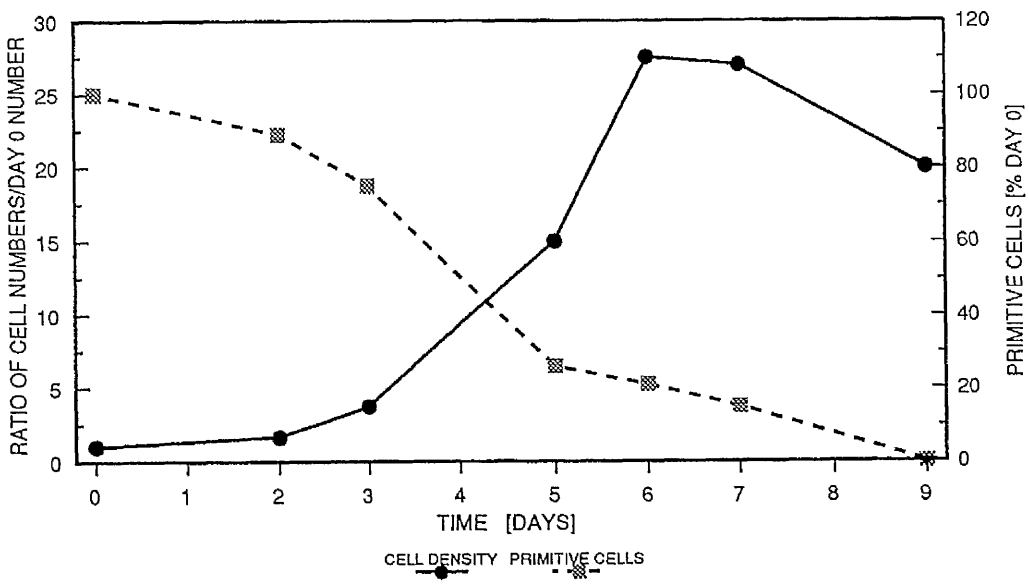


Figure 3.22: Measurement of the cell density and number of primitive cell numbers of FDCP-mix (clone A4) cells during the erythrocytic differentiation in the presence of EPO (3U/mM), IL-3 (1.5U/ml) and haemin (0.2mM). Day 0 value for the cell density was $4x10^4$ cells/ml and for the clonogenic assay was 235 colonies. Cell density was expressed as fold increase of the day 0 value. Numbers of primitive blasts cells was expressed as a percentage of the day 0 number. The cell density was assessed by trypan blue exclusion test. Each point represents one experiment.

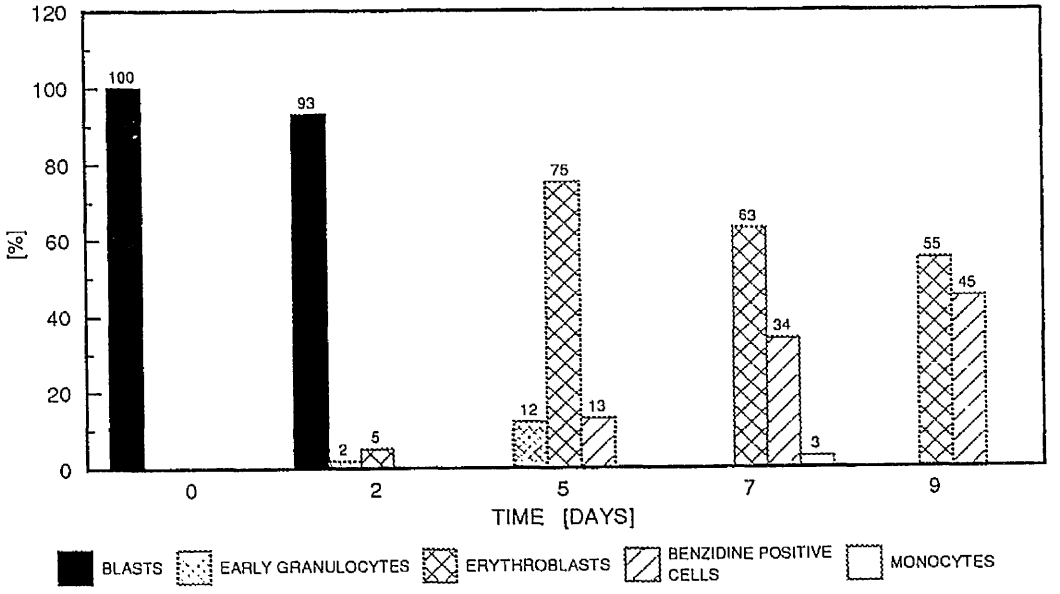


Figure 3.23a: The morphology of FDCP-mix (clone A4) during the erythrocytic differentiation in the presence of EPO (3U/ml), IL-3 (1.5U/ml) and haemin (2mM). The cells were stained with benzidine and May-Grunwald and Giemsa stains. Each point represents one experiment.

Day 7 (Differentiated FDCP-mix cells)

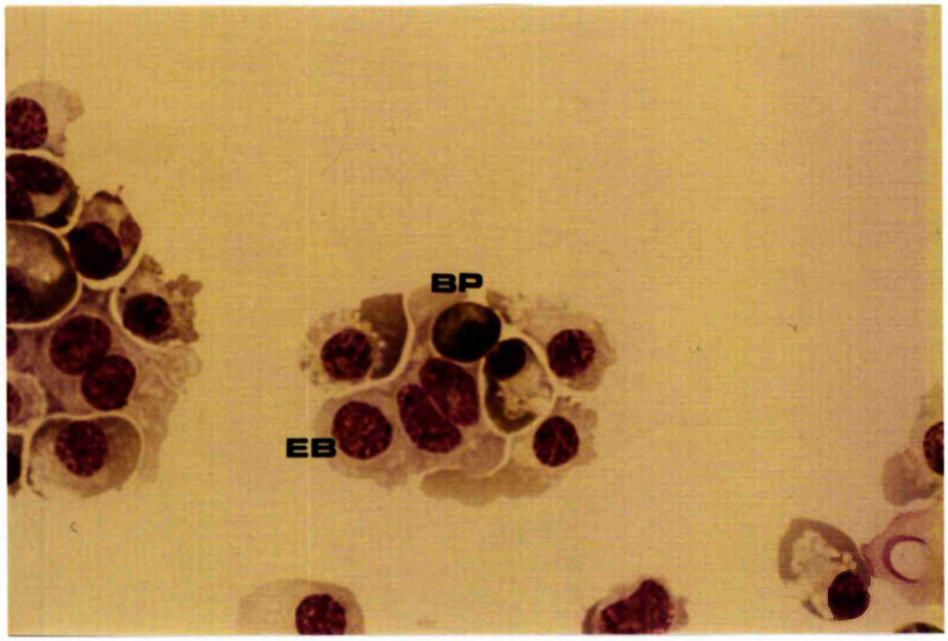


Figure 3.23b: Benzidine staining of FDCP-mix (clone A4) cells at day 7 of the erythrocytic differentiation in the presence of EPO (3U/ml), IL-3 (1.5U/ml) and haemin (0.2mM). At day 7 they were stained with Benzidine and May-Grunwald-Giemsa stains. BP:benzidine positive cells, EB:erythroblasts. Magnification (600x).

of benzidine positive cells, they do not loose the nucleus: Erythrocytic differentiation of FDCP-mix (clone A4) cells give mature cells (33%, figure 23b) that do not contain a nucleus.

3.7.3: Western blot analysis of hsc70 protein

Total protein extracts were analyzed at various times of differentiation. No change in the hsc70 protein expression (Figure 3.24) was observed during the nine days of the erythroid differentiation, yet the growth of these cells was slowing at day 7, as assessed by cell number. No expression of the stress inducible hsp70 was detected during differentiation (Figure 3.24).

This result suggests that in FDCP-mix (clone A4) cells, haemin, even at 0.2mM, does not trigger the stress response as reflected by hsp70 expression.

3.7.4: Northern blot analysis of hsc70, ß-globin, and *c-myc* RNA

Total RNA was analyzed by Northern blotting at various times during the differentiation. No change in the levels of expression of hsc70 RNA was observed (Figure 3.25). The expression of ß-globin RNA can be detected by day 2, increased gradually between days 3-7 and reached a maximum (more than 10 fold) by day 8 (Figure 3.25). The same filter was hybridized with α-actin to assess the equal loading (Figure 3.25). During the differentiation of FDCP-mix cells (clone A4) along the erythrocytic lineage, no change of the level of expression of *c-myc* RNA was observed until day 8.

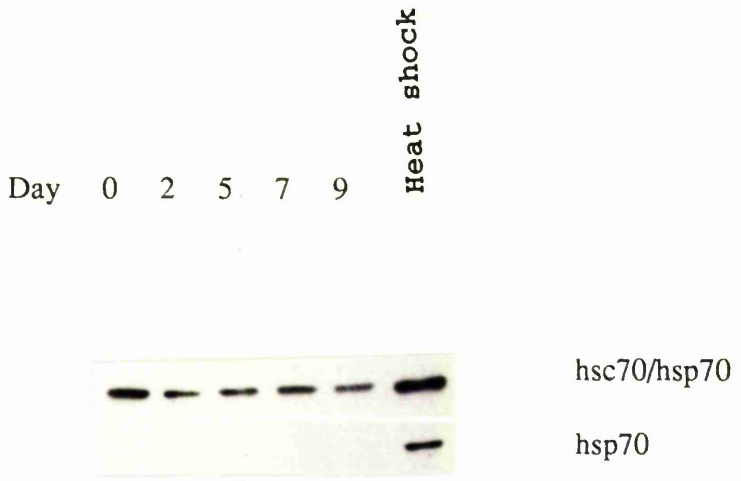


Figure 3.24: Western blot analysis of hsc70/hsp70 from FDCP-mix (clone A4) cells during the erythrocytic differentiation in the presence of EPO (3U/ml), low IL-3 (1.5U/ml) and haemin (0.2mM). Total proteins were resolved by 7.5% PAGE. The same blot was probed successively with monoclonal antibodies 4G4 (hsp70) (shown at the bottom) and 3A3 (hsc70/hsp70) (shown at the top). Lane heat shock consists of FDCP-mix (clone A4) cells heated at 42°C for 1 hour and allowed to recover for 2 hours.
Each lane was loaded with 40μg of total proteins.

