Reduction in p53 synthesis during differentiation of Friend-erythroleukemia cells

Correlation with the commitment to terminal cell division

Ronit Ben -Dori, Dalia Resnitzki and Adi Kimchi*

Department of Virology, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 1 July 1983; revised version received 1 September 1983

The process of cell differentiation in Friend-erythroleukemia cells was accompanied by 80–90% inhibition of p53 synthesis. This decrease was found to be linked to changes in cell-cycle distribution characteristics of the growth arrest program during differentiation rather than to the induction of the globin genes. The shut-off in the expression of p53 always preceded the specific arrest of cells in the G_0/G_1 phase. Interferon did not modulate down the expression of p53 if added to transformed non-induced Friend-erythroleukemia cells; however, it slightly enhanced the extent of reduction in p53 synthesis if added during cell differentiation, thus suggesting a differential effect of interferon between cells at different stages of differentiation

Friend cell *Differentiation* *p53 Synthesis* *Cell cycle* *Interferon*

1. INTRODUCTION

Many transformed cells express high levels of a cellular encoded phosphoprotein termed p53. While p53 was first described in Simian virus 40 (SV40)-transformed cells [1], it has become clear that high levels of p53 expression characterize cells which were transformed by a wide variety of agents [2]. The p53 was detected in normal cells in small amounts and it has been suggested that this protein might have a role in the regulation of the mammalian cell cycle [3–5]. Friend-erythroleukemia cells are virus-transformed erythroid cell precursors that can be induced by dimethylsulfoxide (Me_2SO) and a variety of other agents to express characteristics of terminal erythroid cell differentiation [6]. The induced differentiation is characterized by the coordinated expression of commitment to terminal division [7] and accumulation of the different globin mRNAs, the corresponding hemoglobins, and many other specific proteins. We here report that following induction of differentiation, the rate of p53 synthesis preferentially decreases in correlation with the changes in cell cycle distribution. Interferon, which is considered a cellular growth inhibitor [8,9] does not modulate down the expression of p53 if added to transformed non-induced Friend-erythroleukemia cells; however, it, further reduces the minimal levels of p53 synthesis in differentiating cells.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Cell culture*

The Friend-erythroleukemia cells used originate from clone 745 [10], and were grown as in [11]. Cell density was determined by a model D Coulter counter.

Published by Elsevier Science Publishers B.V.

2.2. *Induction of differentiation*

Exponentially growing Friend-erythroleukemia cells were inoculated at a density of 10^5 cells/ml, and 1.5% (v/v) dimethylsulfoxide (Me_2SO) was added (time 0). Three days later, the culture was diluted 10-fold into fresh medium supplemented with 1.5% Me_2SO in order to prevent the differentiating cells from reaching saturation cell densities [11]. When indicated, cells were induced with Me_2SO at 10^4 cells/ml and the dilution step on day 3 was omitted. Hemoglobin content in cell extracts was measured by the benzidine assay as in [11].

2.3. *Cell-labeling and immunoprecipitation*

10^6–10^7 cells were pulse-labeled for 20 min with [^{35}S]methionine (50 μCi/ml, 1200 Ci/mmol) in methionine-lacking Dulbecco's modified Eagle's medium containing 2% dialyzed fetal calf serum. Cells were washed with phosphate-buffered saline at 4°C and the pellet was extracted in lysis buffer (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.5/100 mM NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1% $NaDodSO_4$) containing protease inhibitors, and clarified for 10 min at $100000 \times g$. Fractions of cell lysate (0.1–0.5 ml) containing 10^7 trichloroacetic acid precipitable cpm were preabsorbed with *Staphylococcus aureus* (formaline fixed) and immunoprecipitated with monoclonal antibodies to p53 (18 h in 4°C). Antigen-antibody complexes were collected following binding to *S. aureus* (1 h in 4°C), then extensively washed with lysis buffer, processed and run on 10% $NaDodSO_4$ polyacrylamide gels. The intensity of the p53 band, from autoradiograms prepared following fluorography of the gels, was measured with Cliniscan scanner and the peak area was calculated.

