EXPRESSION OF THE *p53* ONCOGENE IN ACUTE MYELOBLASTIC LEUKEMIA

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The cellular phosphoprotein, p53, is expressed at high levels in cells transformed by a variety of agents including DNA and RNA viruses, and carcinogens (reviewed in 1 and 2). p53 expression is not restricted to tumor cell lines; fresh human tumor cells from patients with colorectal and mammary tumors exhibit elevated expression of p53 (3). Overproduction of p53 protein in certain cells has been shown (4–6) to confer an enhanced tumorigenic phenotype. The recent demonstration of the capacity of p53 to immortalize primary cells (7) and to cooperate with the activated c-Ha-*ras*-1 oncogene in the transformation of rat embryo fibroblasts (7–9) indicates that the *p53* gene has oncogenic potential, and consequently can be classified as an oncogene. p53 cannot be considered a marker for malignancy, however, since p53 protein synthesis is observed (10–13) after mitogen or serum stimulation of certain quiescent normal cells. Moreover, in the study cited above (3), 4 out of 19 fibroadenomas (benign breast tumors) examined had increased levels of p53.

It is possible that certain oncogene products that confer an establishment/immortalizing phenotype (such as myc, p53, Ela) on primary cultures causing them to grow indefinitely may also play a role during normal and malignant stem cell self-renewal. Self-renewal is the defining property of stem cells, which allows these cells to generate essentially identical progeny in the absence of further differentiation. In this study, we focus on this possibility by examining p53 expression in leukemic blast cells from patients with acute myeloblastic leukemia (AML).[1] AML is a clonal hemopathy that is believed to arise in pluripotent stem cells following multiple carcinogenic events (14–16). It has been proposed that these events give rise to malignant stem cells with the capacity to produce the relatively normal myelopoietic cells and blast cells that characterize this disease.

Leukemic blast populations may be considered a lineage, maintained by a small number of progenitors (17); these are assayed by their capacity to form blast colonies in methylcellulose (18–20), and may be regarded as malignant stem cells

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[1] *Abbreviations used in this paper:* AML, acute myeloblastic leukemia; CM, conditioned medium; LCM, leukocyte-conditioned medium; NET buffer, buffer containing NaCl, EDTA, and Tris, as defined in Materials and Methods; PE2, secondary plating efficiency.

since they possess self-renewal capacity. The capacity of blasts for renewal is usually assayed by replating either single colonies or cells pooled from primary colonies (19). The former method shows marked colony-to-colony variation; in the latter, the plating efficiency of cells from pooled primary colonies (secondary plating efficiency [PE2]) can be considered as the average self-renewal capacity of the leukemic blast stem cells in each patient. A significant correlation has been found between low PE2 values and success in obtaining remission with intensive chemotherapy (21, 22).

Materials and Methods

Cells. Blasts were obtained from the peripheral blood of patients with AML. These patients were compared to a larger historical control consisting of patients seen at our institution in the last three years, and were found to be representative in terms of age, sex, FAB classification, and clinical status. Furthermore, the blast colony assay results obtained using cells from these patients were not significantly different from the historical control population ($p = 0.28$, $n = 82$; Mann-Whitney U test). After isolation, only mononuclear cell populations that were >95% blasts by standard morphological staining were used in this study. Normal marrow cells were obtained from healthy marrow transplantation donors and from recipients, 28 d after transplant. Mononuclear cell fractions were prepared as described previously (20) by separation through Ficoll-Hypaque or Percoll ($\rho = 1.077$ g/ml). The suspension was then depleted of T lymphocytes using a second Ficoll-Hypaque separation after erythrocyte rosette formation. Some normal bone marrow specimens were also depleted of adherent cells. The T lymphocyte–depleted cells were either cultured immediately or preserved by freezing in liquid nitrogen in 10% DMSO, 40% α-MEM, and 50% FCS.

