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Eur J Cancer, Vol. 26, No. 6, pp. 694–698, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00
Pergamon Press plc

Proto-oncogene Expression in Differentiating and Non-differentiating Chronic Myelogenous Leukaemia Cells

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Despite the profound differences between the chronic and blastic phases of chronic myelogenous leukaemia, no differences between chronic and blastic phase cells have been described at the molecular level. Differences have been found in the levels of expression of c-myc, c-myb and p53, which fell when chronic phase cells were cultured, while the levels of expression of the genes were stable when blastic crisis cells were cultured. In contrast c-fms expression increased and MRS expression decreased after culture of chronic or blastic phase cells. The data suggest that the regulation of expression of some genes in blastic crisis cells is unaltered while that of others is disrupted. It is not known whether the failure of c-myc, c-myb and p53 expression to fall during the culture of blastic phase cells is the cause of or a reflection of the failure of these cells to differentiate.
Eur J Cancer, Vol. 26, No. 6, pp. 694—698, 1990.

INTRODUCTION

CHRONIC myelogenous leukaemia (CML) invariably evolves into an acute or blastic phase. During the initial phase, myeloid differentiation is intact while impaired maturation is the hallmark of the blastic phase. Why myeloid maturation fails during the blastic phase is not known. We have established a suspension culture system in which chronic and blastic phase cells behave *in vitro* similarly to their behaviour *in vivo*. The immature cells

from both disease states proliferate well *in vitro* while only immature cells from chronic phase patients differentiate [1]. We report the expression of several proto-oncogenes in chronic and blastic phase CML cells during culture *in vitro*.

MATERIALS AND METHODS

Cells

Cells were prepared as previously described [1]. After initial centrifugation over Ficoll-Hypaque (SG 1.077), T cells were removed [2]. Granulocytes and monocytes were removed with a strong magnet after the cells phagocytized iron filings. The remaining cells were centrifuged over Ficoll-Hypaque (SG 1.063) and the low density cells [3] placed into culture.

Culture and morphological assessment

Cells were suspended at 10^6/ml in RPMI 1640 containing 20% foetal calf serum. The cultures were placed in humidified incubators in 5% CO_2 in air at 37°C. When possible, cultures were harvested on days 2, 4, 7 and 14. One portion of cells was used to assess cell number, the percentage of cells in S phase, the number of clonogenic cells and the percentage of cells containing detectable myc and fos protein, and to prepare slides for differential counting. As in our previous studies, because of the difficulty in distinguishing accurately between myeloblasts, promyelocytes and early myelocytes, all cells containing nucleoli were considered to be 'immature cells'. Cells at the myelocyte stage that did not contain nucleoli and more mature cells were considered to be differentiated [1]. A second portion of cells was used for molecular biological studies.

The proportion of cells in S phase was measured by [^{3}H]thymidine labelling [4]. The proportion of clonogenic cells was assessed in agar [4] with recombinant granulocyte macrophage colony-stimulating factor which was provided by Dr Stephen Clark of the Genetics Institute.

Molecular biological studies

Either simultaneous incubation of Northern blots [5,6] with two or three radiolabelled probes or sequential hybridization with individual probes was done. The blots were washed at high stringency, dried and autoradiographed. The following provided probes: Dr J Battey, myc [7]; Dr T. Curran, fos [8]; Dr P. Rothenberg, myb [9]; Dr C. Stein, histone H_3 [10]; Dr C. Sher, fms [11]; Dr L. Maquat, triose phosphate isomerase (TPI) [12]; Dr M. Oren, p53 [13] and Dr W. Mars, myeloid related sequence (MRS) [14].

