Alterations in Tyrosine Phosphorylation during the Granulocytic Maturation of HL-60 Leukemia Cells

David A. Frank and Alan C. Sartorelli

ABSTRACT

Granulocytic maturation of HL-60 promyelocytic leukemia cells induced by dimethylsulfoxide has been shown to produce a decrease in cellular protein phosphotyrosine residues and increases in both tyrosine kinase and protein phosphotyrosine phosphatase activities (D. A. Frank and A. C. Sartorelli, Biochem. Biophys. Res. Commun., 140: 440-447, 1986). These changes have been shown to not be restricted to dimethylsulfoxide-induced differentiation, since similar changes occur in HL-60 cells initiated with retinoic acid and in HL-60 sublines resistant to dimethylsulfoxide-induced differentiation treated with the retinoid. These regulatory events are not directly coupled to growth arrest, which accompanies terminal maturation, since the anthracycline antibiotics aclacinomycin A and marnelomycin, which induce HL-60 differentiation, cause these changes in phosphotyrosine metabolism, while Adriamycin, at a level which produces an equivalent degree of growth inhibition but does not initiate the maturation of HL-60 cells, does not. Furthermore, an HL-60 subline deficient in hypoxanthine-guanine phosphoribosyltransferase, which differentiates in the presence of 6-thioguanine, produced a decrease in phosphotyrosine residues and increases in tyrosine kinase and phosphotyrosine phosphatase activities in response to the purine antimetabolite, while the parental HL-60 line, in which 6-thioguanine inhibits cellular proliferation but does not induce maturation, does not exhibit these changes. Finally, similar alterations in phosphotyrosine regulation were exhibited during anthracycline-induced differentiation of the murine myelomonocytic leukemia cell line WEHI-3B D+, supporting the concept that the phenomena measured represent a general response to inducers of the granulocytic differentiation of leukemia cells.

INTRODUCTION

The development of a malignant phenotype may be viewed as a defect in the normal process of differentiation in which the neoplastic cells exhibit a change in the tightly regulated homeostatic balance between proliferation and maturation that occurs in normal cells. The cellular mechanisms which control these events are at the center of an understanding of the genesis of malignancy and may be important in the development of novel approaches to combating cancer. Considerable work has been expended in an effort to understand the factors that regulate cellular proliferation, with significant findings being attained on the role of the expression of oncogenes (see, for example, Ref. 1). These genes have been demonstrated to be involved in the generation of a wide variety of neoplasms. Their protein products fall into several broad classes, one of which are the tyrosine kinases (2).

All of the protein kinases discovered prior to 1980 were known to phosphorylate proteins on serine and threonine residues. In 1980, Hunter and Sefton (3) demonstrated that the protein product of the src oncogene was a tyrosine kinase. In subsequent years, the products of a number of other oncogenes were found to possess such activity (1). Of perhaps even greater significance was the discovery that the receptors of a number of polypeptide growth factors contained a tyrosine kinase domain that was essential for receptor activity, and that fundamental growth processes in a number of systems were associated with an increase in intracellular tyrosine phosphorylation (4-8).

Since it appears that tyrosine phosphorylation is intimately associated with cellular proliferation, the mechanism(s) by which tyrosine phosphorylation is regulated during the maturation process assumes major significance. Terminal differentiation consists of both the acquisition of a mature functional phenotype and the cessation of proliferation. If the phosphorylation of tyrosine residues is important to cellular replication, then it follows that terminal differentiation, representing the programmed cessation of the proliferative state, would be associated with a decline in tyrosine phosphorylation. To explore this hypothesis, we have used the HL-60 promyelocytic leukemia cell line, which can be induced to differentiate along either the monocytic or granulocytic pathways by a number of agents (9-12).