OCI-Ly2 is a human lymphoma cell line obtained from Drs. M. Tweeddale and H. Messner (Ontario Cancer Institute).

Blast Colony Assay. Blast colony formation in culture was obtained as described previously (20). The T lymphocyte–depleted mononuclear cells were mixed with 0.8% methylcellulose in α-MEM supplemented with 20% FCS and 10% PHA-leukocyte conditioned medium (PHA-LCM) and cultured in 35-mm Lux culture dishes at 37°C. After 7 d, the colonies were counted and the dishes were pooled. Cells were washed twice in α-MEM and replated in 0.8% methylcellulose, 20% FCS, and 10% PHA-LCM at a concentration of 10^4 cells per 0.1 ml in a 6-mm microwell of a flat-bottom Linbro tissue culture tray. Secondary colonies were counted after 7 d of incubation, and the values are expressed as the secondary plating efficiency (colonies per 10^4 cells, or PE2).

In Vivo Metabolic Labelling of p53. Because many of the samples had been stored frozen, the cells were thawed and incubated overnight at 37°C in α-MEM containing 10% FCS, with or without PHA-LCM, to allow recovery of metabolic activity. In addition, certain samples, including fresh T lymphocyte–depleted normal mononuclear marrow cells, were incubated for 5 d in medium conditioned by cells of the continuous bladder carcinoma line HTB9 (HTB9-CM) (23) to optimize hematopoietic cell growth before metabolic labelling. PHA-LCM and HTB9-CM were essentially identical in their ability to stimulate the growth in culture of granulocyte/macrophage colonies and erythroid colonies from normal bone marrow, as well as leukemic blast colonies from AML blood (23). ~10^7 cells were labelled for 1 h at 37°C with 0.2 mCi of [^{35}S]methionine in 0.5 ml α-MEM lacking methionine, or for 3 h at 37°C with 1.0 mCi [^{32}P]orthophosphate in 2.0 ml α-MEM lacking phosphate. Cells were then washed with PBS and lysed for 30 min on ice with 0.3 ml lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 8, 0.5 mM PMSF). Lysates were cleared by centrifugation for 1 min, and by the addition of 0.5 ml of a 10% suspension of formalin-treated *Staphylococcus aureus* Cowan 1 cells (Pansorbin; Calbiochem-Behring, San Diego, CA) for 15 min on ice, followed by centrifugation and retention of the supernatant. For [^{35}S]methionine-labelled cells, lysate volumes corresponding to equal numbers of TCA-insoluble radioactivity (10^7 cpm) were immunoprecip-

itated overnight at 10°C with antibody in 400 μl NET gel buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.05% NP-40, 0.02% sodium azide, 0.25% gelatin). Immune complexes were collected on 50 μl *S. aureus* cells as above, washed twice in NET gel buffer, and then eluted into 30 μl sample buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 25 mM Tris, pH 6.8) by heating at 70°C for 10 min. *S. aureus* cells were removed by centrifugation, and samples were loaded onto a 12.5% polyacrylamide gel in the presence of SDS. Electrophoresis was at 35 mA until the blue dye front reached the bottom of the gel. Gels were fixed in 7.5% acetic acid/25% methanol for 30 min before being dried and exposed to Kodak XAR-5 film.

Genomic DNA Analysis. DNA was extracted from blast cells by the method of Blin and Stafford (24) using proteinase K and SDS. Samples (10 μg) were digested to completion with the appropriate restriction enzyme, and separated by electrophoresis on 0.8% agarose gels. The DNA in the gels was transferred to nitrocellulose filters as described by Southern (25). The filter was hybridized using standard conditions (26), with a [^{32}P]-labelled DNA fragment obtained from the human p53 cDNA clone p102 (27) after digestion with Nco I and Bam HI. These enzymes liberate a p53 cDNA insert of 1,410 bp.

