

# Key Regulatory Gene Expression in Erythroleukemia Differentiation

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## ABSTRACT

The characteristics of cellular and molecular mechanisms associated with cell proliferation and differentiation is important to understand malignancy. In this report we characterise a leukemic model, D5A1, to study the action of differentiation agent, cellular events and gene expression of the selected transcription factors. Cells induced with 4 mM hexamethylene bisacetamide (HMBA) caused signs of erythroid differentiation (changes in morphology and size, haemoglobinisation) and cessation of proliferation including accumulation of cells in G0/G1. Treatment with HMBA caused a time-related decrease of tumorigenicity detectable by 48 hour. Northern-blotting showed induction of  $\alpha$ -amino levulinic acid synthase-erythroid (ALAS-E) mRNA at 48 hours and appeared in a strong level subsequently. C-myc (myelocytomatosis) and c-myb (myeloblastoma) mRNA levels decreased transiently in early hours returning to control values by 24 hour and decreased again. Stem cell leukaemia (SCL) and GATA-1 mRNA were markedly down regulated in early hours and then returned back. A later time point, upregulation of GATA-1 and SCL was relevant to maturation phenotype. These data provide a useful model to study the cellular and molecular events in leukomogenesis and action of differentiation therapy in leukaemia. *Iran. Biomed. J. 6 (4): 97-103, 2002*

**Keywords:** Erythroleukaemia, Differentiation, Transcription factors, Oncogene

## INTRODUCTION

Differentiation therapy has been provided an alternative strategy in the treatment of hyperproliferative disorders. This has been highlighted by the use of all trans retinoic acid and vitamin D3 [1]. Successful treatment of acute promyelocytic leukemia and other acute myeloid leukemia has identified several novel approaches to induce differentiation and selective apoptosis [2]. It is possible that triggering an early event in commitment to differentiation can cause loss of malignant properties, even in the absence of full morphological changes. One approach toward understanding of process of haematopoiesis and commitment is characterization of regulatory proteins important for cell specific gene expression [3, 4]. Proto-oncogenes are known to control cell growth, function as negative and positive regulators of terminal cell differentiation, which is associated with inhibition of growth and ultimately apoptosis. The proto-oncogenes myelocytomatosis (c-myc), myeloblastoma (c-myb), c-fos and c-jun, which encode nuclear protein, are modulated in their

expression during cell growth and differentiation [5]. Transcription factors are likely to play pivotal roles in specifying lineage phenotypes. GATA-1 emerged as a key regulatory of globin expression and erythroid-specific gene expression. Stem cell leukemia (SCL) may also be important in erythroid commitment [6]. Although differentiation of erythroid cells requires GATA-1 gene expression, the role of this transcription factor and other member of GATA family at earlier stage of erythroid differentiation are unclear. A fundamental problem exists in defining temporal inter relationship between the GATA-1 protein and other nuclear factor involved in haematopoiesis such as SCL, EKLF and NF-E2 [3]. SCL gene displays a GATA motif in its promoter and may be one target of GATA-1 [7]. In order to investigate cellular and molecular consequences of erythroid differentiation, an erythroleukemia cell line, D5A1, was used for studying leukemic differentiation therapy. Little is known about responses of this cell line to inducers and this cell represented an *in vivo* model as well. The early and late changes in the expression of genes particularly transcription factors genes

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responsible for proliferation, differentiation and lineage determination such as: c-myc, c-myb, GATA-1 and SCL-1 were investigated.

## MATERIALS AND METHODS

**Cell culture and induction.** The leukemic cell line, D5A1, was established as previously described [8]. The cells were maintained in DMEM supplemented with 10% FCS, Penicillin (100 U/ml), Streptomycin (100 µg/ml) and Glutamine (2 mM). Hexamethylene Bisacetamide (HMBA) (4 mM) concentration and was used to induce differentiation. To induce erythroid differentiation, the cells were diluted to  $5 \times 10^4$  per ml into fresh medium and incubated at 37°C for 4-5 days. All chemicals were obtained from Sigma chemical company (Germany) and Gibco BRL (UK).

**Assessment of proliferation and differentiation.** Differentiation was monitored by following changes in cell growth, cell size and haemoglobinisation. Changes in cell number and cell size were monitored using a cell counter model ZF, channelizer C1000 and plotter (Coulter USA). Viability was determined by 0.4% trypan blue exclusion. Morphology and cytochemistry were monitored by Giemsa and Benzidine staining of cytocentrifuge slides (Shandon USA). Haem absorption of the lysate was determined by measuring optical density using a scanning spectrophotometer (Pye Unicam, USA) between 380 and 660 nm. The potential of clonogenic cells was also analysed in semisolid medium containing 2.1% methylcellulose and 25% FCS at  $10^4$  cells per ml. The number of colonies was determined after incubation for 5-6 days using an inverted microscope.