2.4. *Cytofluorometry analysis*

Samples of 10^6 cells were stained with propidium iodide (50 μg/ml; Sigma) in the presence of 0.1% Triton X-100 and 4×10^4 cells were analyzed in a fluorescence activated cell sorter (model FACS II Becton-Dickinson). The fluorescent signals were detected with S-11 photomultiplier tube set at 550 V. The percentage of cells in the different phases of the cell-cycle was calculated from the DNA content distribution of cells.

2.5. *Materials*

Monoclonal antibodies against p53 (PAb 421) were obtained from E. Harlow (ICRF, London) [12], and concentrated by ammonium sulfate precipitation. Mouse β interferon was prepared from Friend-erythroleukemia cells as in [11].

3. RESULTS

3.1. *Decrease in the synthesis and stability of p53 protein during differentiation of Friend-erythroleukemia cells*

Friend-erytrholeukemia cells were labeled for 20 min with [^{35}S]methionine during the exponential growth phase (2×10^6 cells/ml) and the p53 was isolated by immunoprecipitation with monoclonal antibodies against this protein. Fig.1A shows that the erythroleukemia cells expressed relatively high amounts of p53 which comigrated on SDS-polyacrylamide gels with the p53 of the Abelson murine leukemia virus-transformed lymphocytes [13]. Following exposure of these cells to

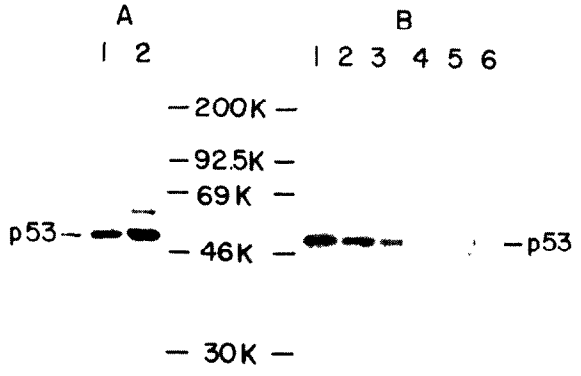

Fig.1. p53 synthesis during differentiation of Friend-erythroleukemia cells (A) Exponentially growing cells (2×10^6 cells/ml) were pulse-labeled for 20 min with [^{35}S]methionine, equal amounts (10^7 cpm) of trichloroacetic acid-insoluble radioactivity were immunoprecipitated with monoclonal antibodies to p53 and analyzed on SDS-polyacrylamide gels, as described in section 2. Lane 1, Friend-erythroleukemia cells; lane 2, Abelson murine leukemia virus-transformed lymphocytes. (B) Friend-erythroleukemia cells were induced to differentiate at 10^5 cells/ml as described in section 2. At different time intervals samples were pulse-labeled, and 10^7 trichloroacetic acid-immunoprecipitated as in (A). Lanes 1–5, days 0, 1, 2, 4 and 7, respectively; lane 6, immunoprecipitation as in lane 1 with normal mouse serum instead of antibodies to p53. M_r-marker proteins are shown between (A) and (B).

the inducer of differentiation (Me_2SO), the rate of p53 synthesis gradually decreased reaching minimal levels at day 7 post-induction (fig.1B). The extent of reduction in the rate of p53 synthesis during cell differentiation varied within a range of 5–10-fold in the different experiments. It should be noted that under the specific conditions of Me_2SO induction performed in this experiment, cell density varied between 10^5 and 2×10^6 cells/ml [11]. However, this parameter by itself, in the absence of Me_2SO, did not influence the rate of p53 synthesis (lane 1, fig. 1A,B).

The half-line of p53 protein before and after induction of differentiation was determined by pulse chase experiments as described in fig.2. In non-induced cells, the half-life of p53 was about 3.5 h and it decreased about 3-fold in the terminally differentiated cells as calculated from the slopes of the curves.