Results

Synthesis of p53 protein was measured in leukemic blast cells from 34 patients with AML. Cells were labelled metabolically with [^{35}S]methionine, and in certain instances with [^{32}P]orthophosphate. Because many of the samples had been stored frozen, the cells were thawed and incubated overnight at 37°C in α-MEM containing 10% FCS to allow recovery of metabolic activity before labelling. In addition, certain samples were incubated for 5 d in HTB9-CM (23) to optimize blast cell growth before metabolic labelling. Radiolabelled p53 was immunoprecipitated from cellular extracts, subjected to electrophoresis in SDS-polyacrylamide gels, and visualized after autoradiography (Fig. 1).

p53 was present in the blast cells of 19 out of 34 AML patients. p53 protein was not detected in lymphocyte- and adherent cell–depleted mononuclear cells from normal bone marrow, or from regenerating marrow from patients with transplants when freshly obtained or after culture under conditions identical to those used to detect p53 in blast cells. We also did not detect p53 protein in proliferating early-passage cultures of human fibroblasts (data not shown). The absence of detectable p53 protein in normal human cells is consistent with numerous previous reports (1, 3, 28–30).

The 15 blast cell preparations in which p53 protein was not detected included blast cells from two patients, which were cultured for 5 d in conditioned medium and shown to be proliferating on the basis of increasing cell number, increasing colony number in the blast colony assay (20) after plating on methylcellulose, and by their DNA-staining (Hoescht) profile on a FACS. Hence, proliferation in the presence of HTB9-CM, which is necessary for the maintenance of AML blast populations in culture, is not sufficient to produce detectable p53 protein synthesis in certain leukemic blast cells.

It is apparent in Fig. 1 that heterogeneity of human p53 protein exists. Patient 1, for example, has a single species of p53, whereas patient 2 has two or possibly three distinct forms of p53. A novel form of heterogeneity was uncovered when samples were screened with two antibodies against p53: an mAb, PAb421 (31), and polyclonal antibody from human serum, P407 (32). p53 protein in the blast cells of patient 3 (Fig. 1) was recognized by the polyclonal serum but not by the PAb421 mAb. We confirmed this observation by labelling the blast cells of this