Detection of proto-oncogene products

Cells were washed and placed onto coverslips coated with alcian blue [15]. The coverslips were fixed with 3.7% formalin and incubated with non-immune rabbit serum in tris-buffered saline for 30 min. A 1:100 dilution of a rabbit polyclonal antibody to the c-myc protein [16] was layered on coverslips, which were incubated for 1 h at 37°C. The coverslips were washed and incubated with biotinylated goat anti-rabbit IgG, washed and incubated with avidin-conjugated horseradish peroxidase (Vecta Stain, Vector Laboratories). Staining was done by incubation with the peroxidase substrate amino-ethyl carbazole. A single investigator (H.P.) evaluated the slides in a single-blinded manner. The same procedure was used to detect c-fos protein in cells. This antibody was prepared by Dr Riobowal [17].

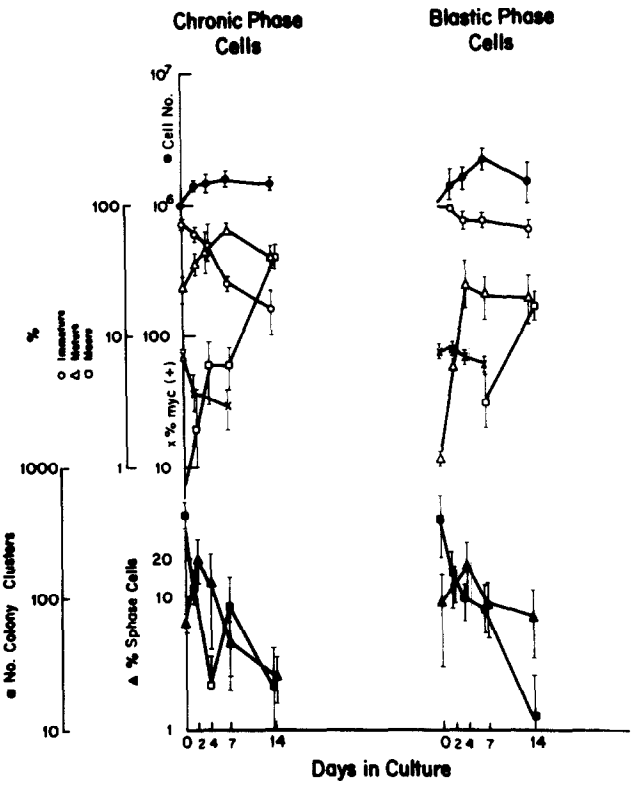

Fig. 1. Behaviour of CML cells during suspension culture (mean, S.D.).

RESULTS

Biological events during culture

For chronic phase cells, 11 independent suspension cultures were studied. Cell numbers progressively increased during the first 7 days of culture, reaching a mean of 1.7 (S.D. 0.2) × 10^6/ml. Cell numbers fell slightly during the 2nd week of culture (Fig. 1). In 3 of the 11 cultures, cell numbers increased only during the first 2–4 days of culture and declined thereafter.

The percentage of cells synthesizing DNA was highest on day 2 and fell thereafter (Fig. 1). The number of cells capable of proliferating and forming clusters or colonies in agar decreased with time (Fig. 1), falling from 443 (106) clonogenic cells per 10^5 cells at time zero to 23 (22) clonogenic cells per 10^5 cells at day 14. The apparent increase in colony number between days 4 and 7 seen in Fig. 1 reflects the fact that only 3 suspension cultures were assayed on day 4 while 2 of the cultures, which contained the highest number of clonogenic cells on day 0, were not assayed on day 4.

The proportion of immature cells fell progressively from 72 (5%) at time zero to 16 (6%) at day 14 (Fig. 1). During the first 7 days, the proportion of mature myeloid cells increased from 23 (5%) to 67 (5%). Between days 7 and 14, the proportion of myeloid cells declined to 41 (7%), these also being replaced by macrophages. Macrophages first appeared on day 2 and progressively increased throughout culture reaching 41 (8%) on day 14. The cells of 1 patient (No. 3, Fig. 2) showed different behaviour in that the percentage of immature cells fell from 74%