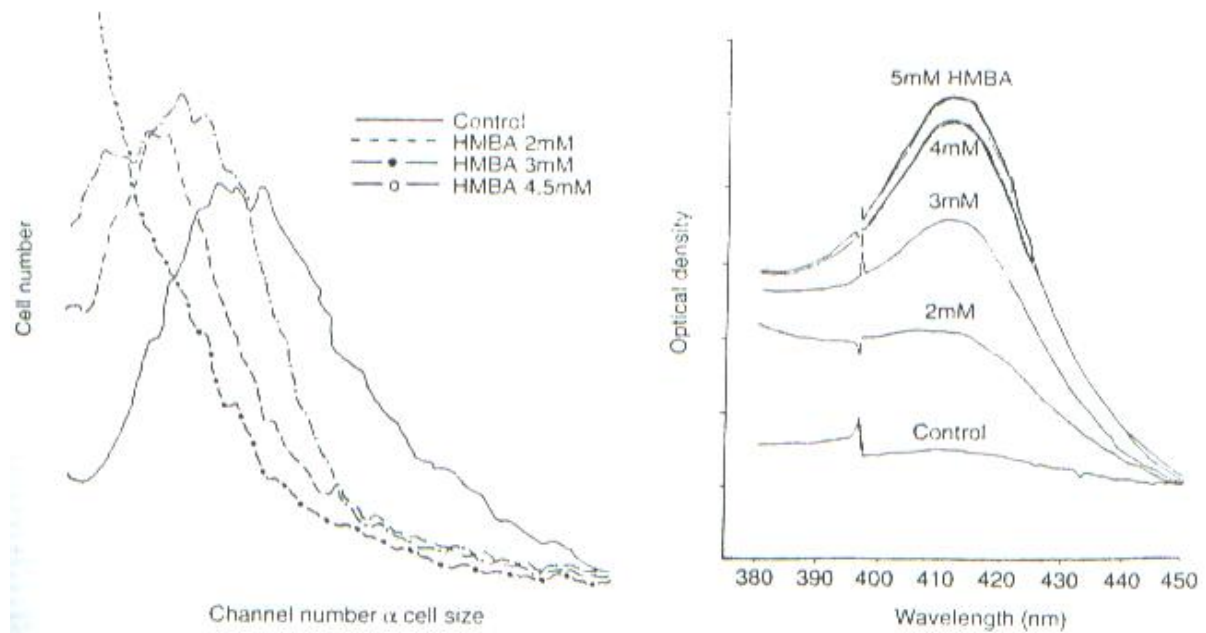
**Cell cycle analysis.** DNA content per cell was determined by staining fixed cells with using the DNA binding fluorochrome, propidium iodide and flow cytometry analysis (Becton Dickinson, USA), as previously described [9].

**In vivo tumorigenicity.** D5A1 cells ( $10^4$  cells) were inoculated subcutaneously into 7-14 days long Evans rats and the time taken to develop tumors was recorded. SPSS used to estimate cumulative tumor development rates by the Kaplan Meier method.

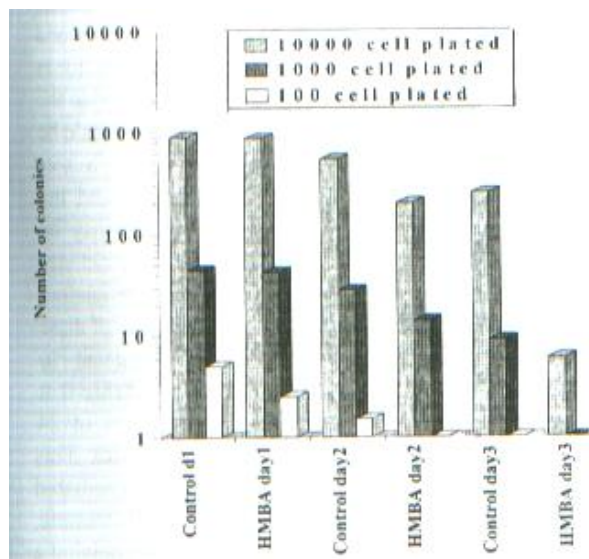
**Northern-blot hybridization.** Gene expression was analysed by Northern-blotting [10]. The induction of mRNA for erythroid specific  $\delta$ -amino levulinic acid synthase was used as early marker of differentiation [11]. The probes were isolated from plasmids by restriction enzyme digestion after extraction from bacteria grown overnight at 37°C in terrific broth using the Qiaprep spin plasmid kit (Qiagen). Probes were labelled by random primed DNA kit (Boehringer Mannheim). The human c-myc probe [12], the viral c-myb probe [13], the rat  $\delta$ -amino levulinic acid synthase-erythroid (ALAS-E) cDNA probe [11], the human genomic  $\beta$ -actin probe [14] were used. Bacterial transformation was used for cloning of SCL cDNA probe [15]. In the case of GATA-1, the probe was prepared from the RT-PCR product.