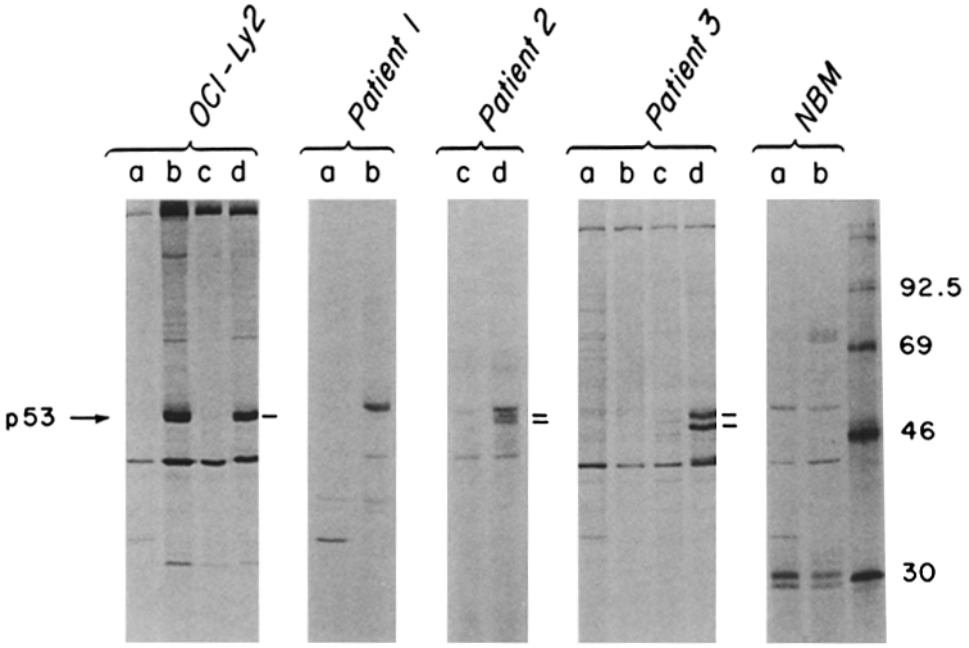

FIGURE 1. Expression of p53 protein in human cells. Blast cells from three patients with AML were obtained as described in Materials and Methods. Blast cells from patient 1 were incubated overnight in α-MEM containing 10% FCS before labelling. Blast cells from patients 2 and 3 were grown in HTB9-CM (23) for 5 d before labelling. OCI-Ly2 is a human lymphoma cell line. Normal bone marrow (*NBM*) consists of Percoll-separated mononuclear cells from marrow obtained from a donor of marrow for transplantation. ~10^7 cells were labelled for 1 h at 37°C with 0.2 mCi of [^{35}S]methionine in 0.5 ml α-MEM lacking methionine. Labelled extracts were immunoprecipitated with the following antibodies: lane *a*, PAb419 (31) (control mAb that recognizes a 35 kD cellular protein); *b*, PAb421 mAb against p53 (31); *c*, human γ globulin; *d*, P407 anti-p53 serum (32).

patient with [^{32}P]orthophosphate and immunoprecipitating the p53 protein, as shown in the autoradiogram in Fig. 2. OCI-Ly2 is a human lymphoma cell line that contains phosphorylated p53 that is recognized by both antibodies, and it is included as a positive control. The p53 protein present in the blast cells of patient 3, however, is only recognized by the P407 serum. Heterogeneity of p53 has been described previously; that observed as differences in mobility is seen in the samples from patients 2 and 3 (Fig. 1) (28, 30). Antigenic heterogeneity has also been observed (11, 33).

P407 serum is polyclonal, and likely contains antibodies to a variety of antigens, in addition to p53. However, both the size and phosphorylated status of the protein recognized by the P407 serum, in the case of patient 3, indicate that the protein is an immunologically altered form of p53 in which the epitope for mAb PAb421 is either missing or masked. Whereas two distinct species of p53 are present in the blast cells of patient 3 after [^{35}S]methionine labelling (Fig. 1), only one species appears to be phosphorylated (Fig. 2). We have no satisfactory explanation for this observation. It is unlikely that the separation achieved in the polyacrylamide gel displayed in Fig. 2 was inadequate to resolve the two species of p53, although this possibility cannot be dismissed. More detailed analysis of

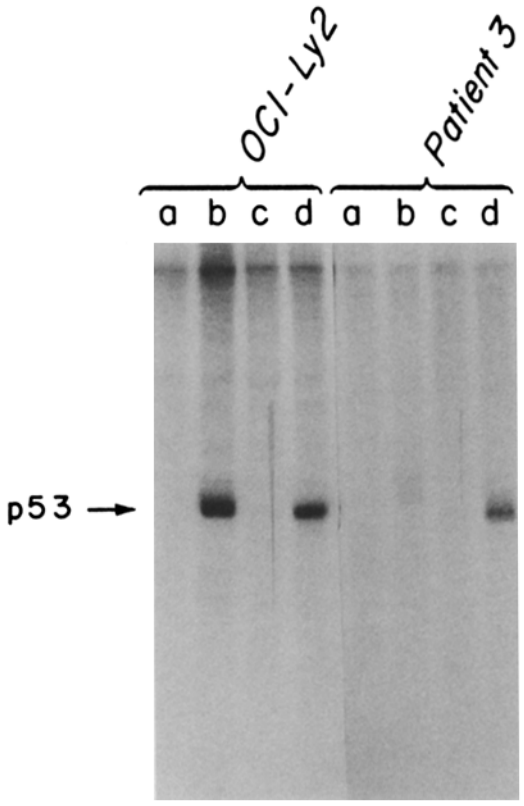

FIGURE 2. Detection of phosphorylated p53 protein in human cells. OCI-Ly2 cells and AML blast cells from patient 3 (see legend to Fig. 1) were labelled for 3 h with 1 mCi [^{32}P]-orthophosphate in 2 ml α-MEM lacking phosphate. Lysate were prepared and proteins immunoprecipitated as described in Materials and Methods. Equal volumes of lysates were subjected to immunoprecipitation. Lane *a*, PAb419 control mAb; *b*, PAb421 mAb against p53; *c*, human γ globulin; *d*, P407 anti-p53 serum.