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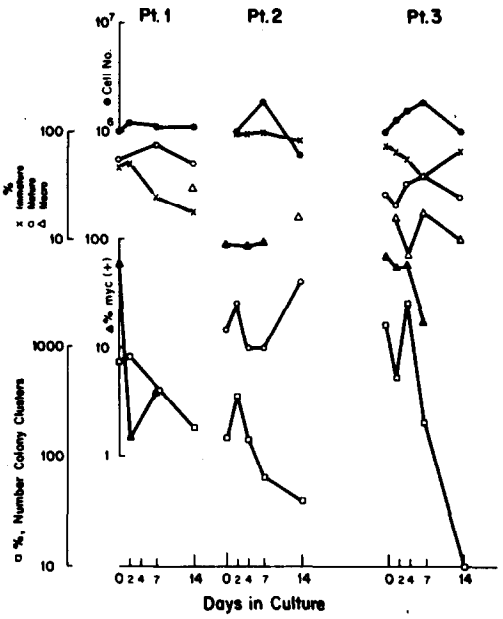

Fig. 2. Biological characteristics of chronic and blastic phase cells *in vitro*. Macro = macrophages.

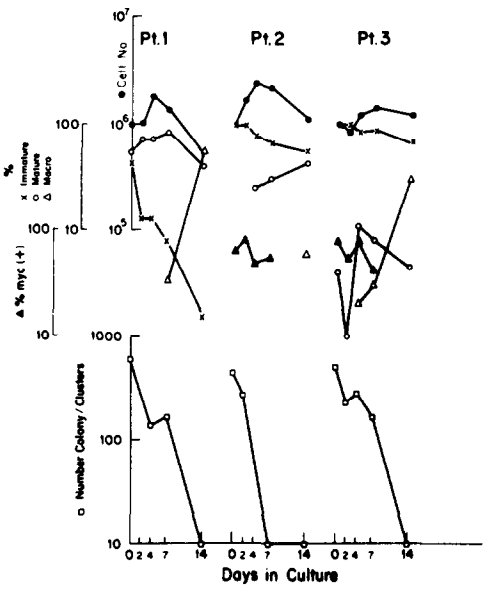

Fig. 4. Biological characteristics of chronic and blastic phase cells *in vitro*. Patient 1 was in chronic phase, 2 and 3 were in blastic phase and 4 was in chronic phase.

at time zero to 38% at day 7, but increased to 66% at day 14. Also, unlike cells of the other 10 patients, it was possible to establish a long-term suspension culture cell line from the short-term culture.

For blastic phase cells, as with the culture of chronic phase cells, the number of cells in blast crisis cultures increased during the first 7 days and then gradually declined in all except 1 culture. Cell numbers increased from 10^6/ml on day 0 to 2.3 (0.4) $\times$ 10^6 on day 7 and fell to 1.5 (0.5) $\times$ 10^6 on day 14 (Fig. 1). The proportion of immature cells also declined in all blastic phase cultures but the decline occurred more gradually and to a lesser extent than that seen in cultures containing chronic phase cells (Fig. 1). At time zero, the proportion of immature cells was 98 (0.7)%, while on day 14, the proportion was 66 (8)%. At each time, the proportion of immature cells was greater in the blastic phase cultures than in the chronic phase cultures ($P < 0.001$). Macrophages first appeared on day 7 and increased in number up to day 14. However, as with myeloid maturation, the proportion of macrophages in 14 day cultures was significantly less than in the chronic phase cultures (17 [4]% vs. 41 [8]%, $P = 0.02$). As with the chronic phase cultures, the percentage of S phase cells was greatest at day 2 of culture and then declined. The number of clonogenic cells also declined with time to the same extent as that in chronic phase cell cultures (Fig. 1).