## RESULTS

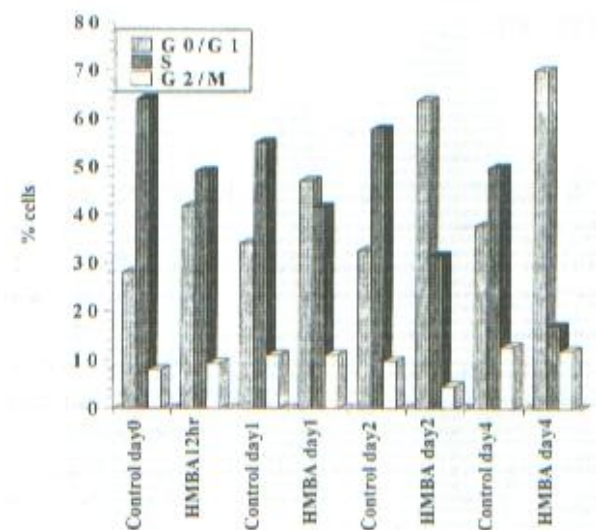
**Effect of differentiation agent on cellular level.** With an initial cell density of  $5 \times 10^4$  cells per ml, the cell number reached a mean value of  $1.56 \times 10^6$  per ml by day 4. Over 95% of cells were viable on day 4. Optimal differentiation induction at 4 mM HMBA using haem absorption of the lysate was chosen since this showed minimal effect on cell viability. On day 4, many of the cells were morphologically similar to polychromatic and late erythroblasts. Cells were benzidine positive (40-50%) and the cell pellet was pinkish-brown. The cells became markedly smaller and nuclear chromatin became condensed (Fig. 1). The clonogenic potential of the cells decreased ( $n = 4$ ) (Fig. 2). Two days pretreatment of HMBA was sufficient to decrease the number of colonies. The clonogenic efficiency of 10,000 and 1,000 cells plated at day 2 were 37% and 49% and at day 3 it was 2.5% and 5%, respectively as compared to the control. Cell cycle analysis showed accumulation of cells in G0/G1 and a decreased percentage of cells in the S phase of cell cycle. After 12 hours, induced cells were already accumulating in G0/G1. After 4 days, most of the cells had accumulated in the G0/G1 phase, concomitantly the number of S cells started to decrease ( $n = 4$ ) (Fig. 3). The cell number reached  $1.9 \times 10^5$  cells per ml on day 4 induced with HMBA. For the first two days of culture, cell number increased and reached a peak between day 2 and 3. From day 3 onwards, the cell number started to decrease.



**Fig. 1.** Cell size and haem absorption by lysate of  $5 \times 10^6$  D5A1 cells induced to differentiate after 4 days cultured with various concentration of HMBA.



**Fig. 2.** The clonogenic potential of D5A1 cells after prior induction in 4 mM HMBA.



**Fig. 3.** Arrest of D5A1 cells in G0/G1 of cell cycle induced to 4 mM HMBA.

***In vivo tumorigenicity.*** Treatment of D5A1 cells with HMBA reduces the capacity to proliferate *in vitro*. Similarly, *in vitro* incubation of the cells with HMBA increased the time taken to develop tumors *in vivo* ( $n = 2$ ) (Table 1). There was a significant delay in tumor development at 48 and 72 hours.