the biochemical properties of p53 protein (for example, by identifying and comparing phosphorylated amino acids in [^{32}P]-labelled p53 proteins from blast cells and OCY-Ly2) may provide insight into the nature and significance of the observed heterogeneity.

To determine whether the absence of p53 expression was the result of rearrangement or loss of the cellular p53 gene, as shown previously in other cellular systems (5, 34, 35), genomic DNA from blast cells was digested with Pvu II, Pst I, Bam HI, or Hind III, and analyzed by the Southern blot technique (25). The probe used for these experiments was a Nco I/Bam HI insert from a cDNA clone, p102, containing human p53-specific sequences (27). A representative autoradiogram is shown in Fig. 3. No differences in the p53 gene were detected at this level of resolution in p53-producing and -nonproducing blast cells. Any alteration occurring at the extreme 5′ end of the p53 gene may have been missed in this analysis, however, since the cDNA clone used as a probe is not full length, and is missing sequences at the 5′ end.

The secondary plating efficiency (PE2) of AML blast populations provides a

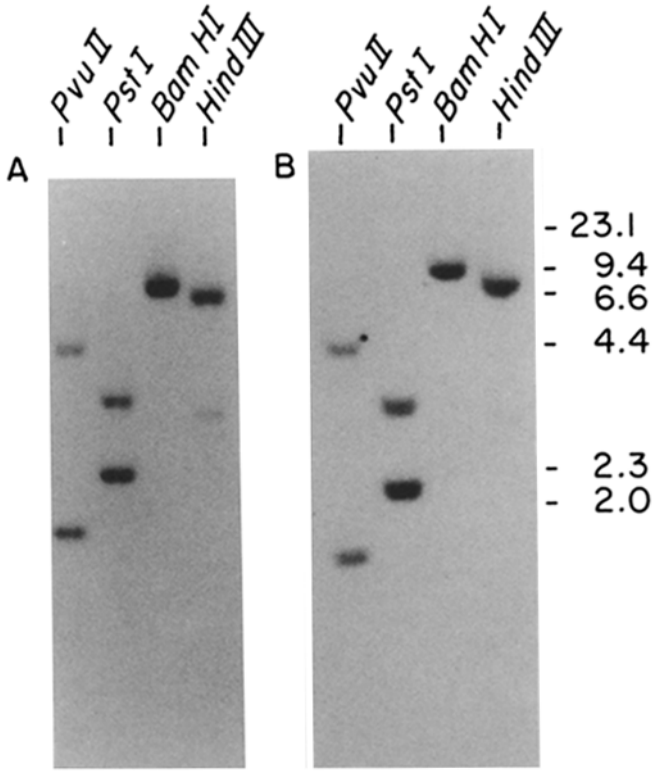

FIGURE 3. Southern blot of genomic DNA from AML blast cells. DNA (10 μg) was digested with restriction endonucleases Pvu II, Pst I, Bam HI, or Hind III, and separated on a 0.8% agarose gel. The DNA in the gel was transferred to a nitrocellulose filter as described by Southern (25). The filter was hybridized to the Nco I/Bam HI fragment of the human p53 cDNA clone p102 located between nucleotides 177 and 1,587 (27). (*A*) Genomic DNA isolated from blast cells of a patient not expressing p53 protein. (*B*) Genomic DNA isolated from cells that do express p53 protein.

measure of the renewal capacity of blast progenitors (19). To examine the possible involvement of p53 in the process of self renewal, the PE2 values of the 19 blast populations positive for p53 expression were determined and compared with the PE2 values of the 15 blast populations where p53 protein was not detected. The extent to which PE2 correlates with p53 synthesis is shown in Fig. 4. A highly significant correlation between p53 expression and a high PE2 value was obtained by the Mann-Whitney U test ($p = 0.0001$).