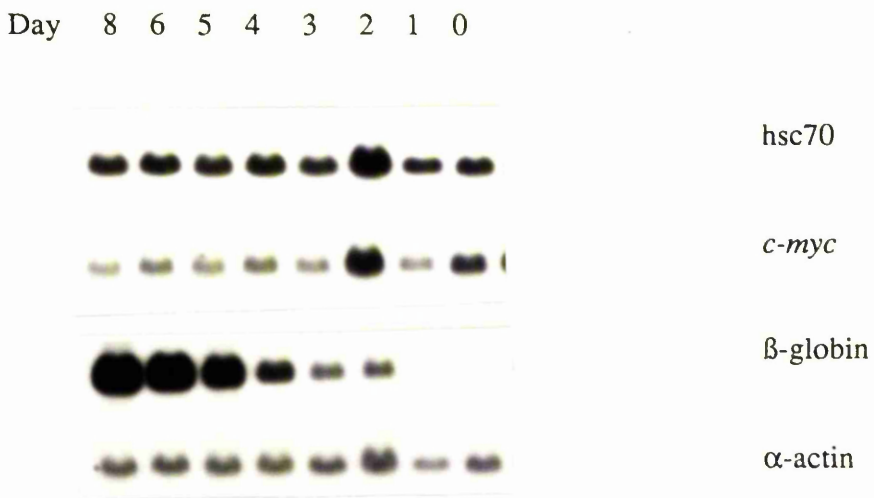


Figure 3.25: Northern blot analysis of total RNA from FDCP-mix (clone A4) cells during the erythrocytic differentiation in the presence of EPO (3U/ml), IL-3 (1.5U/ml) and haemin (0.2mM). Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The blot was hybridized successively with [^{32}P]-labelled probes for hsc70, *c-myc*, ß-globin and α-actin (RNA was a gift from Dr. M. Cross).
Each lane was loaded with 5μg of total RNA.

3.8: ERYTHROCYTIC DIFFERENTIATION OF MEL CELLS

3.8.1: Introduction

MEL cells were differentiated along the erythrocytic lineage by exposure to concentrations of DMSO varying from 1.5% (v/v) to 2% (v/v) (Friend *et al*, 1971; Gusella *et al*, 1976, Wingrove *et al*, 1988). In 1.5% (v/v) or 2% (v/v) of DMSO, MEL cells were differentiated over a period of 4-7 days and reached between 80% and 95% of benzidine positive cells (Friend *et al*, 1971; Rutherford and Weatherall, 1979; Raaphorst *et al*, 1984). Moreover Friend *et al* (1971) showed that MEL cells which were incubated for 4 days with 2% (v/v) DMSO cycled three times regardless if they were further exposed or not to DMSO. Hensold and Housman (1988) showed that the expression of hsc70/hsp70 (not distinguished) decreased after 9 hours and again after 48 hours of differentiation with 1.5% (v/v) DMSO. At 4, 15 and 24 hours, hsc70/hsp70 expression was at the same level as the undifferentiated cells. In these experiments, MEL cells were differentiated using the conditions of Hensold and Housman (1988) to establish whether hsc70 expression changed specifically and to compare it with hsc70 expression using FDCP-mix (clone A4) cells.

3.8.2: DMSO-induced MEL cell differentiation

In these experiments, 1.5% (v/v) DMSO induced most of cells to divide at least 5 fold in 4 days. After day 4, the cell population decreased progressively until the end of the differentiation (Figure 3.26a). By day 3, 5% of the total cell population were producing haemoglobin (as shown by benzidine staining) while by day 7, 95% of cells were benzidine positive. Differentiated MEL cells are not enucleated (Figure 3.26b).

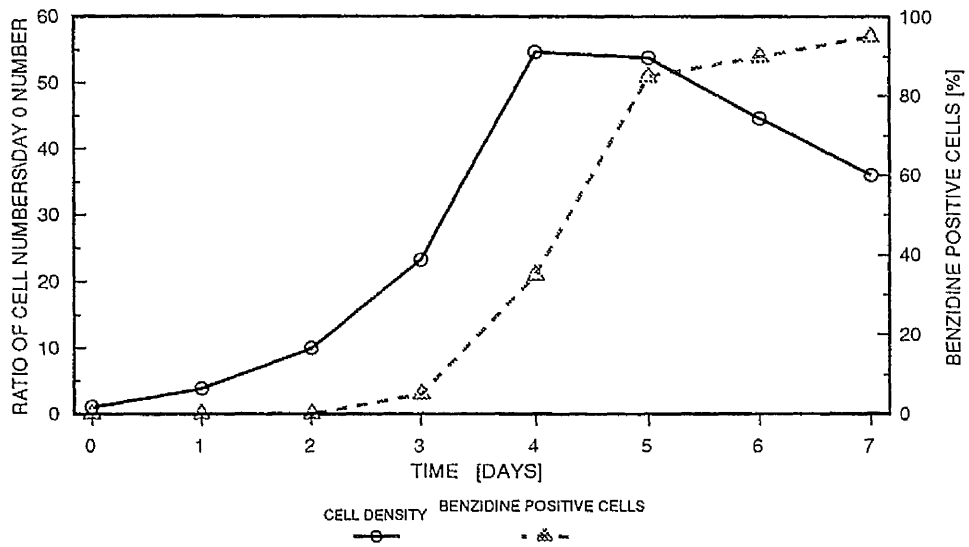
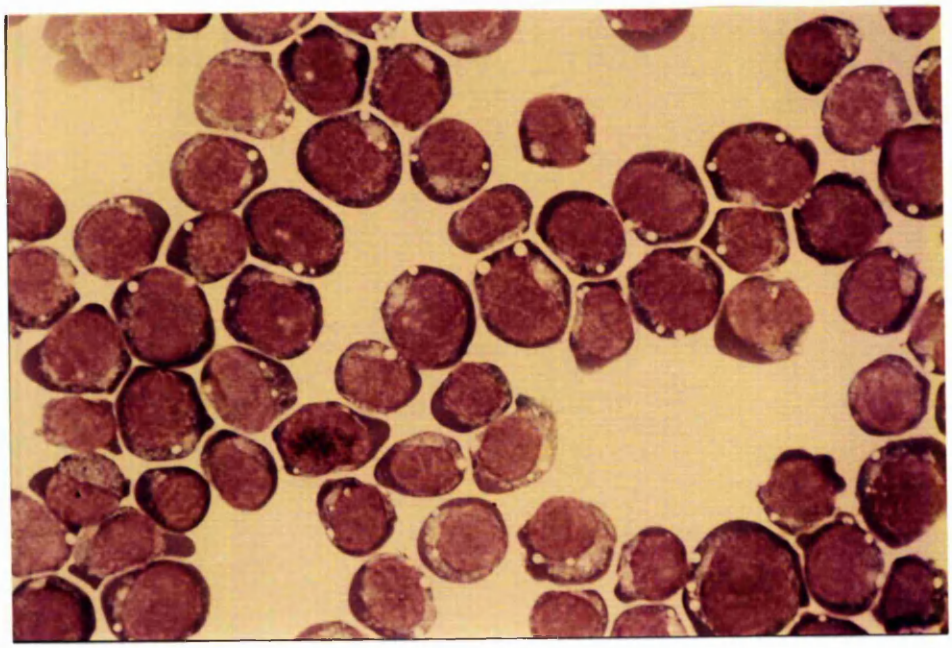


Figure 3.26a: Measurement of the cell density and the number of benzidine positive cells during MEL cell differentiation in the presence of 1.5% (v/v) DMSO. Day 0 value for the cell density was $4x10^4$ cells/ml. Cell density was expressed as fold increase and assessed by trypan blue exclusion test. Numbers of benzidine positive cells were expressed as a percentage. Each point represents the average of three experiments.

Day 0 (Erythroblasts: Undifferentiated MEL cells)



Day 6 (Differentiated MEL cells)

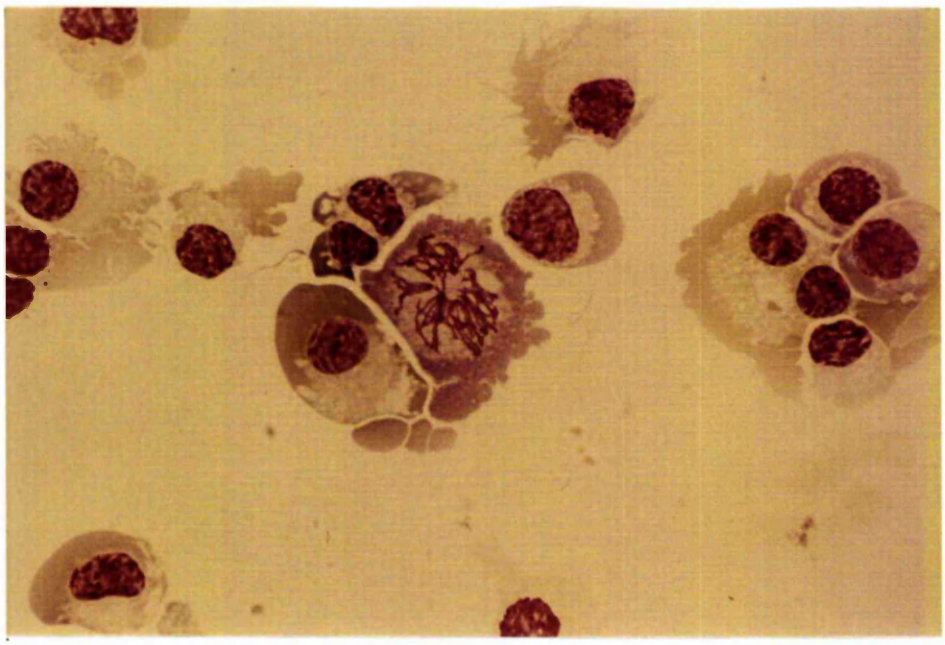


Figure 3.26b: Benzidine staining of MEL cells differentiated in the presence of 1.5% (v/v) DMSO. At days 0 (shown at top) and 6 (shown at bottom) of the differentiation, the cells were stained with benzidine and May-Grunwald and Giemsa solutions. Magnification (600x).

3.8.3: Western blot analysis of hsc70 protein

Western blot analysis demonstrated no change in the level of hsc70 protein expression during the differentiation (Figure 3.27). This result differed from data of Hensold and Housman (1988) who observed decreases in hsc70/hsp70 following 9 and 48 hours of differentiation. The inducible heat shock protein, hsp70, was not expressed during MEL cell differentiation (data not showed). The absence of hsp70 expression is predicted by the data of Hensold *et al* (1990) who showed that MEL cells are unable to express this protein.

3.8.4: Measurement of total protein synthesis during MEL cell differentiation

In this study, DMSO induced MEL cell differentiation resulted in 40% reduction of total protein synthesis as measured by the [^{35}S]-methionine into total protein per cell after 24 hours (Figure 3.28). Following this time, the total level of protein synthesis decreased gradually to reach 14%, by day 6.