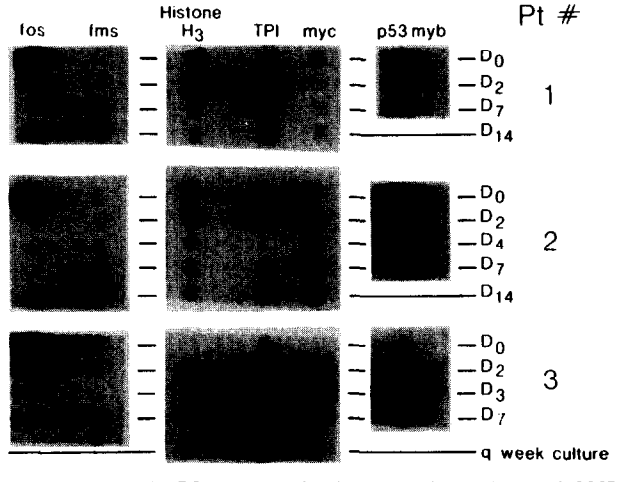

Fig. 3. Changes in RNA transcript levels during culture of CML cells *in vitro*. Patient 1 was in chronic phase, 2 was in blastic phase and 3 was in chronic phase (Ph negative). q week culture for patient 3 represents c-myc, TPI and histone H_3 transcript levels in the suspension culture cell line established from this patient's cells.

Proto-oncogene expression

In chronic phase cells, c-myc RNA levels were serially measured in the cells of 11 suspension cultures (cells from patient 1 were studied twice). Northern blotting showed that initially the immature myeloid cells of chronic phase patients contained low levels of c-myc RNA relative to the c-myc RNA content of blastic phase cells (patient 1 compared with patient 2 in Figs. 2 and 3; patients 1 and 4 compared with patients 2 and 3 in Figs. 4 and 5). The level of c-myc RNA appeared to fall with culture so that transcript levels were lower on days 7 and 14 of culture than on earlier days (patients 1 and 3 in Figs. 2 and 3; patients 1 and 4 in Figs. 4 and 5). In several cultures, the amount of c-myc transcript transiently increased during the first 2 days (patient 3 in Figs. 3 and 4; patient 1 in Figs. 4 and 5), stabilized, and then fell. The proportion of cells containing sufficient c-myc protein to be detected by immunoperoxidase was also assessed. In time zero cultures, the percentage was identical to that of immature cells (71 [5]% vs. 72 [5]%). The percentage of cells staining for c-myc protein fell with time (Figs. 1–5), with the most dramatic fall occurring between days 0 and 2 when the fall in cells containing detectable amounts of c-myc protein was greater than that in morphologically immature cells. On day 2, while there were 61 (7)% immature cells, only 38 (12)% cells stained for c-myc protein. By day 7, the two proportions were similar (25 [3]% and 29 [10]%).

The cells of patient 3 did not follow this pattern. At the RNA level (Fig. 2), a dramatic increase in c-myc RNA occurred during the first 3 days. By day 7, however, c-myc RNA levels fell to approximately the level in the cells of other chronic phase patients. For c-myc (Fig. 3), the percentage of cells staining for

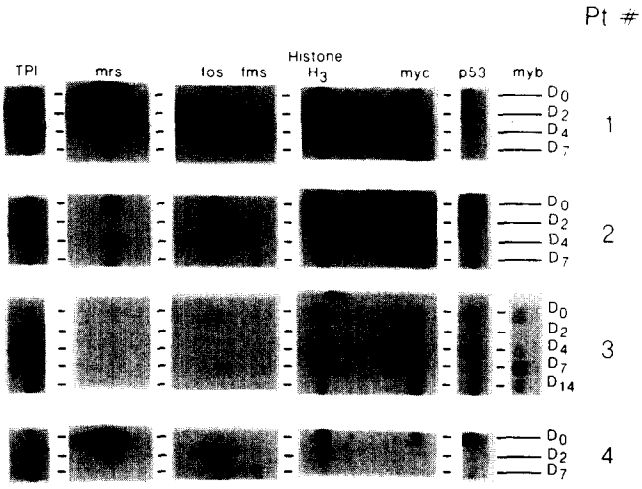

Fig. 5. Changes in RNA transcript levels during culture of CML cells *in vitro*. Patient 1 was in chronic phase, 2 and 3 were in blastic phase and 4 was in chronic phase.