Discussion

Metabolic labelling of cells, followed by lysis and immunoprecipitation was used as an assay to assess p53 protein synthesis in the blast cells of patients with AML. There are a number of limitations in this type of assay, which have been discussed previously in detail (29). In particular, this assay cannot be used to determine the steady-state level of p53, since the half-life of this protein is variable in different cell types. At best, this assay provides a measure for the rate of p53 protein synthesis. The major difficulty with the assay centers on the

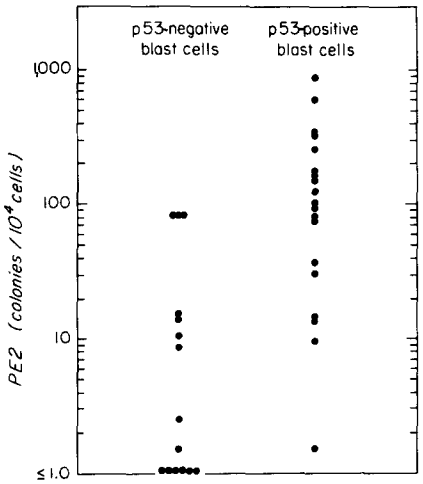

FIGURE 4. PE2 (secondary plating efficiency) of blast cells from 34 AML patients. PE2 was measured in the blast colony assay as described (20). The presence of detectable p53 protein was determined by metabolic labelling as described in Materials and Methods. $p53^+$ and $p53^-$ cells are significantly different with respect to PE2 ($p = 0.0001$; Mann-Whitney U test).

interpretation of negative data. The absence of immunoprecipitable p53 may be the result of any one of the following: inefficient uptake of [^{35}S]methionine, inefficient incorporation of [^{35}S]methionine into protein, large intracellular pools of competing methionine, absence or occlusion of the epitope on p53 to which the antibody is directed.

To eliminate some of these objections, we routinely immunoprecipitate aliquots of lysate containing equal numbers of TCA-insoluble counts. Furthermore, we routinely perform a control immunoprecipitation with mAb PAb419 (31) that recognizes a ubiquitous 35 kD protein (p35) (36). The presence of p35 on autoradiograms ensures that labelling artifacts have not occurred. In Fig. 1, p35 is present in each of the lanes where this antibody was used. We show that normal bone marrow cells do incorporate [^{35}S]methionine into p35 under the same conditions that fail to radiolabel p53. We recognize the possibility that normal bone marrow cells, and certain blast cells where p53 cannot be detected, may express p53 at levels too low to be detected by this assay. If p53 protein turnover was accelerated in these cells, we would likely not detect it using the metabolic labelling protocol described here.

To compare the sensitivity of metabolic labelling with other assays for p53, cellular extracts from $p53^+$ leukemic blast cells were prepared and examined for p53 by Western blotting (37) and by a solid-phase RIA for p53 (29). In neither case was a p53 signal detected (data not shown), indicating that the steady-state level of p53 protein remains low even in leukemic blast cells that are clearly expressing the protein. Hence, for an unstable protein like p53, measurement of synthesis by metabolic labelling is more sensitive than measurement of steady state level.

The metabolic labelling studies allow us to draw several conclusions: (*a*) p53 is expressed in fresh leukemic blast cells, (*b*) normal myeloid cells present in bone

marrow do not synthesize detectable p53, (*c*) human p53 protein exhibits structural heterogeneity on SDS-polyacrylamide gels, (*d*) heterogeneity of p53 expression exists in blast cells from AML patients, and (*e*) this heterogeneity in p53 expression shows a striking correlation with the secondary plating efficiency of blast cells in the blast colony assay.