3.8.5: Northern blot analysis of hsc70, ß-globin and α-actin RNA

Northern analysis of total RNA isolated from cells at various time points in the first 48 hours, showed that hsc70 expression did not change during differentiation (Figure 3.29). ß-globin expression (marker of the differentiation) was detectable at day 0 and increased by day 2 (2-5 fold), reached a maximum by day 4 and decreased slightly by days 6-7 (2 fold) (Figure 3.29). Equal loadings of RNA was confirmed using an α-actin gene probe (Figure 3.29).

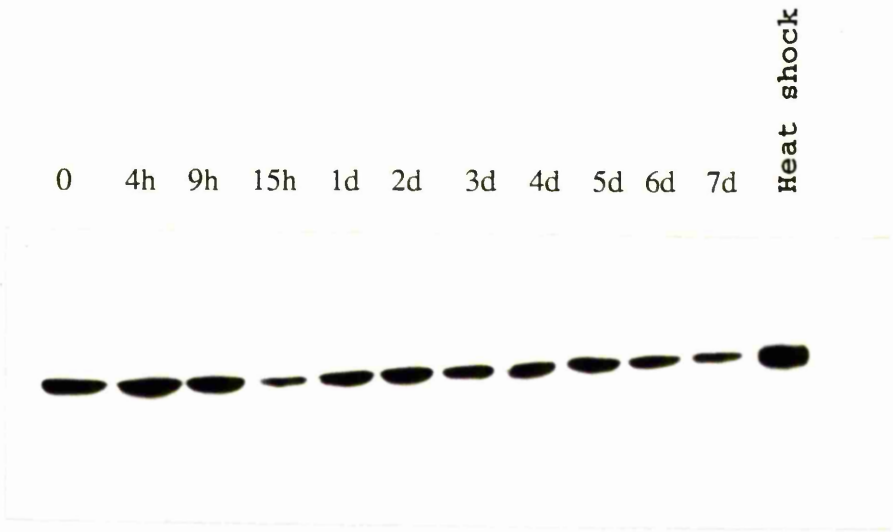


Figure 3.27: Western blot analysis of the total proteins from MEL cells during the 168 hours of differentiation in the presence of 1.5% (v/v) DMSO. Total proteins were resolved by 7.5% PAGE. The filter was probed successively with monoclonal antibody 3A3 (hsc70/hsp70). Time of differentiation is expressed in hour (h) or day (d). Lane heat shock consists of total protein from FDCP-mix (clone A4) cells heat shocked at 42°C for 1 hour and allowed to recover for 2 hours.
Each lane was loaded with 40µg of total proteins.

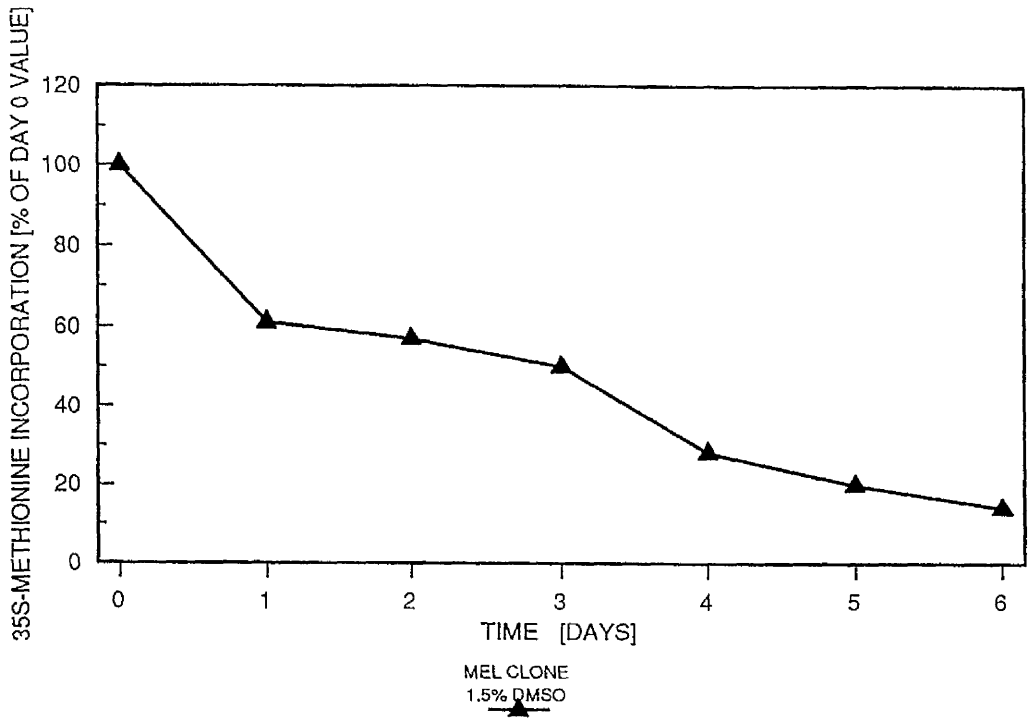


Figure 3.28: Decrease of incorporation of [^{35}S]-methionine during MEL cell differentiation. *In vitro* incorporation of [^{35}S]-methionine was performed during 6 days of DMSO [1.5% (v/v)]-induced differentiation. Each point represents the average of three experiments.

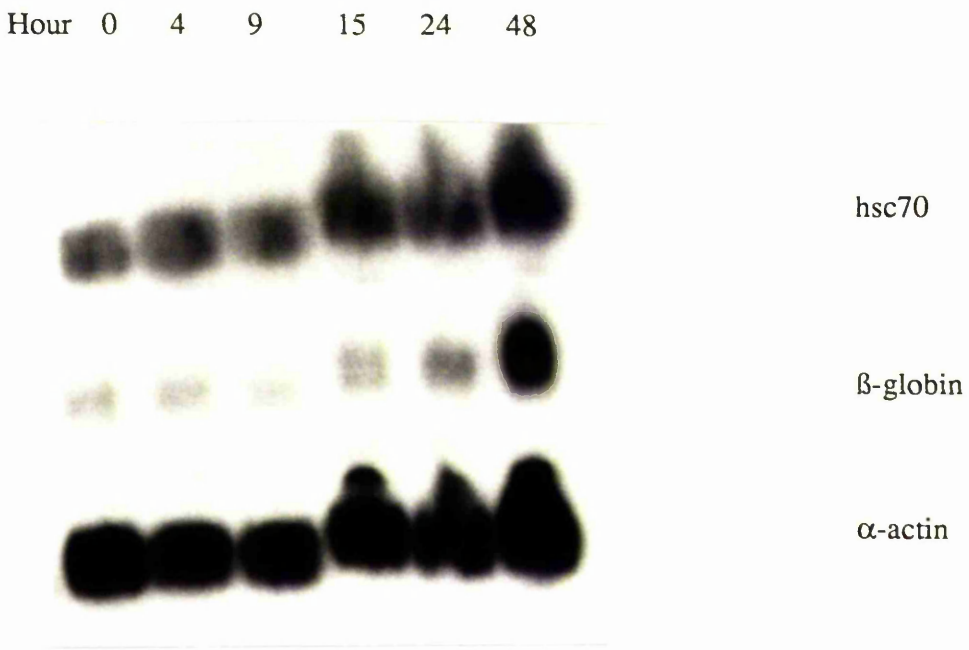


Figure 3.29: Northern blot analysis of the total RNA from MEL cells during the first 48 hours of the differentiation in the presence of 1.5% (v/v) DMSO. Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The same Northern blot was hybridized successively with [^{32}P]-labelled probes for hsc70, ß-globin and α-actin. Each lane was loaded with 5µg of total RNA.

3.8.6: Does *c-myc* expression change during MEL cell differentiation?

Previous data (Coppola *et al*, 1989) show that following treatment of MEL cells with DMSO, *c-myc* expression falls rapidly in the first 24 hours and then returns to basal level after 48 hours. Other workers reported a progressive decrease in *c-myc* expression, as terminally differentiated benzidine-positive cells accumulated after 24 hours (Lachman and Skoultchi, 1984). Coppola and Cole (1986) had previously shown that following transfection of MEL cells with retrovirus containing the *c-myc* gene, the overexpression of *c-myc* protein blocked DMSO-induced differentiation, suggesting the early decrease in *c-myc* expression may trigger the differentiation. On the other hand, it has been reported that during MEL cell differentiation (with DMSO), the early decline in *c-myc* expression has no significant effect on the cellular concentration of *c-myc* protein (Wingrove *et al*, 1988). The same workers reported that *in vitro* translation of the *c-myc* RNA resulted in synthesis of two isoforms of the *c-myc* protein and proposed that one isoform may play a role during cell growth and the other during differentiation. However MEL cells treated with EPO show an increase in *c-myc* RNA, but do not differentiate: A result which suggests that the changes in *c-myc* expression could also be a consequence of the differentiation inducer.

In the study presented here, the *c-myc* expression decreased after 4 hours of differentiation before returning to basal levels after 24 hours. Following a further 144 hours of differentiation, the constitutive expression of *c-myc* had not changed significantly, when compared to untreated cells.

3.8.7: Conclusions: Erythrocytic differentiation of MEL cells

Friend *et al* (1971) reported that the differentiation of MEL cells with 2% (v/v) DMSO cells was accompanied by proliferation, with cells cycling 3 times during the 4 days of differentiation. In the experiment presented here, proliferation and differentiation of MEL cells were also coupled for 4 days (Table 3.5). In 4 days, there were a 5 fold increase in cell number, and thereafter most of cells stop proliferating and complete the differentiation to erythrocytes (benzidine positives).

At both protein and RNA levels, no change in hsc70 expression was observed (Table 3.5). These data on MEL cells do not correspond to the work of Hensold and Housman (1988). They observed an early fall in the synthesis of a 70kDa heat shock protein (it was not possible to discriminate hsc70 from hsp70 at that time) and RNA levels following 9 hours of incubation, returning to the basal level of expression by 24 hours, and decreasing again after 48 hours of differentiation. They did not show the level of hsc70/hsp70 expression after 48 hours of differentiation. The probable explanation for this observation is that the MEL cells in these experiment were a different subclone. For several reasons, the results suggest this conclusion: firstly the time of the differentiation of MEL cells, to have a score of 95% of cells stained benzidine positive, was 7 days rather than the 3 days reported by Hensold and Housman (1988). Secondly, unlike the data of Hensold (1990), hsp70 RNA accumulates under normal growth in the MEL cells. Other studies on hsp70 RNA confirmed that it is not detectable in non stressed cells because hsp70 RNA is stable only after stress (Theodorakis and Morimoto, 1987). The sequences in the 3'untranslated region (UTR) of the hsp70 message play a critical role in this regulation. It has been shown in *Drosophila* cells that the rapid degradation of hsp70 messages at normal temperatures operated through recognition of the hsp70 3' UTR (Petersen and Lindquist, 1989). It may be possible that the probes I used to detect hsp70 RNA also detected hsc70 RNA, in both heat shocked and normal MEL cells.