***Molecular events.*** ALAS-E mRNA as an early marker of differentiation became detectable in day 2 to give a strong signal on day 4 ( $n = 4$ ). The level of c-myc and ALAS-E mRNA expression during different time exposure with HMBA was compared with control. The data showed that within 2 hours

**Table 1.** Tumor development of D5A1 cells incubated in vitro with 4 mM HMBA.

Cell line injected	Tumorigenicity	Mean survival (days)
Control	7/7 (100)	19
HMBA (24 h)	8/8 (100)	22
HMBA (48 h)	10/11 (91)	31
HMBA (72 h)	6/10 (60)	42

*P* value compared to control: 24 h = 0.0133, 48 h < 0.001 and 72 h < 0.001.

c-myc mRNA completely disappeared but at 12 hours it started to be expressed again and at 24 hours had returned to near baseline level of control. At 48 hours, c-myc mRNA decreased again and ALAS-E mRNA was expressed ( $n = 4$ ) (Fig. 4A). The result on the level of c-myb expression during treatment with HMBA showed that in 6 hours c-myb mRNA had completely disappeared but at 12 hours appeared again. At 48 hours, the level of c-myb mRNA decreased ( $n = 2$ ) (Fig. 4B). The result on the level of GATA-1 mRNA expression in cells treated with HMBA showed that the level of mRNA in the early hours was down regulated and in 6 hours disappeared and in 24 and 48 hours returned back and expressed higher than control (Fig. 4C). The level of SCL mRNA expression in cells treated with HMBA showed that the level of mRNA in the early hours was down regulated and in 4 and 6 hours almost disappeared, and in 24 and 48 hours returned back and expressed higher than control (Fig. 4D).

## DISCUSSION

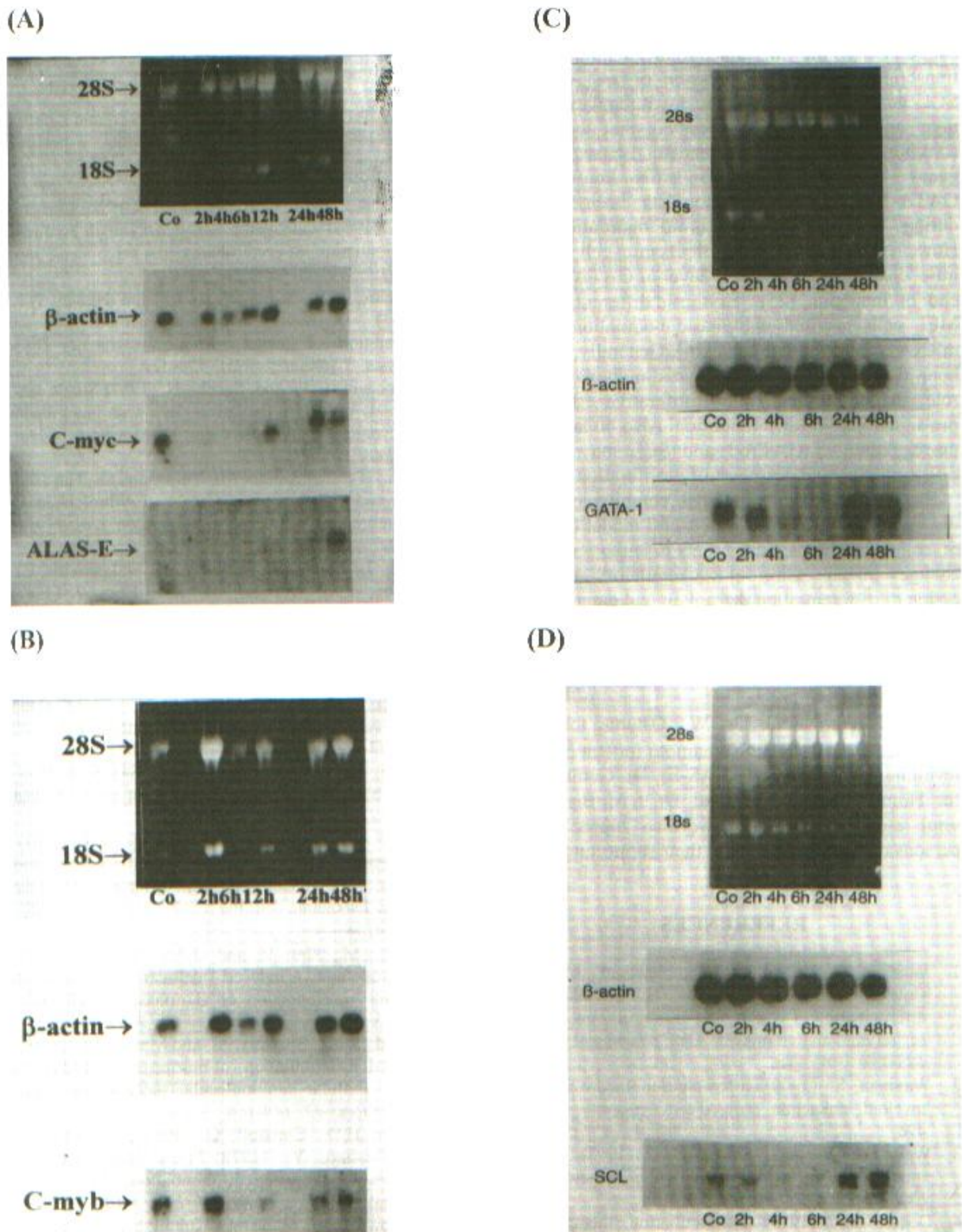
A novel model for studying the erythroid development is described. Exposure of erythroid to HMBA for 24-48 hours resulted in commitment to erythroid maturation and reduction in proliferative potential. A time and dose-dependent inhibition of proliferation and differentiation agent was observed. Reduction in clonogenic capacity was not apparent until 48 hours exposure to inducer, although an effect on cell cycle was already detectable after 12 hours. Exposure to inducer over different time periods revealed a significant ability of such treatment to inhibit tumor development, at least partially, by its effect on causing cells to exit the cell cycle. Studies have shown that the cellular decision to proliferate and differentiation is related to the phase of the cell cycle and exiting from cell cycle is a prerequisite for maturation [16].