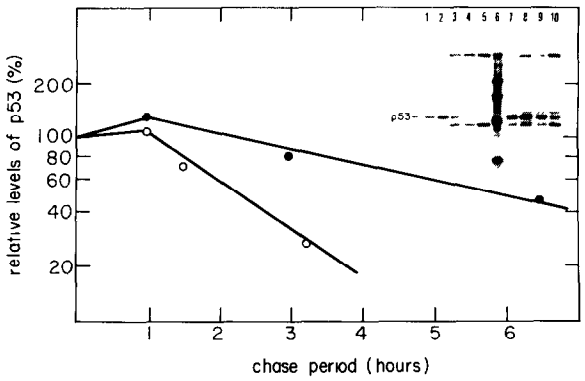

Fig.2. Pulse chase analysis of p53 in control non-induced and Me_2SO-induced Friend cells. Exponentially growing control cells (10^6 cells/ml) and differentiated cells at day 7 post-induction (2×10^6 cells/ml) were pulse-labeled as in fig.1. At the end of the pulse, the cells were washed extensively with methionine containing medium free of the radioisotope and incubated in the same medium for the indicated chase periods. Equal amounts of trichloroacetic acid-insoluble radioactivity were immunoprecipitated, analyzed on gels and the autoradiograms scanned as in section 2. Levels of the p53 are expressed as a percentage of the corresponding 0 h chase sample. (●—●) control cells; (○—○) differentiated cells. Insert: lanes 1–5, samples from differentiated cells at 0-, 1-, 1.5-, 3.25- and 6-h chase periods, respectively; lanes 7–10, samples from control cells at 0-, 1-, 3- and 6.5-h chase periods, respectively; lane 6, M_r marker proteins as in fig.1.

3.2. *Reduction in the rate of p53 synthesis is correlated with the commitment to terminal cell division during the process of differentiation*

We have here determined the distribution of cells within the different phases of the cell-cycle by cytofluorometric analysis of DNA content of the cells. Fig.3 shows that in exponentially growing cells (time 0), 65% of cells were found at the replicative phases of the cell cycle, i.e., S + G_2 + M phases, and this value gradually decreased following induction of differentiation. It can therefore be concluded that the typical decline in the rate of cell growth reported to take during differentiation [11] results from specific accumulation of cells at the resting phase of the cell cycle; i.e., the G_0/G_1 phase. In this respect, it was of interest to determine whether the reduction in p53 synthesis is preferentially linked to this defined program of growth arrest rather than to the other pathways which operate during cell differentiation. Fig.4. shows that exposure of cells to Me_2SO at low cell density (10^4 cells/ml) delayed the changes in cell-cycle distribution by 24 h as compared to the conventional induction at 10^5 cells/ml, whereas the

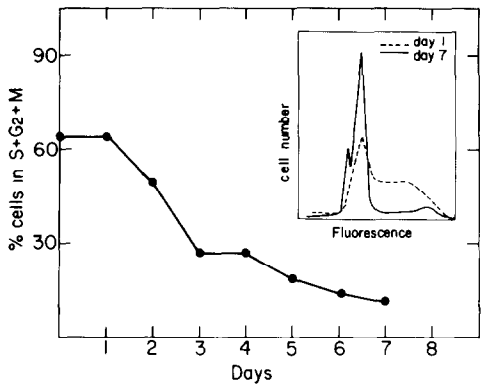

Fig.3. Changes in cell-cycle distribution during differentiation of Friend-erythroleukemia cells. Cells were induced by 1.5% Me_2SO at 10^5 cells/ml and samples were removed for cytofluorometric analysis at different days following induction as in section 2. The percentage of cells found at the S + G_2 + M phases of the cell-cycle was calculated from the DNA distribution profile of cells; two of them are illustrated in the insert (days 1 and 7 of differentiation). The first peak represents the number of cells with G_0/G_1 DNA content, the second peak presents cells with G_2/M DNA content, and between the two peaks are represented cells in the S stage.