It has been reported that the level of expression of *c-myc* RNA decreased sharply before the first 24 hours of differentiation of MEL cells with DMSO, returned to the level of basal expression at 24 hours until 48 hours (Coppola *et al*, 1989; Hensold and Housman, 1988), to decrease below the basal level until the end of the differentiation (Lachman and Skoultchi, 1984). These laboratories reported a decrease in *c-myc* RNA expression in differentiated MEL cells, but none of them showed any data, nor quantify the decrease of *c-myc* RNA. However, in the work presented here, no major change (only 2-3 fold decrease) in *c-myc* RNA expression was detected at day 7, in terminal differentiated MEL cells (erythrocytes), when compared to untreated cells (Table 3.5).

Hours	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	ß-globin RNA	Benzidine positive (%)	Cell growth Ratio of cell numbers/day 0 number
0	BE	BE	BE	BE	0	1.0
4	BE	BE	↓↓↓↓	BE	0	1.2
9	BE	BE	↓↓	BE	0	1.9
15	BE	BE	BE	BE	0	2.3
24	BE	BE	BE	BE	0	3.8
48	BE	BE	BE	↑↑	0	10.0
72	BE	BE	↓↓	↑↑↑	5	23.3
96	BE	BE	↓↓	↑↑↑↑	35	54.7
120	BE	BE	↓↓	↑↑↑↑	85	53.8
144	BE	BE	↓↓	↑↑↑	90	44.6
168	BE	BE	↓↓	↑↑↑	95	36.0

Table 3.5: Summary of erythrocytic differentiation of MEL cells in the presence of 1.5% (v/v) DMSO.
Increase of expression: ↑↑ (2-5 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold).
Decrease of expression:↓↓ (2-3 fold), ↓↓↓↓ (>10 fold).

3.9: ERYTHROCYTIC DIFFERENTIATION OF FDCP-mix (*bcl-2*, clone 2J) CELLS

3.9.1: Introduction

Hsc70 expression at both protein and RNA levels did not change significantly in either the erythrocyte differentiation of FDCP-mix (clone A4) or MEL cells. In both of these systems differentiation is coupled to proliferation. It was therefore of interest to determine if the absence of growth factors or chemical stimuli during differentiation would affect the expression of hsc70. This was achieved by using FDCP-mix cells transfected with the human *bcl-2* gene (clone 2J), which have been shown to be erythroid differentiation in the absence of growth factor and without cell division (Fairbairn *et al*, 1993).

3.9.2: Measurement of cell density, primitive cell numbers and morphology

After removing the IL-3 from the culture medium of FDCP-mix (*bcl-2*, clone 2J) cells, the cell population showed 40-50% increase in cell number on day 1 compared to day 0, which then remained constant until day 6 (Figure 3.30). Colony-forming cells (Figure 3.30), which were measured each day during differentiation, demonstrated a rapid decrease to 31% of clonogenic cells after 3 days. The morphology of the cell populations during differentiation is shown in figures 3.31a and 3.31b and confirmed data of Fairbairn *et al* (1993)

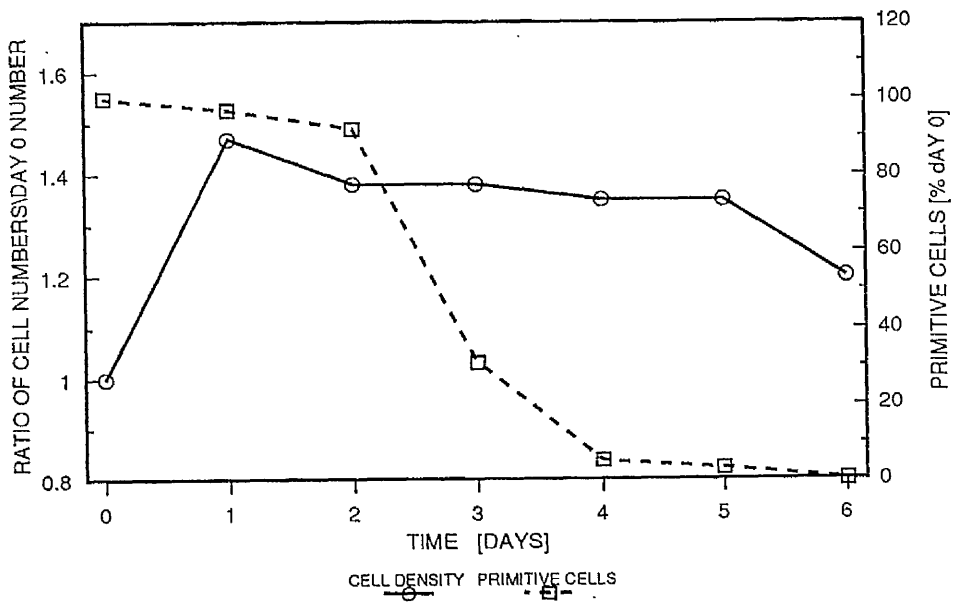


Figure 3.30: Measurement of the cell density and primitive cell numbers of FDCP-mix (*bcl-2*, clone 2J) cells during the erythrocytic differentiation in the absence of growth factors and haemin. Day 0 value for the cell density was $5x10^5$ cells/ml, and for the clonogenic assays, 195 colonies. Cell density was expressed as fold increase of the day 0 value. Numbers of primitive blast cells was expressed as a percentage of the day 0 number. The cell density was assessed by trypan blue exclusion test. Each point represents the average of three experiments.

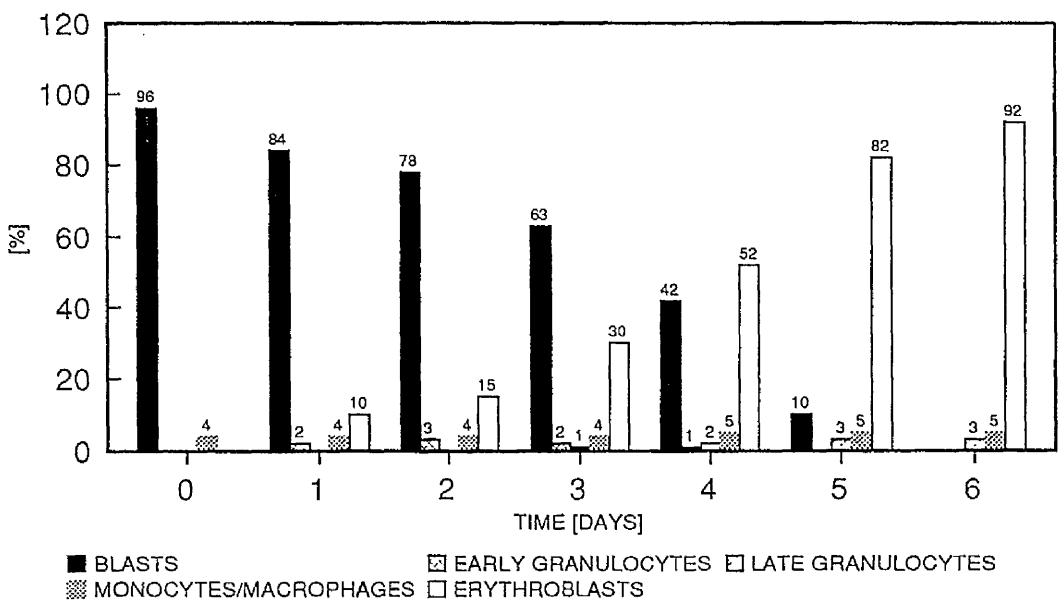
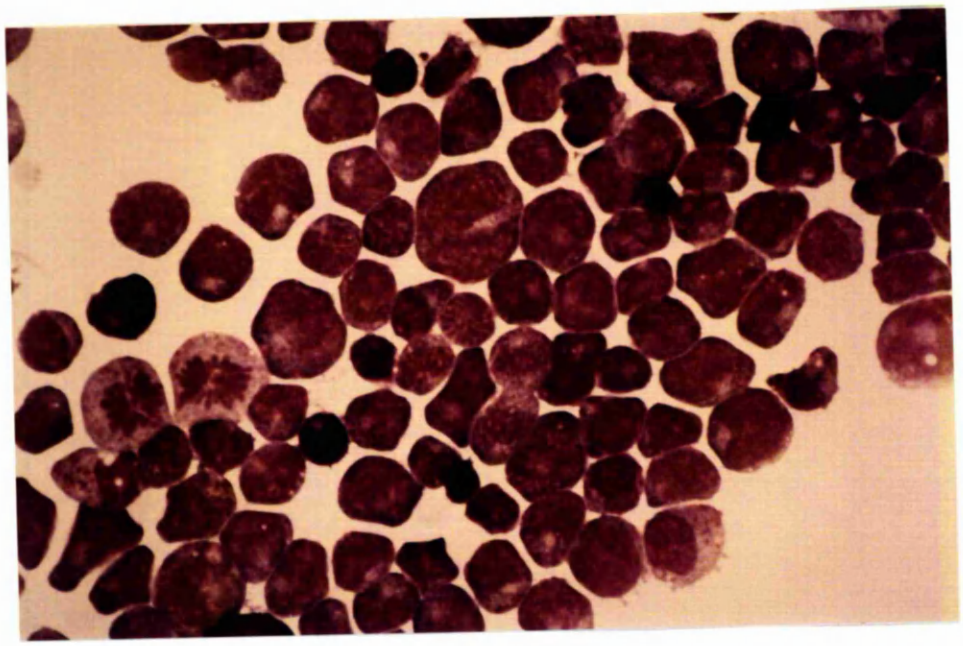


Figure 3.31a: The morphology of FDCP-mix (*bcl-2*, clone 2J) during the erythrocytic differentiation in the absence of growth factors. The cells were stained with benzidine and May-Grunwald-Giemsa stains. Each point represents the average of three experiments.