In earlier preliminary experiments, we have shown that during both granulocytic and monocytic maturation, induced by DMSO1 and 12-O-tetradecanoylphorbol-13-acetate, respectively, a decrease in the percentage of cellular phosphoaminoacid residues represented by phosphotyrosine of approximately 10-fold occurred (13, 14). This phenomenon was accompanied by a 3-fold increase in tyrosine kinase activity during granulocytic maturation and a 2-fold elevation in this enzymic activity upon induction of monocytic differentiation. Protein phosphotyrosine phosphatase activity, the enzyme activity responsible for removing phosphate from phosphotyrosine residues, increased even more markedly in the differentiated state, being elevated 7-fold with granulocytic maturation and 11-fold with monocytic differentiation. Thus, the fall in total cellular phosphotyrosine residues with maturation induced by either DMSO or 12-O-tetradecanoylphorbol-13-acetate was associated with increased tyrosine kinase activity and an even greater elevation of protein phosphotyrosine phosphatase activity (14).

Since terminal differentiation is intimately associated with growth cessation, it was not possible to conclude from our earlier study with DMSO and 12-O-tetradecanoylphorbol-13-acetate that the observed changes in phosphotyrosine metabolism were due to the absence of proliferation or to the differentiation process per se. The current report, which uses a variety of inducers of granulocytic differentiation in different cell lines, was designed to address this issue.

MATERIALS AND METHODS

Materials. Radioactive isotopes were purchased from Amersham Corp., Arlington Heights, IL. Tissue culture media and serum were

1Received 6/16/87; revised 9/28/87; accepted 10/2/87.
2The abbreviations used are: DMSO, dimethylsulfoxide; RA, retinoic acid; GAT, polyglutamate:alanine:tyrosine, 6:3:1); NBT, nitroblue tetrazolium; FBS, fetal bovine serum; TCA, trichloroacetic acid; ADR, Adriamycin; ACM, aclacinomycin A; MCM, marcelloymycin; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; P-tyr, phosphotyrosine; NTE, 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, and 1 mM EDTA.

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obtained from Grand Island Biological Co., Grand Island, NY. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise noted.

Culture Conditions. HL-60 promyelocytic leukemia cells were a gift from Dr. R. C. Gallo of the National Cancer Institute. Cells were grown in RPMI Medium 1640 supplemented with 10% (v/v) heat-inactivated FBS (56°C for 30 min) at 37°C in a humidified 95% air/5% CO2 atmosphere. Cells were seeded at a level of 2 x 10^5 cells/ml before being passed by dilution into fresh medium to a concentration of 2 x 10^5 cells/ml. All studies used cells that were between passages 32 and 60.

DMSO-resistant sublines of HL-60 (HL-60/DMSO Y1 and HL-60/DMSO Y2) were derived by the repeated passage of cells in growth medium containing 1.2% (v/v) DMSO and were continuously maintained in this environment.

An HL-60 mutant subline lacking HGPRT, designated HL-60/HGPRT⁻, was developed by mutagenesis and selection (15) and passed in RPMI Medium 1640 containing 10% FBS.

A431 cells were obtained from the American Type Culture Collection (Rockville, MD). These cells were seeded at 0.5 to 1.0 x 10^6 cells/100 cm² and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS in a humidified 95% air/5% CO2 incubator at 37°C. Cells were seeded at a level of 1 x 10^5 cells/ml.

Induction of Differentiation. Granulocytic differentiation was induced in HL-60 cells by treatment with 1 mM RA, 50 nM aclacinomycin A, or 40 nM marcellomycin. The differentiation of HL-60/DMSO Y1 and Y2 cells was induced by exposure to 1 µM RA, and HL-60/HGPRT⁻ cells were initiated by exposure to 300 µM 6-thioguanine. Differentiation of WEHI-3B D⁺ cells was induced by treatment with either 50 nM aclacinomycin A, 20 nM Adriamycin, or 30 nM marcellomycin.

Granulocytic differentiation was measured by the ability of cells to reduce NBT (16).