this protein was maintained through day 4 and then fell. During this time, cell numbers increased and the number of clonogenic cells stayed high. These changes parallelled the changes in the proportion of immature cells. Unfortunately, day 14 RNA studies were not possible.

p53 RNA levels were also measured in 9 chronic phase cultures. RNA levels followed the same pattern of changes as seen for c-myc RNA, falling with differentiation. c-myb transcript levels were measured in the cells of 3 chronic phase suspension cultures. RNA levels parallelled p53 and c-myc RNA levels. Histone H_3 RNA levels were measured in every study. Levels were uniformly high during the first few days of culture and declined thereafter.

RNA transcripts of c-fms were initially detectable in 6 of 10 cultures and appeared after 2–4 days of incubation in the remaining 4 cultures. Transcript levels increased with time in 9 of 10 studies (Figs. 2–5). There was no relation between the level of c-fms RNA and the proportion of macrophages present. c-fos transcripts were detectable at time zero in every specimen studied. Transcript levels fell during the first 7 days of culture in 5 of 10 cultures, increased in 2 cultures, and did not change in 3 cultures. There was no relation between c-fos transcript levels and either c-fms RNA levels or the proportion of macrophages. The percentage of cells that stained with anti-fos antibodies was initially 62 (18)%. As with c-myc expression, cells containing detectable fos protein fell as the cells differentiated. After 2 days of culture, the percentage of cells staining for fos protein was 31 (12)% and, after 7 days of culture, 43 (10)%. Hence, as with c-myc protein, the greatest decrease in cells containing detectable c-fos protein occurred during the first 2 days. There was no relation between fos RNA levels and the proportion of cells containing sufficient fos protein to be detectable by immunoperoxidase. MRS transcripts were detectable in 7 of 9 cultures at time zero and declined in all during culture.

In blastic crisis cultures, Northern blotting was done on suspension cultures established from 4 patients with myeloid blastic crisis, with 1 patient's cells being studied twice with a 2 month interval. c-myc RNA levels were initially high (Figs. 2 and 3). In contrast to the fall in c-myc transcript levels in the chronic phase cell cultures, c-myc RNA levels remained more or less constant (patient 2 in Figs. 2 and 3, patients 2 and 3 in Figs. 4 and 5). Similarly, the proportion of cells staining for c-myc protein fell only slightly 76 [7]% initially, 61 [9]% on day 7 [Fig. 1]). The proportion of cells containing c-myc protein was the same among chronic phase and blastic phase immature cells (71 [5]% and 76 [7]%). Additionally, the amount of c-myc protein in the cells as determined by the intensity of staining was also indistinguishable among the immature cells (data not shown).

As seen in chronic phase cultures, p53 and c-myb transcript levels paralleled c-myc RNA levels (Figs. 2–5). Unlike the chronic phase cell cultures, in one half of the blastic phase cultures there was no significant decline in histone H3 or TPI RNA levels during culture. c-fms and MRS transcript levels behaved similarly to those of the chronic phase cell cultures. c-fms levels were undetectable at time zero in 4 cultures, and appeared and increased with time in 3 of the 4 cultures. In the fifth culture, c-fms was detectable initially and increased with time. 4 cultures were assessed for MRS RNA levels at time zero and transcripts were detectable in all before declining.

c-fos transcripts were detectable in all 5 cultures at all times, at levels similar to those in chronic phase cultures. In 2 of 5 cultures, transcript levels declined while in 3 cultures they did not change. Initially, 82 [13]% of cells contained detectable c-fos protein, which fell to 73 [4]% on day 7. As was the case for c-myc protein, the proportion of cells containing detectable c-fos protein at time zero in blastic and chronic phase cultures was (62 [18]% vs. 82 [13]%, not significant). When assayed at 7 days the percentage of cells staining for c-fos protein was greater in the blastic phase cultures 73 [47]% vs. 43 [10]%, $P = 0.05$). However, there was no relation between c-fos RNA levels in the culture and the proportion of individual cells containing c-fos.