Day 0 (blasts: Undifferentiated cells)



Day 6 (erythroblasts)

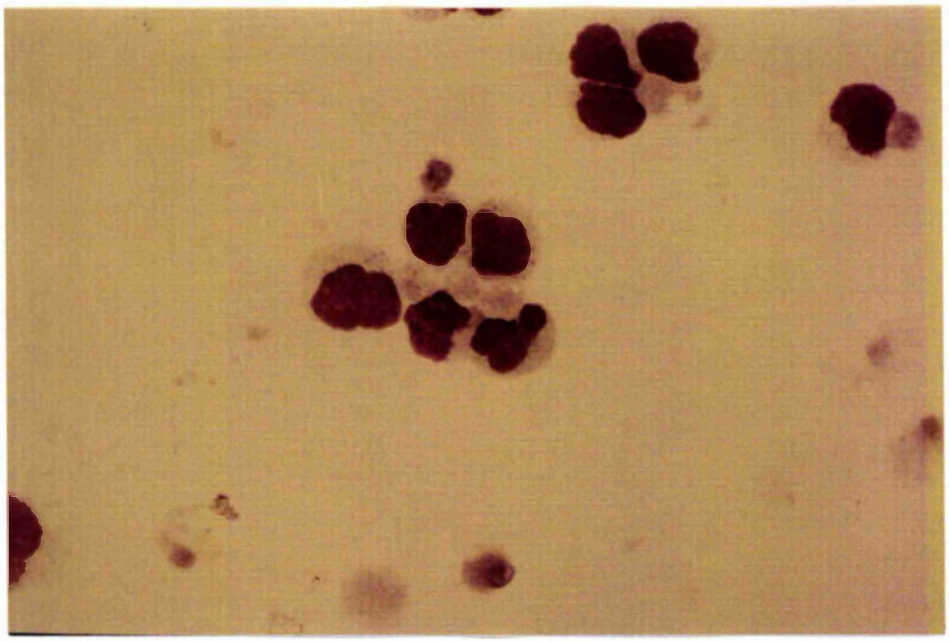


Figure 3.31b: Benzidine staining of FDCP-mix (*bcl-2*, clone 2J) during the erythrocytic differentiation in the absence of growth factors and haemin. The cells were stained at days 0 (shown at top) and 6 (shown at bottom) with benzidine and May-Grunwald and Giemsa stains. Magnification (600x).

3.9.3: Western blot analysis of hsc70, *bcl-2*, globin and actin proteins

Western blot analysis of total protein extracts showed that hsc70 expression decreased by day 1 and remained at low levels until day 6 (Figure 3.32). During the time course, the expression of the inducible hsp70 was not detected. Interestingly, no globin protein was observed at any time in the differentiation time course, yet controls (day 9 of the erythrocytic differentiation of FDCP-mix (clone A4) and MEL cell differentiation (day 7) confirmed the transfer of protein and the specificity of the antibody against mouse globin (Figure 3.32). Human *bcl-2* expression also decreased rapidly but was still detectable by day 6. Equal loading was determined by the actin expression (Figure 3.32).

3.9.4: Northern blot analysis of hsc70, ß-globin, *c-myc* and α-actin RNA

Expression of hsc70 did not change during the 9 days of differentiation (Figure 3.33), *c-myc* expression decreased dramatically (more than 10 fold) at day 1, when most cells stop dividing, and remained barely detectable until the end of the differentiation (Figure 3.33). The constitutive expression of the ß-globin RNA was detectable at day 0, reached a maximum of expression at day 4 (more than 10 fold) and then decreased slightly (Figure 3.33). Equal loading between samples was assessed using an α-actin gene probe (Figure 3.33).

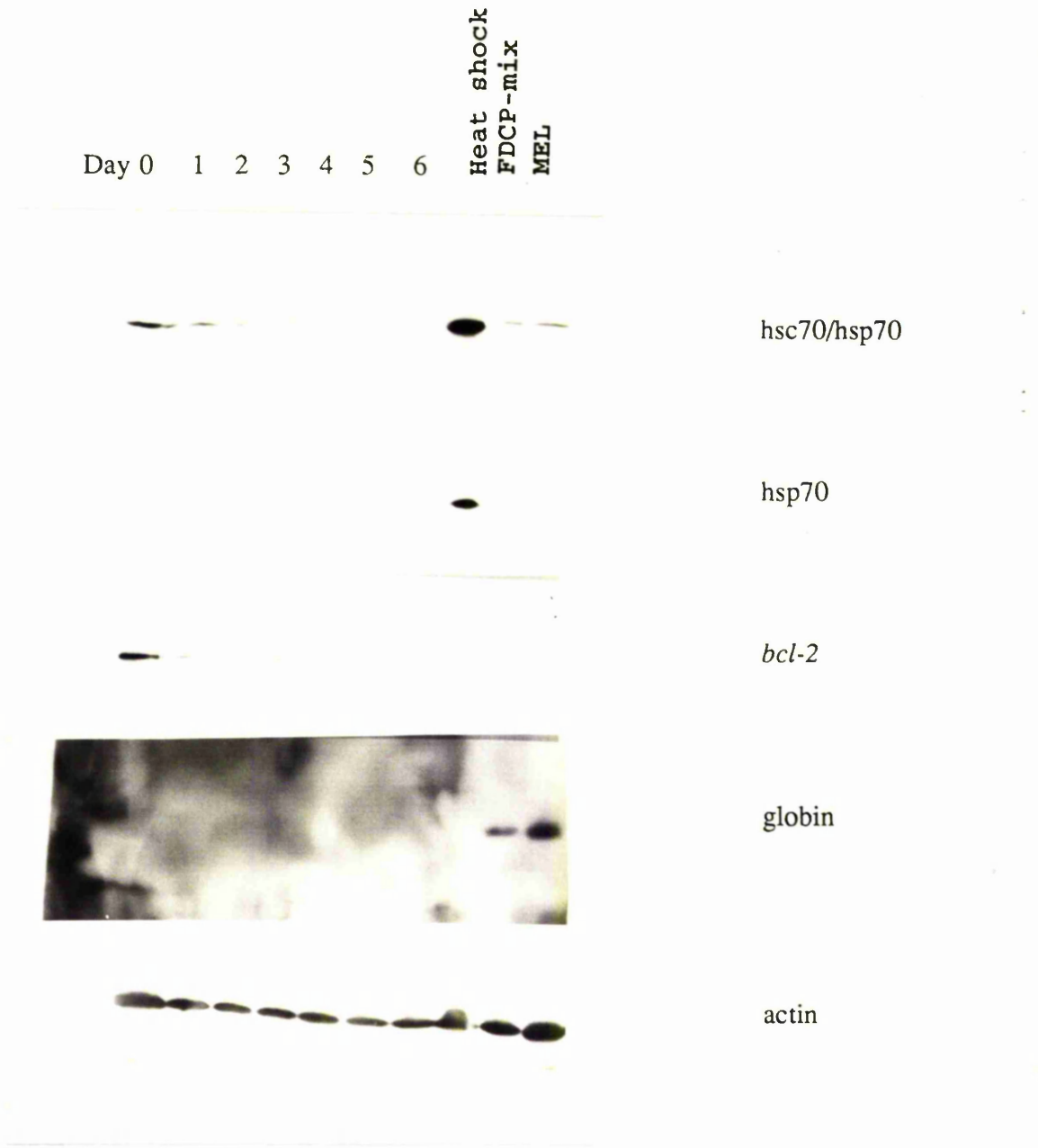


Figure 3.32: Western blot analysis of the total proteins from FDCP-mix (*bcl-2*, clone 2J) during the erythrocytic differentiation in the absence of growth factors and haemin. Total proteins were resolved by 7.5% or 12.5% PAGE. The blot (7.5%) was probed successively with monoclonal antibodies anti hsp70 (4G4), hsc70/hsp70 (3A3) and actin. Another blot (12.5%) was probed successively with polyclonal antibody anti globin and monoclonal antibody anti human *bcl-2*. Lane heat shock consists of total protein extracted from FDCP-mix (clone A4) cells heat shocked for 1 hour at 42°C and allowed to recover for 2 hours. Lane FDCP-mix consists of total proteins extracted at day 9 from erythrocytic FDCP-mix (clone A4) cells (positive control) and lane MEL consists of total proteins extracted at day 6 from erythrocytic MEL cells (positive control).
Each lane was loaded with 40μg of total proteins.

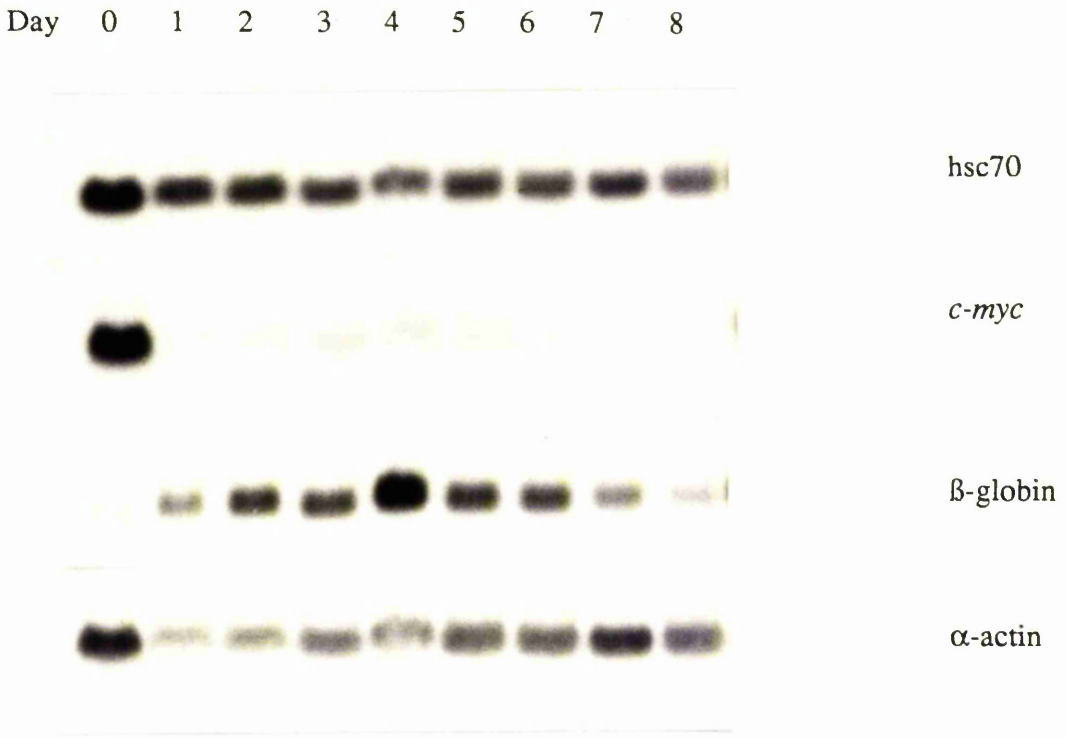


Figure 3.33: Northern blot analysis of total RNA from FDCP-mix (bcl-2, clone 2J) cells during the erythrocytic differentiation in the absence of growth factors and haemin. Total RNA was resolved on formaldehyde-agarose (1%) gel and transferred to Hybond-N. The same blot was hybridized successively with [^{32}P]-labelled probes for hsc70, *c-myc*, ß-globin and α-actin. Each lane was loaded with 5μg of total RNA.

