Constitutive Expression of p53 Protein in Enriched Normal Human Blast Cell Populations

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Previous studies by others using metabolic labeling, cell lysis, and immunoprecipitation have reported elevated levels of p53 protein in blast cells derived from patients with acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML), whereas p53 protein was not detected in normal light-density bone marrow cells. In this report, using the same detection methods, we confirm the negligible expression of p53 protein in normal light density marrow cells. However, we find clearly significant levels of p53 protein expression in enriched normal human marrow blast populations. Furthermore, using a panel of p53 specific monoclonal antibodies, we find the p53 protein constitutively synthesized by normal marrow blasts has the immunologic pheno-
type identified by PAb240 that reportedly recognizes a common conformational-dependent epitope on mutant p53. We have also found that the p53 immunologic subclass identified by PAb240 exists in normal human circulating lymphocytes either resting, serum starved, or PHA activated. In summary, it is clear that (1) normal marrow blast populations provide the appropriate control for assessing the levels of p53 protein expression in leukemic blast cells; and (2) PAb240 cannot be used to distinguish p53 mutated at the DNA level from normal p53 in fresh human hematopoietic cells.

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MATERIALS AND METHODS

Normal human bone marrow and peripheral blood specimens. After appropriate Human Protection Committee validation and informed consent, posterior iliac crest bone marrow aspirates and peripheral blood specimens were obtained from normal healthy volunteers. The procedures used to obtain the enriched marrow blast populations and the nonadherent blood lymphocyte populations were as previously described in detail. Antip53 MoAbs. Saturating amounts of four anti-p53 murine MoAbs were used. PAb421 and PAb1801 were obtained from Oncogene Science (Manhasset, NY). PAb421 recognizes conformationally independent epitopes located on the C terminus of the p53 protein, whereas PAb1801 recognizes conformationally independent epitopes located on the N terminus. PAb240 (Oncogene Science) recognizes a common conformational-dependent epitope on mutant p53. PAb1620 (kindly provided by Dr Jo Milner, Cambridge University, UK) recognizes a common conformational-dependent epitope on wild-type p53.

Cell lines and culture conditions. Cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO2 in air. The Daudi cell line, derived from a patient with Burkitt's lymphoma, was used as a positive control. HL60, derived from a patient with promyelocytic leukemia, which has a deletion of the p53 gene, served as a negative control. MDA-MB-231, a malignant breast cell line, served as a positive control for PAb1620.

Metabolic labeling and immunoprecipitation. Cells were incubated for 2 hours in methionine-free medium before labeling for 1 to 2 hours at 37°C with 1 mCi [35S]-methionine (New England Nuclear, Boston, MA) in 1 mL of methionine-free IMDM contain-

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Submitted September 18, 1991; accepted December 17, 1991.

Supported by National Cancer Institute Grants No. CA20194 and RO1CA28704, The Associazione Italiana per la Ricerca sul Cancro (Genoa, Italy), The Enid A. Haupt Charitable Trust, The Samuel and May Rudin Foundation, and The United Leukemia Fund.

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0006-4971/92/7908-0014$3.00/0


ing 10% dialyzed heat-inactivated FCS (GIBCO Laboratories, Life Technologies, Inc, Grand Island, NY). Cells were washed twice with IMDM and lysed for 30 minutes at 4°C in 1 mL lysis buffer (0.5% [vol/vol] Nonidet P-40, 0.5% [vol/vol] Triton X-100, 250 mmol/L sucrose, 150 mmol/L NaCl, 50 mmol/L HEPES pH 7.5 [Fisher Scientific Ltd, Springfield, NJ]) containing protease and phosphatase inhibitors (20 μg/mL of aprotinin, leupeptin, pepstatin, trypsin inhibitor [STI], 1 mmol/L phenylmethylsulfonyl fluoride [PMSF; Boehringer Mannheim Biochemicals, Indianapolis, IN], 25 mmol/L benzamidine, 1 mmol/L sodium orthovanadate [Na3VO4], 10 mmol/L sodium fluoride [NaF], 80 mmol/L β-glycerophosphate, 20 mmol/L ethylene glycol-bis [β-aminethoxy] ethyl] N,N,N',N'-tetraacetic acid [EGTA; Sigma Chemical Co, St Louis, MO]).

Lysates were cleared by centrifugation for 30 minutes at 10,000 g.

The supernatant was preabsorbed with protein-G-agarose (GIBCO) and protein-A-sepharose (Sigma) and equivalent amounts of TCA-insoluble counts (106 to 107 cpm) were immunoprecipitated for 2 hours. Immune complexes were collected on Protein-A-sepharose (Sigma) and protein-G-agarose for 1 hour, washed five times with lysis buffer, and eluted into 30 μL of sample buffer by heating at 90°C for 10 minutes. The samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% acrylamide gel. After SDS-PAGE, gels were soaked in Entensify (DuPont Co Biotechnology Systems, Wilmington, DE), dried, and exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY).