Phosphoaminoacid Analysis. Protein and cellular phosphoaminoacids were quantitated by the method of Cooper et al. (17). Proteins and peptides labeled with [32P] in tyrosine kinase assays were precipitated in ice-cold 10% (w/v) TCA, resuspended in 6 N HCl, and hydrolyzed for 90 min at 110°C. HCl in these materials was removed by vacuum centrifugation, and the pellet was resuspended in 5 µl of thin-layer electrophoresis buffer (pyridine:glacial acetic acid:water, 10:10:1890, v/v/v, pH 3.5) containing 0.5 µg each of unlabeled phosphoserine, phosphothreonine, and P-tyr. Samples were applied to cellulose-coated (250-µm) glass plates and separated by electrophoresis using 800 V for 45 min at 4°C. The plates were dried, the standards visualized with ninhydrin, the spots removed by scraping, and radioactivity was determined by liquid scintillation spectrometry.

To assess whole cell phosphoaminoacid distribution, cells were labeled with [32P]orthophosphate (0.5-1.0 mCi) for 16 h in RPMI Medium 1640 lacking phosphate, but supplemented with 4% FBS, penicillin (50 U/ml), gentamicin (50 µg/ml), and glutamine (2 mM). Cells were collected by centrifugation, washed twice with phosphate-buffered saline, and lysed in lysis buffer (1% sodium dodecyl sulfate (w/v), 5 mM EDTA, 150 mM NaCl, 100 units/ml of kallikrein inhibitory protinin, 1 mM phenylmethylsulfonyl fluoride, 10 µM Tris-HCl, pH 7.4) with vigorous shaking for 20 min at 4°C. Unbroken cells and debris were removed by centrifugation (20,000 x g for 20 min at 2°C). Nucleic acids, phospholipids, and low-molecular-weight compounds were subsequently removed by phenol extraction and precipitation. The supernatant from the centrifugation was mixed with 0.4 ml of NTE and 0.4 ml of NTE-saturated phenol at room temperature. The solutions were mixed vigorously and centrifuged at 12,000 x g for 1 min. The phenol layer was extracted once again with 0.8 ml of NTE, then mixed with 13 ml of water and 2 ml of 100% (w/v) TCA, and allowed to stand at 2°C for 1 h. The precipitate was recovered by centrifugation (10,000 x g for 10 min at 2°C), extracted with 5 ml of chloroform:methanol, 2:1 (v/v), and centrifuged as before. The pellet was then hydrolyzed and subjected to electrophoresis as described above, except that electrophoresis at pH 3.5 was preceded by electrophoresis at pH 1.9 (formic acid:acetic acid:water, 50:156:1794, v/v) in a perpendicular direction. This additional step was needed to separate P-tyr from uridine monophosphate. Spots were then isolated and quantitated as previously described. Since this technique involves degradation of the phosphoaminoacids, it cannot give quantitative data on total levels, only relative amounts (17).

Tyrosine Kinase Assay. Tyrosine phosphorylation of artificial substrates was measured by a modification of the method of Braun et al. (18). Ten to 20 µg of cellular particulate protein (14) were added to tyrosine kinase buffer containing 20 mM N-2-hydroxyethylpiperaziner-N'-'-2-ethanesulfonic acid (pH 7.4), 12 mM MnCl₂, 10 µM ZnCl₂, and 0.5% (v/v) Nonidet P-40 with or without substrate (GAT, average M, 25,000, at a final concentration of 1 mg/ml). After 3 min at 22°C, the reaction was initiated by the addition of 25 µM [γ-32P]ATP (3 Ci/mm). The reaction was terminated by the addition of 7 µl of unlabeled ATP (10 mM). Fifty µl of the mixture were then applied to a 1 cm x 1 cm-square of Whatman 3MM filter paper, which was washed and counted as described by Corbin and Reimann (19), except that the TCA washes contained 10 mM sodium pyrophosphate. In each case, radioactivity was assessed by liquid scintillation spectrometry, and net phosphorylation represented the difference between tubes with and without substrate.