3.10: DISCUSSION: ERYTHROCYTIC DIFFERENTIATION OF FDCP-mix CLONES AND MEL CELLS

3.10.1: Possible role of hsc70

In reticulocytes or erythrocytes, hsp70/hsc70 may regulate a protein involved in differentiation, such as the haeme regulated kinase (HRI). It has been demonstrated using rabbit reticulocyte lysates, that a complex of proteins including p56, hsp90 and hsc73 regulates the activation of the HRI (Matts and Hurst, 1992; Matts *et al*, 1992; Figure 3.34), which in turn regulates globin synthesis. In the presence of haemin, this enzyme (HRI) is inactivated by a complex formed by haemin, hsp90, hsc73 (similar to hsc70) and p56 proteins, and globin is synthesized. In the absence of haemin or hsc73, HRI phosphorylates the α-subunit of the eukaryotic initiation factor eIF-2α (Fagard and London, 1981; Trachsel *et al*, 1978). The phosphorylation of this initiation factor results in the arrest in the initiation of translation of the globin transcripts. Further study on HRI in a mouse cell line, MEL supports this *in vitro* experiment using rabbit reticulocyte lysates (Sarre *et al*, 1989). These authors observed that differentiated MEL cells respond to iron depletion with a shut-off of protein synthesis, whilst non differentiated MEL cells do not. Therefore the high constitutive expression of hsc70, which is observed during the erythroid differentiation of MEL (section 3.8.7; Table 3.5), FDCP-mix (Table 3.6) cells, and in purified human red cells (Gromov and Celis, 1991) suggests that its expression may still be necessary to chaperon some specific intracellular proteins such as the haeme-regulated kinase. Other proteins may be chaperoned by hsc70 in erythrocytes, and thus further investigations may be necessary to identify these proteins.

These observations contrast the erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J), where no hsc70 or globin expression was detected (Figure 3.32), confirming that expression of both proteins may be associated.

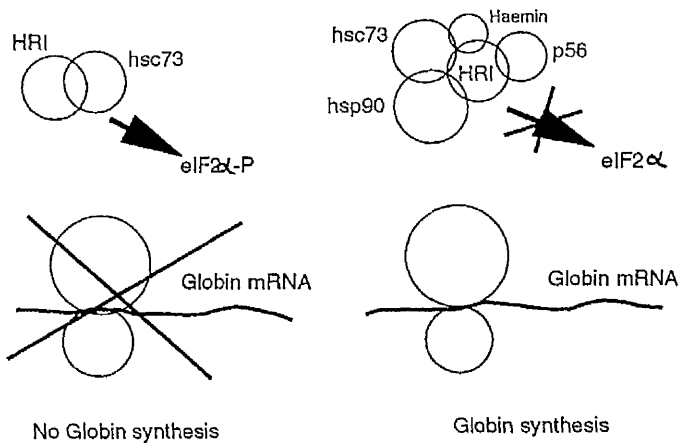


Figure 3.34: Regulation of the synthesis of globin protein (Matts *et al*, 1992). The complex hsc73-haeme kinase regulated (HRI) phosphorylates the initiation factor eIF2α. The phosphorylation blocks the globin synthesis. In the presence of haemin, a complex of proteins (hsp90, hsc73 and p56) inactivates HRI. The initiation factor is not phosphorylated and therefore globin synthesis occurs. When hs73 is removed, globin synthesis is stopped.

Day	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	ß-globin RNA	Morphology (%)	Primitive cells (% day 0)	Cell growth Ratio of cell numbers/day 0 number
0	BE	BE	BE	-	B:100	100	1.0
2	BE	BE	BE	↑	B:93,EG:2 EB:5	70	2.5
5	BE	BE	BE	↑↑↑	EG:12,EB:75 BP:13	10	6.5
7	BE	BE	BE	↑↑↑↑	EB:63,BP:34 M:3	0	7.5
9	BE	BE	BE	↑↑↑↑	EB:55,BP:45	0	6.2

Table 3.6: Summary of erythrocytic differentiation of FDCP-mix (clone A4) cells in the presence of EPO (3U/ml), Il-3 (1.5U/ml) and haemin (0.2mM). B:blasts, EG:early granulocytes, EB:erythroblasts, BP:benzidine positive, M:monocytes/macrophages, BE:basal expression. Increase of expression: ↑ (<2 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold), - (not detected).

3.10.2: Stress and erythrocytic differentiation of haemopoietic cells

In contrast to the differentiation of FDCP-mix clones [clone 2J (*bcl-2*) and clone A4] and MEL cells (see following section) along the erythrocytic lineage, where no hsp70 expression was observed, Singh and Yu (1984) showed an increase of a 70,000 molecular weight heat shock protein and its transcripts during the first 24 hours of the erythroblast differentiation of a human K562 leukaemic cell line induced with haemin. Theodorakis *et al* (1989) identified an increase in the inducible heat shock protein, hsp70 during K562 differentiation, in the presence of the same concentration of haemin. This increase in hsp70 expression was accompanied by an increase in the binding of a stress inducible transcription factor, heat shock transcription factor (HSF) on the heat shock element (HSF) of hsp70 promoter (section 1.2.2). This result suggests that the presence of haemin (30μM) may stress K562 cells, resulting in the expression of the specific stress protein, hsp70. In the presence of a higher concentration of haemin (0.2mM), the erythrocytic differentiation of FDCP-mix (clone A4) cells, did not induce hsp70 expression, suggesting that K562 cells may be more sensitive to haemin than FDCP-mix cells. The absence of hsp70 expression during the erythrocytic differentiation of FDCP-mix clones [clone 2J (*bcl-2*) and clone A4] and MEL cells, shows that its expression is not required to differentiate these cell lines. It may also be suggested that the increase of hsp70 expression observed during the first 24 hours of haemin-induced K562 differentiation, is triggered by an early increase in globin synthesis (from days 0 to 4), the number of benzidine positive K562 cells increasing sharply after day 2 (Cioe *et al*, 1981). In FDCP-mix and MEL cells, the number of benzidine positive cells increased at a later stage, days 5 and 3 respectively (Tables 3.5 and 3.6). The later appearance time of benzidine positive cells is probably due to the complexity of the formation of haemoglobin (α and ß chains) in these cell lines. In contrast to FDCP-mix and MEL cells, K562 cells synthesize mainly an embryonic haemoglobin (α chain) (Rutherford *et al*, 1981; Cioe *et al*, 1981). In previous work, it has

been reported that hsc70 and hsp70 have similar role in the cells (Bassford and Collier, 1988; Beckmann et al, 1990; Hartl and Neupert, 1990). In contrast to hsc70, hsp70 has no intron (Dworniczak and Mirault, 1987), and thus it may be suggested that hsp70 can be expressed more rapidly than hsc70 to respond faster to a need in the environment such as globin synthesis. For these reasons, globin expression may be "sensed" by K562 cells as a stress, leading to an increase in hsp70 expression in these cells.

3.10.3: Cell survival and proliferation leading to differentiation

EPO has been suggested to stimulate proliferation and increase the amplification of the pre-erythropoietin responsive cells (pre ERC) compartment (reviewed by Lord, 1976; Wangenheim et al, 1977) thus accounting for the extra cell divisions calculated by Lejtha *et al* (1971). The EPO dependent period extends from at least the colony-forming unit-erythroid (CFU-E) stage to the stage at which haemoglobin synthesis begins (Stephenson *et al*, 1971; Koury and Bondurant, 1988). Cells infected with Friend virus anaemia-inducing (FVA) and cultured without EPO did not proliferate, contained fragmented DNA (Koury *et al*, 1990). These authors suggest that EPO controls erythrocyte production by retarding DNA cleavage, thereby altering the course of erythroid progenitor cells toward cell death.

The work presented here confirms that during erythrocytic differentiation, EPO is necessary for cell proliferation and the synthesis of haemoglobin in FDCP-mix (clone A4) cells (Figures 3.22 and 3.32). During erythrocytic differentiation of MEL cells, in the absence of growth factors, but in the presence of 1.5% (v/v) DMSO, proliferation was coupled to globin synthesis. In contrast, erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J), in the absence of growth factors, did not lead to cell proliferation and globin synthesis (Table 3.7), suggesting; (1) that the survival signal promoted by *bcl-2* did not allow the cells to be fully mature, and (2) EPO is necessary to complete the differentiation

into mature erythrocytes. These also results show that proliferation is required to synthesize haemoglobin and differentiate haemopoietic cells, thus confirming the work of other done in other laboratories (Lord, 1976; Wangenheim *et al*, 1977; Lejtha *et al*, 1971).

Hsc70 expression, a hypothesized marker of cell survival (section 3.5.3.2), did not decrease during the erythrocytic differentiation of FDCP-mix (clone A4), in the presence of growth factors. In contrast, during the erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J), in the absence of growth factors, a dramatic decrease of hsc70 expression was observed, and yet *bcl-2*, another survival signal discussed earlier, was expressed in this cell line. This result confirmed previous observations (section 3.5.3), which showed that in contrast to normal haemopoietic cells, survival signals such as hsc70, in *bcl-2* transfected cells may be downregulated during erythrocytic differentiation. This suggests that EPO stimulated cell survival, but signalling pathway is probably not similar to that by *bcl-2* in *bcl-2* transfected FDCP-mix cells. The downregulation of a survival signal such as occurs during differentiation of FDCP-mix (*bcl-2*, clone 2J) cells, in the absence of growth factors, may be due directly or indirectly to human *bcl-2* expression; This remains to be elucidated.

3.10.4: Regulation of hsc70

3.10.4.1: Transcriptional or translational regulation of hsc70

During erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J), in the absence of growth factors, hsc70 protein expression decreased dramatically in contrast to hsc70 RNA, which did not change. The erythrocytic differentiation confirms previous experiments (section 3.5.5.1), showing that hsc70 is regulated at the translational or post-translational levels, suggesting that the regulation of hsc70 is not similar to hsp70, which is transcriptionally activated (section 1.2.2).