In Murine Erythroleukaemia (MEL) cells induced to Dimethyl sulphoxide (DMSO) and HMBA after several cell divisions all arrested in G1 phase [17]. The loss of clonogenicity with DMSO showed that

erythroleukemic cells are less leukemogenic. For malignancy tests mice inoculated with cells exposed to DMSO for 72 and 96 hours had slower-growing tumors than the mice injected with untreated cells [18]. D5A1 cells showed early induction of the erythroid-specific ALAS-E. It is interesting that the pattern of change in this enzyme was somewhat similar to that of seen in MEL cells but there was important difference. In MEL cells, the mRNA species was detectable in uninduced cells [11] suggesting that MEL cells are already committed.

To further investigate molecular events, the modulation of key regulatory gene expression induced was studied. It has not been fully established whether modulated expression of nuclear protooncogenes represents obligate events in regulation of cell differentiation and whether the function of a protooncogene relates more to proliferation, or to differentiation [19]. Biphasic down regulation of c-myc and c-myb in early and late hours of the induction may be a critical factor in commitment and expression of genes related to the differentiated phenotype. The early decline could be involved as antiproliferative effect and a pre-signaling for process of differentiation and the eventual switching off of myc and myb may be responsible for cessation of proliferative activity accompanying induction of differentiation. However, the modulations of expression of these genes are not sufficient for commitment to differentiation. This is indicated by evidence that accumulation of cells in G0/G1 occurred in 24 hours, when c-myc and c-myb returned back. This observation suggests that another inducer-mediated step is required to facilitate cells to differentiate. The changes in myc and myb followed somewhat a similar pattern seen in MEL cells and HL60. Both cells showed a decrease in c-myc RNA when induced to differentiation. C-myb mRNA showed a distinct biphasic decrease. These studies suggest that the continued suppression of c-myb is critical for commitment to terminal cell division [20-23].

GATA-1 and SCL mRNA markedly down regulated in the early hour and returned back. At later time points however mRNA levels were greater than those present at the beginning. Changes



**Fig. 4.** The level of c-myc, ALAS-E, c-myb, GATA1, and SCL mRNA during incubation of D5A1 cells with 4 mM HMBA as detected by Northern-blot. **(A)**, the level of c-myc and ALAS-E mRNA; **(B)**, the level of c-myb mRNA; **(C)**, the level of GATA-1 mRNA; and **(D)**, the level of SCL mRNA.



in SCL and GATA-1 were also similar; however the similarity in both gene expressions occurred suggests that they are linked to differentiation. The transcription arrest of expression of GATA-1 and SCL genes are similar to the down regulation of c-myc and c-myb. It is possible that there is a common regulatory mechanism between these genes. A parallel decrease in early hours in both GATA-1 and SCL expression has been observed during normal human erythroid maturation [24]. However, in human erythroleukemic cell lines, a little change is detected in level of GATA-1 at the end of induction [25]. Erythroid differentiation of MEL cells resulted in biphasic modulation of SCL mRNA similar to our cells [26]. Generally, in early hour down regulation of c-myc, c-myb may have a role in commitment, anti-proliferative effect and pre-signalling of differentiation. Also, in late phase up-regulation of GATA-1 and SCL may promote differentiation and cessation of proliferation.

These data demonstrate that combination of *in vitro* and *in vivo* provides a useful model for study of cellular and molecular events in leukomogenesis, changes in expression of key regulatory genes and action of differentiation therapy in leukemia.

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