Protein Phosphotyrosine Phosphatase Assay. Protein phosphotyrosine phosphatase was measured using a modification of the method of Shrinfer and Brautigan (20). The substrate tyrosine-[32P]GAT, was prepared by incubating 20 to 30 µg of A431 cell particulate fraction protein with 1 to 2 mg of GAT, 100 µM [γ-32P]ATP, 10 mM epidermal growth factor (Collaborative, Research, Lexington, MA), and 25 µM Na₃VO₄, in a total volume of 1 ml of tyrosine kinase buffer. The reaction was allowed to proceed overnight at 22°C and was terminated by the addition of 111 µl of 100% (w/v) TCA. A precipitate was allowed to form at 4°C for 1 h and the pellet was collected by centrifugation at 12,000 x g for 5 min. The pellet was washed 3 times in 10% TCA at room temperature and was solubilized in 100 µl of 1 N NaOH. Two ml of protein phosphotyrosine phosphatase buffer (50 mM N-2-hydroxyethylpiperaziner-N'-'-2-ethanesulfonic acid, pH 7.0, and 25 mM 2-mercaptoethanol) were added and the solution was diaphragmatically stirred (M, 10,000 cutoff; Amicon Corp., Danvers, MA) at 5,000 x g for 30 min against the buffer until the [32P] in the filtrate was 1% of that of the reaction mixture (i.e., greater than 99% of the label was incorporated in GAT). One to 2 nmol of phosphate were incorporated per milligram of GAT (2 x 3 x 10⁻³ mol phosphate/mol tyrosine). The retained material was then collected and designated the protein phosphotyrosine phosphatase substrate.

Protein phosphotyrosine phosphate activity was measured by the release of [32P]orthophosphate from tyrosine-[32P]GAT in a 50-µl reaction mixture containing approximately 10,000 cpm of substrate and 10 to 20 µg of cellular particulate protein in protein phosphotyrosine phosphatase buffer containing 0.5 mg/ml of bovine serum albumin at 37°C. The reaction was initiated by the addition of the substrate and was allowed to proceed for one min, at which time the reaction was terminated by the addition of 50 µl of 20% (v/v) TCA. The reaction products were digested with 100 µg of proteinase K for 5 min. Fifty µl of the supernatant were mixed with 5 ml of Hydrofluor (National Diagnostics, Sommerville, NJ) and radioactivity therein was determined with a scintillation spectrometer. Specific activity was represented as the difference in the amount of radioactivity released in the presence and absence of cellular protein.

RESULTS

That the granulocytic differentiation of HL-60 leukemia cells induced by DMSO is accompanied by a decrease in phosphotyrosine residues and an increase in intracellular tyrosine kinase
and phosphotyrosine phosphatase activities (13, 14) is not specific for the polar-planar solvent, but is part of the programmed series of events associated with the maturation process, is supported by a number of experiments. This includes the finding that the treatment of HL-60 cells for 6 days with 1 μM RA, a potent inducer of granulocytic maturation (11), resulted in greater than 80% of the cells becoming functionally mature, as assessed by their ability to reduce NBT (Table 1). With the attainment of the mature phenotype, the proportion of phosphoamino acids represented by phosphotyrosine decreased from 1.5 to 0.3%. This event was accompanied by a 2.5-fold increase in tyrosine kinase activity and in a 5-fold increase in protein phosphotyrosine phosphatase activity (Table 1). The mature cells decreased in volume and protein content by approximately 45% compared to the untreated cells, and thus the changes are accordingly smaller on a per cell basis. When untreated cells were allowed to reach plateau density, they had a 35% decrease in cellular protein, yet tyrosine kinase and phosphotyrosine phosphatase activities were unchanged when normalized to protein concentration, excluding changes in cell size as a cause of these phenomena. Thus, RA-induced differentiation led to changes in phosphotyrosine regulation that were analogous to those produced by DMSO, implying that these changes are associated with the differentiation process.