Elsewhere (section 3.5.5.1), it was suggested that *c-myc* may not regulate hsc70 RNA expression. The differentiation of FDCP-mix (*bcl-2*, clone 2J), in the absence of

Day	Hsc70 protein	Hsc70 RNA	*c-myc* RNA	Globin protein	ß-globin RNA	Morphology (%)	Primitive cells (% day 0)	Cell growth Ratio of cell numbers/day 0 number
0	BE	BE	BE	-	BE	B:96,M:4	100	1.0
1	↓↓↓	BE	↓↓↓↓	-	↑↑	B:84,EG:2 EB:10,M:4	97	1.4
2	↓↓↓	BE	↓↓↓↓	-	↑↑	B:78,EG:3 EB:15,M:4	92	1.4
3	↓↓↓	BE	↓↓↓↓	-	↑↑↑	B:63,EG:2 LG:1,EB:30 M:4	31	1.4
4	↓↓↓	BE	↓↓↓↓	-	↑↑↑↑	B:42,EG:1 LG:2,EB:50 M:5	5	1.3
5	↓↓↓	BE	↓↓↓↓	-	↑↑↑	B:10,LG:3 EB:82,M:5	3	1.3
6	↓↓↓	BE	↓↓↓↓	-	↑↑↑	EB:92,M:5 LG:3	0	1.2

Table 3.7: Summary of the erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J) in the absence of growth factors. B:blasts, EB:erythroblasts, LG:late granulocytes, M:monocytes/macrophages, BE:basal expression. Increase of expression:↑↑ (2-5 fold), ↑↑↑ (5-10 fold), ↑↑↑↑ (>10 fold). Decrease of expression:↓↓↓ (5-10 fold), ↓↓↓↓ (>10 fold). (-):not detected

growth factors, showed that in contrast to hsc70 RNA expression, *c-myc* RNA decreased severely, suggesting that the expression of both genes is not co-ordinately regulated. In MEL cells treated with EPO and cycloheximide, a protein synthesis inhibitor, an increase in *c-myc* RNA expression was reported (Chern *et al*, 1991). However MEL cells which are treated with EPO do not differentiate (Rifkind and Marks, 1978; Chern *et al*, 1991). Moreover in mouse spleen cells rich in erythroid progenitors and in HEL cells, EPO and haemin have been demonstrated to increase the level of *c-myc* expression within 24 hours of stimulation (Spangler and Sytkowski, 1992; Griffin *et al*, 1990). These data show that EPO may activate *c-myc* and hsc70 expression.

3.10.4.2: Role of EPO and haemin

During the erythrocytic differentiation of FDCP-mix (clone A4), in the presence of EPO, IL-3 and haemin, hsc70 protein expression did not change (Table 3.6). In contrast, a dramatic decrease of hsc70 protein expression was observed by day 1 of the differentiation of FDCP-mix (*bcl-2*, clone 2J), in the absence of growth factors. It has been suggested previously that IL-3 does not influence hsc70 protein expression (section 3.5.5.4), suggesting that during erythrocytic differentiation, EPO and/or haemin may affect hsc70 protein expression. In order to study the possible role of EPO and haemin in hsc70 expression, FDCP-mix (*bcl-2*, clone 2J) cells were incubated with EPO and/or haemin. No benzidine positive cells were obtained in the presence of EPO and/or haemin and Western blot analysis showed that, in contrast to FDCP-mix (clone A4) and MEL cells, no globin protein was synthesized by FDCP-mix (*bcl-2*, clone 2J) in the presence or absence of EPO and/or haemin (Figure 3.32; Table 3.7). Whilst no globin protein was detected, globin RNA expression increased during FDCP-mix (*bcl-2*, clone 2J), suggesting that globin expression was blocked at the translational level. The absence of globin synthesis in these cells suggests that following transfection human *bcl-2* in FDCP-mix (clone A4), this cell

line has been blocked in the erythrocytic pathway and therefore can not be differentiated until the erythrocytic stage (formation of haemoglobin and enucleation). However it is acknowledged that differentiation potential of the cell line may have changed following several months in culture.

3.10.4.3: Cell proliferation

During the erythrocytic differentiation of FDCP-mix (clone A4) and MEL cells, differentiation was coupled to proliferation for 5-7 and 4 days respectively, and after, a decrease in cell number was observed until the end of the differentiation (sections 3.7.2 and 3.8.2). However, following the decrease in cell number, hsc70 expression did not change. As has already been reported elsewhere (section 3.5.5.3), these results indicate that hsc70 expression is not associated with cell proliferation. (see the end of section 3.10.4.3).

3.10.4.4: Is hsc70 expression associated with total protein synthesis?

In order to determine whether the change in hsc70 expression was associated with changes in total protein synthesis, the incorporation of [^{35}S]-methionine into the total proteins fraction was analyzed only during MEL cell differentiation: There was a 40% decrease in incorporation of the radio-labelled amino-acid over the time course of differentiation (Figure 3.28).

Other laboratories have also observed a reduction in protein synthesis during MEL cell differentiation with DMSO (Parker and Housman, 1985; Lannigan *et al*, 1986). After 5 days of MEL cell differentiation, total proteins were labelled with [^{35}S]-methionine, cytoplasmic proteins were extracted, separated by two-dimensional electrophoresis and detected by fluorography (Parker and Housman, 1985). Fluorograms of the two-dimensional gels were analyzed and 330 spots (from small to high molecular weights) were compared to fluorograms of total labelled proteins extracted from undifferentiated cells,

also separated in two-dimensional gels. These results showed that 25 to 59% of the proteins were reduced in level of expression, whilst 10 to 29% were increased. These results indicate that a reduction in protein complexity was occurring in the cytoplasm during differentiation.

These results confirmed previous experiments in this work (section 3.4.5.5) suggesting that there is no association between hsc70 expression and total protein synthesis during differentiation. The constant level of hsc70 expression may also reflect an increase in hsc70 protein stability during differentiation rather than a constant hsc70 synthesis. The stability of hsc70 protein has never been investigated during differentiation, and therefore represents a possible field of investigation for explaining the constant amount of hsc70 protein during the erythrocytic differentiation of haemopoietic cells.

(conclusion from section 3.10.4.3). However, for all these differentiations, cell cycle analysis should be done. If the cells were distributed in different phases of the cell cycle during differentiation, the kinetics of hsc70 expression would be difficult to measure.

3.11: FINAL DISCUSSION

3.11.1: Hsp70 expression, stress and differentiation of haemopoietic cells

Following a stress, both eukaryotic and prokaryotic cells express a family of proteins, termed heat shock proteins (hsps) (section 1.2.1.4). During the macrophage (Twomey *et al*, 1993) and erythrocyte (Singh and Yu, 1984; Theodorakis *et al*, 1989) differentiation of leukaemic cell lines, an increase in hsp70 expression was observed within 24 hours of differentiation, suggesting that hsp70 and/or the associated stress may trigger the differentiation. However, it is not clear whether hsp70 expression is a consequence of the toxic effect of the chemical inducers used to differentiate these cells, or the trigger of the differentiation. In order to establish the generality of these changes in stress during differentiation, an IL-3-dependent cell line FDCP-mix was differentiated along different lineages using growth factors, physiological inducers of haemopoietic cells. The differentiation of FDCP-mix clones along the granulocyte, macrophage, or erythrocyte lineages were carried out using various of changes in growth factors, changes which were thought to be potentially stressful for FDCP-mix clones. For example, all the differentiations were performed by transferring cells from a high concentration of IL-3 to a low concentration of IL-3, or no IL-3, plus the appropriate growth factors. For FDCP-mix cells, IL-3 is a survival factor, and in the absence of IL-3, cells die by apoptosis (Williams *et al*, 1990). The measurement of possible stress during the progression of different differentiation pathways was assessed by the detection of hsp70 expression. Hsp70 expression was analyzed by immunoprobing the Western blot. These results showed that no hsp70 expression was detected during differentiation of FDCP-mix clones along three different lineages, suggesting that stress does not trigger differentiation of normal haemopoietic cells. However, in contrast to normal haemopoietic cells, the differentiation of some leukaemic cells (K562 or U932) with some anti-cancer drugs such as haemin or

retinoic acid, may be triggered by a stress and/or an increase of hsp70 expression. How could a stress induce the differentiation of leukaemic cells? After a heat shock, heat shock transcription factors (HSF) bind to the hsp70 promoter to activate its transcription, and also to key regulatory loci such as steroid receptor genes to repress their expression (Westwood *et al*, 1991). How could HSF binding lead to activation of some genes and to repression of others? Westwood *et al* (1991) suggested that potential differences in the HSF-binding sequence between positive and negative *cis*-elements could result in structural alterations that change the activity of the transcription factor. At the protein level, the two major changes occurring following a stress are, a decrease in the total protein synthesis and an increase in hsp70 expression, both changes which may be regulated by HSF. During the erythrocytic and macrophagic differentiations of leukaemic cells, a decrease in the general protein synthesis (Fibach *et al*, 1985) and an increase in hsp70 expression were also observed, suggesting that HSF and/or hsp70 may trigger differentiation.

The results presented here, indicate that stress does not trigger differentiation of normal haemopoietic cells as no hsp70 expression was also detected during the differentiation of FDCP-mix clones. Therefore the decrease in the total protein synthesis observed during differentiation of FDCP-mix clones was unlikely to be caused by HSF activation, otherwise hsp70 expression would have increased.

3.11.2: Hsc70 protein expression is lineage-dependent

In this work, it has been shown that changes in hsc70 protein expression were associated with the lineage of differentiation of normal haemopoietic cell lines. During granulocytic differentiation with or without growth factors (LCM, low IL-3) of FDCP-mix clones, hsc70 protein expression decreased dramatically before (in the absence of growth factors) or after (in the presence of growth factors) the maturation of cells (Table 3.8). These results suggest that the decrease in the expression of hsc70 protein did not trigger

the granulocytic differentiation of these cell lines. My results do not agree the work of Beere *et al* (1993a) on HL-60 who suggested that the dramatic decrease in hsc70 protein expression observed before the maturation of the cells along the granulocytic differentiation, may trigger the differentiation. Further experiments suggested that hsc70 protein expression did not decrease in mature macrophage and erythrocytic FDCP-mix and MEL cells, compared to undifferentiated cells.

The results reported in this work confirmed several other studies, which showed a high constitutive expression of 70kDa heat shock proteins in various cells: hsc73 in mature macrophages (Twomey *et al*, 1993), hsp70 in K562 erythrocytic cells (Theodorakis *et al*, 1989), hsp70 in adult reticulocytes (Banerji *et al*, 1987), and hscx70 in mature human erythrocytes (Gromov and Celis, 1991). Therefore, this study indicates that hsc70 protein expression is lineage dependent.