**Cell cycle analysis.** Cell cycle studies were performed as described previously. The mean cell cycle parameters for the enriched blast populations were: G0/G1, 76%; S 20%, G2/M 4%. Representative cell cycle parameters for the peripheral blood lymphocytes are shown in Fig 1.

**RESULTS**

**p53 expression in normal human bone marrow cells.** Light density marrow cells (<1.075 g/cm3) and enriched marrow blast populations from six normal donors were metabolically labeled with [35S]-methionine and p53 immunoprecipitated from cell lysates using MoAbs PAb421, PAb1801, PAb240, and PAb1620 (Fig 2). Daudi cells were used as a positive control and HL60 cells, lacking p53 protein, were used as a negative control. As expected, no p53 protein was detected in HL60 cells (Fig 2, lane A) regardless of which p53 specific MoAb was used, while p53 was readily detectable in Daudi cells (Fig 2, lane B). The p53 protein synthesized by Daudi cells was recognized by PAb421 as well as by PAb240, which reportedly recognizes a conformational epitope specific for mutant p53, but was not reactive with PAb1620, which reportedly recognizes a conformational epitope specific for wild-type p53 protein. The synthesis of negligible amounts of p53 protein in normal light density marrow cells (Fig 2, lane C) is consistent with previous reports. However, p53 was clearly detected in the enriched blast cell population (Fig 2, lane D). As expected, p53 protein synthesized by normal blasts was detected by PAb421 and PAb1801 (not shown), whereas, unexpectedly, it was immunoprecipitated by PAb240 and not by PAb1620.

To confirm the activity of PAb1620, p53 was immunoprecipitated from lysates obtained from the human malignant breast cell line, MDA-MB-231, previously reported to be reactive with PAb1620 (Fig 3). p53 from MDA-MB-231 cells was immunoprecipitated by all four antibodies, indicating that this cell line contains at least two different immunologic subsets of p53.

To demonstrate that the 53K protein in the PAb240 immunoprecipitations is indeed p53, an additional control experiment was performed (not shown). Blast cells were labeled with [35S]-Methionine, lysed, and submitted to three successive rounds of immunoprecipitation with either PAb421, PAb240, or PAb1620. No 53K protein was immunoprecipitated when the final PAb421 supernatant was reimmunoprecipitated with PAb240. This was also true when the final PAb240 supernatant was immunoprecipitated with PAb421. However, p53 was immunoprecipitated from the final PAb1620 supernatant by either PAb240 or PAb421.

**p53 expression in normal human peripheral blood lymphocytes.** Having made the quite surprising observation that the p53 protein synthesized by normal human marrow blast cells was immunologically recognized by PAb240, the same studies were performed on lymphocytes before and after phytohemagglutinin (PHA) stimulation. In light of the intrinsic high proliferative capacity of normal marrow blasts, we were interested in looking at the p53 immunologic phenotype expressed by resting and PHA-activated normal lymphocytes. Thus lymphocytes were metabolically

**Fig 1.** Expression of p53 protein in fresh normal human circulating resting and PHA-stimulated lymphocytes. After 0 hours (lane A), 24 hours (lane B), 48 hours (lane C), and 72 hours (lane D) of PHA stimulation, lymphocytes were metabolically labeled for 2 hours, lysed, and equal amounts of TCA-precipitable counts (6 x 106 cpm) were immunoprecipitated with normal mouse IgG (control), and PAbs 240 and 1620. The gel was exposed to X-ray film for 6 hours at all time points. Lane A (0 hours, PAb 240) shows a faint p53 band that was intensified by longer exposures (not shown). The cell cycle parameters are shown for each time point.
After 60 minutes of labeling with [35S]-methionine, the cells were lysed and equal amounts of TCA-precipitable counts (7 x 10^4 cpm equivalent to 15 x 10^4 light-density cells and 1 x 10^4 blast cells) were immunoprecipitated with three different MoAbs to p53. HL60 cells (lane A) were used as a negative control; Daudi cells (lane B) were used as a positive control; light-density marrow cells (lane C); and marrow blast cells (lane D). Lane B was exposed to X-ray film for overnight and lanes A, C, and D for 3 days.