If, as we have suggested (17), the blasts of AML are an abnormal lineage, it is difficult to find an appropriate normal control population for comparison. If blasts have normal counterparts, as would be implied by models postulating blocked differentiation (see, for example, 38), these counterparts would be present as very rare primitive cells in normal marrow. From either viewpoint, normal marrow mononuclear cells, depleted of T lymphocytes, may be the most suitable control population. These cells were cultured, moreover, in conditioned medium that supports cell proliferation and is known to contain hematopoietic growth factors including granulocyte/macrophage colony stimulating factor (GM-CSF), and erythroid-potentiating activity (EPA) (23). For all the above reasons, we consider our inability to detect p53 in normal marrow cells and in certain leukemic blast cell populations using mAb (PAb421) and polyclonal antibody (P407) to be significant and not artifactual. The presence of p53 in some leukemic blast populations and the absence of detectable p53 in normal myelopoietic cells is consistent with the model that AML blast cell populations form an independent lineage characterized by abnormal differentiation and possibly by abnormal gene expression (17).

The mechanism for regulating p53 expression in leukemic blast cells and for generating the heterogeneity in expression of p53 that is observed is not understood. In our initial attempts to characterize this heterogeneity, we examined the structure of the p53 gene in blast cells and found no differences between p53-producing and -nonproducing cells. In experiments using mouse and human cell lines, evidence has been obtained that the levels of p53 protein are not correlated with the amount of p53 mRNA, indicating that the amount of p53 protein in these cells is regulated at the posttranscriptional level, perhaps through changes in protein stability (27, 39–41). Experiments are presently in progress to examine p53 mRNA levels in blast cells to determine whether the findings are similar to those obtained using cell lines.

An immunologically distinct form of p53, not displaying the epitope for mAb PAb421 (31) was identified in the blast cells of one patient. Previously, Milner (11) showed that the p53 protein molecules in quiescent mouse lymphocytes do not display the epitope for PAb421, whereas the p53 protein in proliferating lymphocytes do. Wolf et al. (33) have also identified an immunologically distinct form of p53 that does not express the PAb421 epitope. This epitope has been mapped to the carboxyl-terminus on p53 (42). The significance of this alteration on p53 is not clear.

The highly significant correlation that is observed between p53 expression and the secondary plating efficiency (PE2) of leukemic blast cells suggests that p53 may play a role in events occurring in the process of self renewal. Expression of p53 has been shown to extend the life span of primary cells in culture, and also to transform these cells in collaboration with activated Ha-*ras* (7–9). Taken together, these data support the suggestion of Buick and Pollak (43) that cellular

immortalization may represent a deregulation of the self renewal process in stem cells through inappropriate expression of certain cellular oncogenes.

Exposure of blast cells in culture to a number of agents has been shown to change their PE2 value (44). 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) and 5-azacytidine increase PE2, whereas interferon and cytosine arabinoside decrease PE2. If p53 is involved in the process of self renewal, then we would expect these agents to affect p53 expression in the same way as they affect PE2. Experiments along these lines are presently in progress. More definitive proof must await the direct genetic manipulation of p53 expression in leukemic blast cells and demonstration that the PE2 value of these cells can be regulated.

Summary

We have investigated whether the *p53* oncogene is expressed in the blast cells of patients with acute myeloblastic leukemia. p53 protein was detected in the blast cells of 19 out of 34 patients, but not in normal myelopoietic cells. We find a highly significant correlation between p53 protein synthesis in leukemic blast cells and the secondary plating efficiency of these cells ($p = 0.0001$). The latter provides an estimate for the self renewal capacity of progenitor cells in the blast population. These data indicate that p53 may be involved in leukemic stem cell renewal.

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