3.11.3: Role of hsc70 in survival and life span of haemopoietic cells

3.11.3.1: Growth factor stimulation and cell survival

Following a stress such as heat shock, cells expressing hsc70/hsp70 survive a longer time than cells which do not express these hsps (section 1.2.1.2), suggesting that hsps are involved in cell survival. For example, in yeast, it was demonstrated that the hsp70 genes family (SSA1,2,3,4) are strictly necessary for cell survival, their deletion stops basic functions such as protein translocation through the endoplasmic reticulum and mitochondria, leading yeasts to a lost of viability (section 3.5.3.2). In the work presented here, and elsewhere (Hamilton *et al*, 1988; Koury and Bondurant, 1990; Bennet *et al*, 1992), haemopoietic growth factors have been reported to maintain survival of progenitors and/or mature cells. During differentiation of FDCP-mix clones, in the presence of growth factors, hsc70 protein expression was found to represent a good marker for cell survival in mature haemopoietic cells. Why is the level of hsc70 expression very low in granulocytes

Cell line	decreases of hsc70 protein (>10 fold) (day)	Hsc70 RNA	*c-myc* RNA	Lysozyme protein	Lysozyme RNA	Morphology (%)	Primitive cells (% day 0)	Exponential cell growth
A4/LCM/IL-3	6	↓↓↓↓	↓↓↓↓	ND	↑↑	EG:51 LG:37 M:12	21	stop
1B/LCM/IL-3	6	↓↓	↓↓	↑↑↑	↑↑↑↑	EG:35 LG:61,M:4	8	stop
1B/LCM/-IL-3	6	↓↓	↓↓↓↓	↑↑↑	↑↑↑↑	EG:31 LG:65,M:4	1	stop
1B/-LCM/-IL-3	1	BE	↓↓↓↓	BE	↑↑	B:55,EG:37 E:5,M:3	90	stop
A4/L cells CM (M-CSF)	No change	ND	ND	ND	ND	M:98,LG:2	ND	N/A

Table 3.7: Summary of granulocytic and macrophage differentiations of FDCP-mix clones in the presence or not of LCM and/or IL-3 (1.5U/ml). Data corresponding to the day of the decrease of the level of expression of hsc70 protein (>10 fold). B:blasts, EG:early granulocytes, LG:late granulocytes, M:monocytes/macrophages, BE:basalexpression. Decrease of expression:↓↓ (2-5 fold), ↓↓↓ (5-10 fold), ↓↓↓↓ (>10 fold). Increase of expression:↑ (1-2 fold), ↑↑ (2-5 fold), ↑↑↑↑ (>10 fold). ND:not determined, NA:non applicable

(decrease of 5-10 fold) and high in erythrocytes and macrophages (no change, compared to undifferentiated cells)? It has been shown that mature granulocytes have a half-life varying between 6 and 18 hours (Dresh *et al*, 1969; Rubinson *et al*, 1976). In contrast, alveolar macrophages and erythrocytes have a life span of approximately 81 days (Thomas *et al*, 1976) and 4 months (Harris, 1978) respectively. The studies on haemopoietic life span suggest that hsc70 protein expression may be required in macrophages and erythrocytes to chaperon some intracellular proteins (sections 1.2.3.1 and 3.10.1) or to be involved in protein degradation (section 1.2.3.2), assisting their basic cellular functions. For example it has been demonstrated in rabbit reticulocyte lysate that hsc73 (constitutive 70kDa) and hsp90 were involved in the regulation of the globin protein synthesis. When hsc73 was removed from the lysate, no globin protein was synthesized. This result shows that the constitutive expression of hsps is necessary to synthesize globin protein. Moreover erythrophagocytosis has been shown to induce several hsps such as hsp70 and hsp90 (Clerget and Polla, 1990). The constitutive expression of hsc70 seems to be associated with some cellular functions in mature erythrocytes and macrophages. No such observations have been shown in mature granulocytes.

3.11.3.2: Following transfection of the human bcl-2 gene

In the absence of growth factors, FDCP-mix (*bcl-2*, clone 1B) was shown to delay apoptosis (Fairbairn *et al*, 1993), but did not delay the decrease in hsc70 expression during granulocytic differentiation (Table 3.8), as was observed during the differentiation in the presence of growth factors. These results suggested that the disparate survival signals are generated by over expression of *bcl-2* or growth factor stimulation of FDCP-mix cells.

3.11.4: Regulation of hsc70 expression

3.11.4.1: Translational regulation or self-degradation of hsc70

By Northern blot analysis, it was shown in this work that hsc70 expression was not transcriptionally regulated during differentiation of FDCP-mix clones (section 3.5.5.1). During granulocytic differentiation, the decrease in hsc70 protein expression is regulated either at the translational level, or due to an increase in hsc70 protein degradation. Analyses of total protein by silver staining and pulse chase labelling with [^{35}S]-methionine, followed by separation on first-dimensional SDS polyacrylamide gel electrophoresis, did not show any significant change in the total protein pattern during differentiation (data not shown). However as mentioned previously (section 3.5.5.2) hsc70 expression was not associated to the general protein synthesis during erythrocytic differentiation of MEL cells (section 3.7.4).

Only hsp70 stability has been investigated in *Drosophila*, mouse (L-929) and hamster (CHO) cell lines (Mitchell *et al*, 1985). It was shown that degradation of the 70-kDa hsp occurs by autolytic cleavage based on the structural potential. For example, 70-kDa hsp contains at the carboxyl end, the appropriate arrangement of serine, cysteine, histidine, and aspartic acid residues to become a "serine" type protease if it were folded properly. These results suggest that during the granulocytic differentiation of haemopoietic cells, the decrease in hsc70 expression may be due to a specific self-degradation, which would occur following a change in conformation of the hsc70 protein. If this decrease in hsc70 protein expression occurs progressively during differentiation (in a few days), this hypothesis would be less appropriate to the circumstance, but the decrease in hsc70 expression observed during the granulocytic differentiation of FDCP-mix clones occurs dramatically in a short time (within 24 hours), suggesting that hsc70 may be self-degraded. To my knowledge, there is no published data regarding the stability of hsc70 protein during the granulocytic differentiation.

3.11.4.2: Effect of growth factors on hsc70 protein expression

Earlier studies have shown that growth factors may have the effect of activating hsp70/hsc70 protein expression. For example, IL-2 activates hsc70/hsp70 (not distinguished) expression in T-lymphocytes (Farrar *et al* (1988). Rat pheochromocytoma PC12 cells, in the presence of nerve growth factor (NGF), acquire the properties of sympathetic and sensory neurons). Following stimulation of PC12 cells with NGF, hsp70/hsc70 (not distinguished) promoters are activated and bind a nuclear complex, the SRE-2 element (SRE:serum response element) (Visvader *et al*, 1988).

The progressive removal of the IL-3 from IL-3 growth-dependent cell line FDCP-mix did not change hsc70 expression (sections 3.2.5 and 3.5.5.4), suggesting that IL-3 and cell growth do not regulate hsc70 expression. GM-CSF and G-CSF are thought to activate hsc70 protein expression in proliferating cells (section 3.5.5.4) during the granulocytic differentiation, but when FDCP-mix cells stop proliferating, hsc70 protein expression is down-regulated, suggesting that G-CSF and GM-CSF do not activate hsc70 when cells stop dividing. In contrast to GM-CSF and G-CSF, M-CSF is suggested to activate hsc70 protein in non dividing FDCP-mix cells during differentiation (sections 3.5.5.4.2 and 3.6.3.2). In this work, EPO was also thought to up-regulate hsc70 in the presence or absence of cell proliferation (section 3.10.4.2). This result may show that EPO does not regulate hsc70 expression through the cell cycle. However in the work presented here, hsc70 expression was shown not to be regulated at the transcriptional level, suggesting that growth factors rather than regulate hsc70 at the translational level, may do so at the translational or post-translational level. The mechanism of this translational or post-translational regulation mechanism has still to be determined.

3.11.5: Further directions

During macrophagic or erythrocytic differentiations of haemopoietic cells, hsc70

expression did not change. However the role of hsc70, as a "chaperon" (see section 1.2.3) may change in macrophages or erythrocytes, compared to undifferentiated cells (blasts). In erythrocytes, it has been suggested that a specific kinase is associated with several hsps to regulate the synthesis of globin protein (section 3.10.1). Does hsc70 protein chaperon another protein during the erythrocytic differentiation? Upon erythrophagocytosis, the expression of various hsps increased. What is the role of these hsps during erythrophagocytosis? These results suggest also that the localization of hsc70 may change following macrophagic and erythrocytic differentiation, and thus hsc70 could be localized by immunochemistry. Immunoprecipitation studies could be performed during differentiation to identify possible proteins which may be chaperoned and would therefore co-precipitate.

During the granulocytic differentiation of FDCP-mix clones (sections 3.5.5.1 and 3.10.4.1) and the erythrocytic differentiation of FDCP-mix (*bcl-2*, clone 2J) cells, it was shown that the decrease in hsc70 expression may be translationally regulated. The regulation of hsc70 could be determined by *in vitro* translation (using [^{35}S]-methionine) of total mRNA extracted during differentiation, followed by an immunoprecipitation with monoclonal antibodies to hsc70 protein, and separation by SDS PAGE. If hsc70 is regulated at the translational level, a decrease in immunoprecipitated hsc70 should be observed. On the other hand, if hsc70 is regulated post-translationally, cells could be labelled with [^{35}S]-methionine (time 0), treated with appropriate differentiating growth factor conditions (without radiolabelled amino-acid), and hsc70 could be immunoprecipitated with monoclonal antibody to hsc70, at the day hsc70 expression decreased. This pulse chase approach should measure possible hsc70 degradation during differentiation.

In this work, it was also suggested that hsc70 expression may be activated in macrophages and erythrocytes upon M-CSF and EPO stimulations respectively. Following

purification of monocytes or erythrocytes from human peripheral blood, these cells could be cultured in growth factor free medium and hsc70 protein expression measured, and compared to monocytes and erythrocytes which were cultured in the presence of growth factors. If hsc70 expression decreases in monocytes and erythrocytes cultured in the absence of growth factors, the life span of these cells could be assessed, and compared to the life span of the same cells cultured in the presence of growth factors.

My results suggest that FDCP-mix (clone A4) cells is not a multipotent cell line, but is composed of a mixed population of cells which do not respond to the same growth factors. For example, during the macrophage differentiation of FDCP-mix (clone A4) cells, up to 80% of the cell population die than undergo differentiation (up to 20%). These results may suggest that at least 80% of FDCP-mix cells do not respond to M-CSF, perhaps due to the absence of receptors at its cell surface for this growth factor. Therefore, several experiments should be done to analyse the cell population of FDCP-mix cells during differentiation. First, morphological studies on single cells should be done to determine the percentage of cells which differentiate in the presence of the appropriate growth factors. Second, the presence of GM-CSF, G-CSF, M-CSF and EPO receptors should be examined by binding assays during differentiations to determine the percentage of the cell population of FDCP-mix (clone A4) cells which express at its cell surface these specific receptors.

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