labeled before PHA stimulation (0 hours) and at 24, 48, and 72 hours after PHA stimulation, and immunoprecipitated at these time points with control normal mouse IgG, PAb240, and PAb1620 (Fig 1). PAb240 immunoprecipitated p53 from lysates of unstimulated (0 hours) and PHA-stimulated lymphocytes, whereas PAb1620 failed to detect p53 in either resting or PHA-activated lymphocytes. The same results were obtained in a second study (data not shown) using lymphocytes from another normal donor. It should be noted that in the second study, the lymphocytes were cultured in both the presence and absence (ie, 0.5% bovine serum albumin [BSA]) of FCS for 24 hours before the addition of PHA and p53 was still only detected by PAb421 and PAb240 but not by PAb1620. Serum starvation was performed in light of a previous study by Milne that showed that different immunologic subclasses of p53 exist in unstimulated and Con A-stimulated murine splenic lymphocytes cultured in the absence of FCS.

DISCUSSION

In the present study, we have confirmed the previous observation that negligible amounts of p53 protein are present in normal human light-density marrow cells. However, in addition, we have demonstrated that constitutively expressed p53 protein can be clearly detected in as few as 1 x 10^4 cells derived from highly enriched normal marrow blast populations.

Elevated levels of p53 protein have been reported in ALL and AML blast populations. However, our study is the first to demonstrate significant expression of p53 in normal hematopoietic blast populations. It is clear that this latter population is the appropriate normal control for determining whether leukemic blasts have elevated or reduced expression of p53 protein.

That significant expression of p53 protein was detected in normal blast populations compared with negligible amounts in the light-density marrow populations from which the blasts were derived suggests that expression of p53 is correlated with the proliferative potential of the cell population. Evidence supporting this are the recent findings that p53 levels progressively increase from G1 → S → G2,M while nonstimulated cells express p53 to a lesser degree.

A quite unexpected finding was that essentially all of the p53 protein synthesized by normal human enriched marrow blasts and normal human resting and activated peripheral lymphocytes had an immunologic phenotype recognized by PAb240. PAb240 has been proposed to identify a common conformational epitope specific for mutant p53 and that immunoprecipitation of p53 by PAb240 is diagnostic of mutation in both murine and human systems. By the present methods used, we were unable to detect a p53 protein species immunoreactive with PAb1620 in human hematopoietic blast populations, resting, serum-starved, or PHA-activated lymphocytes. PAb1620 has been reported to be reactive with a conformational epitope specific for wild-type p53 protein and is unreactive with mutated p53. Because the marrow and blood specimens for our studies were obtained from eight normal healthy donors, this precludes the possibility that the conformation of the p53 protein recognized by PAb240 found in each of the eight normal hematopoietic cell populations was caused by mutations of the p53 gene. A plausible explanation for our findings is offered by the studies of Milner et al, which provide evidence that wild-type p53 is an allosteric protein that can exist in at least two conformations and that the function of the p53 protein in normal cell proliferation.
is conformation dependent. Based on their evidence, it was further suggested that wild-type p53, when in the conformation identified by PAb1620, functions as a suppressor of cell proliferation and that wild-type p53 in a conformation identified by PAb240 (not brought about by genetic mutations) functions to transiently release the suppressor effect of p53, allowing for cell proliferation. Further evidence to support the immunologic data is the demonstration by two-dimensional gel electrophoresis of multiple subspecies of human wild-type p53.7

In summary, normal human hematopoietic cells contain p53 protein in a conformational state identified by PAb240. In light of our observations and Milner’s studies, a possible explanation could be that the majority of human normal circulating lymphocytes and enriched marrow blast cells contain an activated conformation of p53, and that p53 protein in the wild-type suppressor conformation (identified by PAb1620), having a short half-life, exists in minute quantities that cannot be detected. Based on our findings, it appears that PAb240 cannot be used to distinguish p53 mutated at the DNA level from normal p53 in fresh human hematopoietic cells. The significance of our findings will have to await further detailed studies on the biologic functions and immunologic characterization of the p53 protein in human hematopoietic cells.

NOTE ADDED IN PROOF

In light of a recent publication by Wiman et al8 (which appeared after submission of this report) showing that the Namalwa cell line derived from a Burkitt lymphoma patient was strongly positive for PAb1801 and unreactive with PAb240, we obtained the Namalwa cell line and performed a series of immunoprecipitations under the conditions described in our report. We were interested in obtaining an hematopoietic cell line that was unreactive with PAb240 and possibly reactive with PAb1620. We also found the p53 from the Namalwa cell line to be strongly reactive with PAb1801, but in addition, we found strong reactivity with PAb1620. However, faint reactivity was observed with PAb240.

ACKNOWLEDGMENT

We thank Dr B. Robert Franza, Jr, for his advice and encouragement, and Su Demeritt for preparation of the manuscript.

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