DISCUSSION

We used a suspension culture system in which the behaviour of immature CNL cells closely approximates their behaviour *in vivo* in man. Immature myeloid cells from chronic or blastic phase patients proliferate for more than 1 week in this system and the extent of differentiation *in vitro* parallels that *in vivo*. The ability of chronic myelogenous leukaemia cells to proliferate in suspension culture in the absence of exogenous growth factors may be a reflection of the frequent constitutive expression of the interleukin 1β genes in these cells [18]. The small number of cells that differentiate in the blastic phase cultures could represent the differentiation of residual chronic phase cells or the process which produces the maturation arrest characteristics of blastic crisis may be 'leaky', permitting some blastic phase cells to differentiate. Cell proliferation, as assayed by the number of cells synthesizing DNA, peaked after 2–4 days, correlating with the most rapid increase in cell numbers [1].

The expression of four genes was similar in the chronic and blastic phase cultures. Histone H_3 RNA levels were highest during the first 2–4 days, corresponding to the period of rapid cell proliferation. TPI RNA levels also tended to be higher during this period and in general were well maintained, indicating that the energy metabolism of the myeloid cells is maintained throughout culture.

C-fms and MRS transcript levels followed the same pattern of change during culture of chronic and blastic phase cells, with the former increasing and the latter falling. The increase in c-fms expression occurred as macrophages appear in the cultures. This observation differs from a previous study in which it was reported that c-fms expression fell as monocytes evolved into tissue macrophages [19]. The fall in MRS expression is compatible with previous reports that this gene is expressed at the

highest level in promyelocytes and myelocytes [20] and falls as cells become granulocytes. Substantial differences in level of morphologic maturation are noted when chronic and blastic cell cultures are compared.

The changes in MRS and c-fms expression parallelled the progressive fall in the clonogenicity of cells in both the chronic and blastic phase cultures, suggesting that all may be part of the coordinated changes which occur during normal myeloid maturation. These data therefore suggest that while blastic crisis cells do not mature morphologically, maturation of some cellular processes occurs.

The second group of genes includes c-myc, c-myb and p53. These genes were coordinated in expression, with their transcript levels falling during myeloid differentiation. The fall in expression at the RNA level was most apparent after 7 days of culture. Of interest was the decline in c-myc expression at the protein level during the first 2 days of culture, before morphological evidence of maturation is discernible and before RNA transcript levels fall. This decline is reminiscent of the molecular events during the differentiation of HL60 cells [21, 22]. In contrast, the level of expression of c-myc, c-myb and p53 as maintained in blastic crisis cultures, parallelling the failure of these cells to differentiate.

Studies of c-fos expression at the transcript and protein level produced conflicting results. No consistent change in transcript level was observed during culture whether or not differentiation occurred. At the protein level, however, the pattern of c-fos expression closely parallelled that of c-myc, declining in chronic phase cultures but not changing in blastic phase cultures. There was no relation between fos RNA transcript levels in populations of cells and the proportion of individual cells containing sufficient amounts of c-fos protein to be detected by immunoperoxidase. This dissociation between c-fos transcript and protein levels has been noted by others [19,23].

Our findings are compatible with the hypothesis that the transition of the chronic phase of CML to the blastic phase is the result of the deregulation of expression of proto-oncogenes coding for nucleoproteins [24,25] and with the observations that the constitutive expression of c-myc and c-myb block differentiation [26,27]. The constitutive expression of the *myc* gene prevents differentiation but not commitment [26]. Studies are in progress with antisense techniques to establish if the failure of c-myc and c-myb to fall during the culture of blastic crisis cells is the cause or a reflection of the failure of these cells to differentiate. Finally, our data suggest that post-transcriptional and post-translational regulation of gene expression both play an important role in the regulation of myeloid differentiation.

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Acknowledgements—We thank the National Cancer Institute for support (CA 41285).