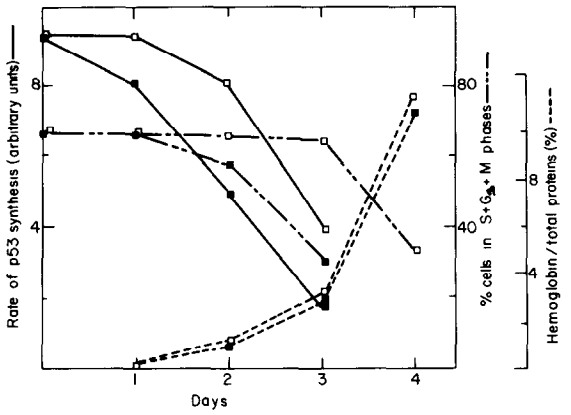

Fig.4. Kinetics of the changes in cell-cycle distribution, p53 synthesis and hemoglobin accumulation following Me_2SO induction at low cell density; comparison with the conventional conditions of differentiation. Friend cells were induced by 1.5% Me_2SO at 10^4 cells/ml (□) and 10^5 cells/ml (■) as described in section 2. At different time intervals, samples were pulse-labeled with [^{25}S]methionine and the rate of p53 synthesis was determined by densitometric tracing of the autoradiogram as in section 2. Another portion of cells was used for cytofluorometric analysis as in fig.3. Hemoglobin was determined in 20 μg cell extract. (——) Rate of p53 synthesis expressed in arbitrary units; (— · — ·) percentage of cells found at replicative phases $S + G_2 + M$; (- - - - -) hemoglobin total proteins (%).

kinetics of hemoglobin accumulation were not changed. Very interestingly, the decrease in the rate of p53 synthesis also depended on cell concentration and a similar delay of 24 h was detected in the kinetics of reduction in p53 synthesis if induction started at a low cell density (fig.4). The decline in the expression of p53 during cell differentiation seems, therefore, to be linked to the program of growth arrest rather than to the induction of the globin genes. Fig.4 also shows that in both conditions of induction of differentiation the reduction in the rate of p53 always preceded this specific change in cell-cycle distribution.

3.3. *Effects of interferon on cell growth and on the expression of p53 in Friend-erythroleukemia cells*

We have exposed Friend-erythroleukemia cells to interferon in order to study whether interferon changes the expression of p53 protein under conditions where it inhibits cell growth. Table 1 shows that the treatment of transformed non-induced cells with 1000 Units/ml of interferon resulted in growth inhibition without affecting either the rate of p53 synthesis or the distribution of cells within the different phases of the cell cycle. The response of differentiating Friend-erythroleukemia cells to interferon was, however, completely different. Me_2SO-induced cells were 100-times more sensitive to the antigrowth effect of interferon than non-induced cells and 10 Units/ml were sufficient to cause 50% growth inhibition. In addition, low doses of interferon further reduced the minimal levels of p53 synthesis measured at the end of the in vitro process of cell differentiation by 30%, and

Table 1

Effect of interferon on cell growth, cell-cycle distribution and the rate of p53 synthesis in Friend-erythroleukemia cells

	Differentiating cells		Non-induced cells	
IFN (units/ml)[a]	1	10	10	1000
Growth inhibition (%)[b]	25	50	none	45
Decrease in the ratio $S + G_2 + M/G_1$ phases (%)	35	53	none	none
Inhibition of p53 synthesis (%)	32	35	none	none

[a] Interferon was added either to differentiating cells at day 4 post Me_2SO induction or to exponentially growing non-induced cells. The different parameters were measured 72 h later, except for p53 synthesis which was determined 48 h after exposure to interferon. The data are expressed as percentage inhibition of the values measured in the corresponding cells not treated with interferon

[b] Growth was determined by monitoring cell density as in section 2

also reduced the portion of cells found at the replicative phases at that time (table 1).

4. DISCUSSION

We here report that the process of cell differentiation in Friend-erythroleukemia cells is accompanied by 80–90% inhibition of p53 synthesis. The change in the turnover of the p53 protein is, however, less dramatic and the half-line decreases from 3.5 h in non-induced cells to about 1 h in terminally differentiated cells. Based on these two observations, we suggest that a marked decline in the intracellular level of p53 takes place during cell differentiation. Several mechanisms which regulate the levels of p53 in different cell systems were previously described, such as stabilization of p53 protein in SV40-transformed cells by its association with large T-antigen [1], or changes in the amount of translatable p53 mRNA following differentiation of embryonal carcinoma cells [14]. It will be of interest of study in the Friend cells whether transcriptional and/or post-transcriptional events are being subjected to shut-off mechanisms during differentiation, an approach which can be studied by using the p53 cDNA clones which were recently isolated [15].

It has been previously suggested that p53 is involved in the regulation of the mammalian cell-cycle based on experiments performed on G_0-arrested lymphocytes [2], normal kidney epithelium cells [3] and Swiss 3T3 cells [4]. In the latter case, microinjection of antibodies directed against p53 into the nuclei of quiescent 3T3 cells inhibited their entry into the S phase of the cell cycle. According to the detailed kinetics presented here, reduction in p53 synthesis starts at a short-time interval following Me_2SO induction and precedes the typical arrest in growth which is characterized by accumulation of cells at the G_0/G_1 phase of the cell cycle. We here show that these two events are closely linked and it remains to be determined whether causal relationships exist between the decline in p53 levels and the specific cell-cycle arrest. Variants of erythroleukemia cells inducible for homoglobin synthesis without commitment to terminal cell division have been recently isolated [16], and it will be of interest to test whether the shut-off mechanism of p53 synthesis is operating in these cells.

Another feature which characterizes the shut-off in p53 synthesis following Me_2SO induction is its dependence on cell concentration. Starting induction at cell density which was 10-times lower than the conventional conditions of induction delayed the kinetics of decline in p53 synthesis by 24 h (time period which allows 2.3 cell doublings). One possibility to interpret these data is to assume that, unlike the induction of the globin genes, the shut-off in p53 synthesis is not directly induced by Me_2SO, but is rather modulated by extracellular factors secreted by the differentiating cells which have to reach certain threshold levels in order to be effective. We have previously reported that Friend-erythroleukemia cells secrete interferon during differentiation [11]. However, since this interferon is produced at a late stage following induction (days 5–7) and the addition of neutralizing antibodies against this specific interferon did not affect the rate of p53 synthesis (not shown), it is unlikely that the spontaneous interferon could modulate the main reduction of p53 synthesis. In addition, the data in this work clearly demonstrate that exogenous preparations of interferon do not modulate down the expression of p53 in transformed non-induced cells even if added at high doses which inhibit cell growth but can, however, further reduce the minimal levels of p53 synthesis taking place during differentiation. It would be of interest to study in the future whether other negative growth factors besides interferon are being secreted in these systems of cell differentiation.

ACKNOWLEDGEMENTS

We thank Dr E. Harlow for the supply of monoclonal antibodies against p53, and Drs M. Oren and V. Rotter for helpful discussions. Work was supported by the Leukemia Research Foundation, Chicago. A.K. is an incumbent of the Louis and Ida Rich Career Development Chair.

REFERENCES

[1] Linzer, D.I.H. and Levine, A.J. (1979) Cell 17, 43–52.
[2] Advances in Viral Oncology (1982) (Klein, G., ed.) vol. 2., Raven, New York.
[3] Milner, J. and Milner S. (1981) Virology 112, 785–788.

[4] Dippold, W.G., Jay, G., DeLeo, A.B., Khoury, G. and Old, L.J. (1981) Proc. Natl. Acad. Sci. USA 78, 1695–1699.
[5] Mercer, W.E., Nelson, D., DeLeo, A.B., Old, L.J. and Baserga, R. (1982) Proc. Natl. Acad. Sci. USA 79, 6309–6312.
[6] Marks, P.A. and Rifkind, R.A. (1978) Annu. Rev. Biochem. 47, 419–448.
[7] Gusella, J., Geller, R., Clarke, B., Weeks, V. and Housman, D. (1976) Cell 9, 221–229.
[8] Taylor-Papadimitrou, J. (1980) in: Interferon 2 (Gresser, I. ed) pp.13–46, Academic Press, New York.
[9] Kimchi, A., Shure, H., Lapidot, Y., Rapoport, S., Panet, A. and Revel, M. (1981) FEBS Lett. 134, 212–216.
[10] Friend, C., Scher, W., Holland, J.G. and Sato, T. (1971) Proc. Natl. Acad. Sci. USA 68, 378–382.
[11] Friedman-Einat, M., Revel, M. and Kimchi, A. (1982) Mol. Cell. Biol. 2, 1472–1480.
[12] Harlow, E., Crawford, L.V., Pim, D.C. and Williamson, N.H. (1981) J. Virol. 39, 861–869.
[13] Rotter, V., Boss, M.A. and Baltimore, D. (1981) J. Virol. 38, 336–346.
[14] Oren, M., Reich, N.C. and Levine, A.J. (1982) Mol. Cell. Biol. 2, 443–449.
[15] Oren, M. and Levine, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 56–59.
[16] Marks, P.A., Chen, Z., Banks, J. and Rifkind, R.A. (1983) Proc. Natl. Acad. Sci. USA 80, 2281–2284.