DMSO is known to directly stimulate tyrosine kinase activity in at least one system (21). Although DMSO, in concentrations up to 20%, had no measurable effect on HL-60 leukemia cell tyrosine kinase activity (data not shown), it was important to provide further evidence that an action of the polar-planar solvent not associated with maturation per se was responsible for the observed changes in phosphotyrosine regulation. To accomplish this, two HL-60 sublines (HL-60/DMSO Y1 and HL-60/DMSO Y2) resistant to DMSO-induced differentiation were developed by selection in DMSO-containing medium by the procedure described in “Materials and Methods.” Both sublines exhibited a dependency on DMSO (1.2%) for growth, but were resistant to the differentiation-inducing properties of the polar-planar solvent (Fig. 1). However, upon the addition of 1 μM RA, these clones expressed a differentiated phenotype analogous to that of the parental line, indicating that they had not lost the ability to express the mature state. These sublines had a base-line phosphotyrosine content somewhat lower than that of parental cells (Table 2). Following RA-induced differentiation, these levels were lowered in a manner comparable to that occurring in mature parental HL-60 cells. The increase in tyrosine kinase and protein phosphotyrosine phosphatase activities that accompanied the differentiated phenotype in these cells was similar to that obtained in mature parental HL-60 cells (Fig. 2). These findings provide additional support to the concept that the changes in P-tyr content and tyrosine kinase and phosphotyrosine phosphatase activities that occurred as a consequence of DMSO-induced maturation are specific for the differentiation process and are probably not the result of an unrelated action by the polar-planar solvent.

A cardinal feature of terminal differentiation is the cessation of cellular proliferation. Thus, the changes observed in P-tyr regulation may be a reflection of inhibition of growth rather than of the differentiation process per se, although this possibility seemed unlikely, since cells which stopped proliferating by entrance into the plateau phase did not exhibit alterations in P-tyr regulation. Nonetheless, to address this question further the tumor-inhibitory anthracyclines were used. Two members of this antibiotic class, ACM and MCM, induce the granulocytic differentiation of HL-60 cells, whereas ADR produces only cytotoxicity (22). Concentrations of these three drugs were selected which caused almost equal inhibition of cellular replication; in agreement with previous results (22), under these conditions, only ACM and MCM induced the differentiation of the HL-60 leukemia (Fig. 3). In addition, ACM and MCM caused a reduction in P-tyr levels (Table 3), a 2-fold increase in tyrosine kinase activity, and about a 4-fold increase in protein phosphotyrosine phosphatase activity (Fig. 4). ADR-treated cells displayed no change in tyrosine kinase activity and only a small increase in protein phosphotyrosine phosphatase activity. Thus, although these anthracycline antibiotics inhibited cellular replication to a similar extent, only the two that induced differ-

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Table 1 Changes in tyrosine phosphorylation in HL-60 leukemia cells induced to differentiate by RA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NBT*</th>
<th>% of P-tyr residues</th>
<th>Tyrosine kinase activity (pmol/min/mg protein)</th>
<th>Phosphotyrosine phosphatase activity (P-released/min/mg protein; cpm × 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 ± 0</td>
<td>1.5 ± 0.2</td>
<td>38 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>RA</td>
<td>78 ± 4</td>
<td>0.3 ± 0.1</td>
<td>102 ± 5</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

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Table 2 Phosphoaminoacid distribution in HL-60/DMSO Y1 and Y2 leukemia cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>P-tyr</th>
<th>P-ser</th>
<th>P-thr</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60/DMSO Y1</td>
<td>−RA</td>
<td>1.0 ± 0.2</td>
<td>89.4 ± 1.1</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>0.1 ± 0.1</td>
<td>89.7 ± 1.0</td>
<td>10.2 ± 0.3</td>
</tr>
<tr>
<td>HL-60/DMSO Y2</td>
<td>−RA</td>
<td>1.1 ± 0.2</td>
<td>89.9 ± 1.0</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>0.1 ± 0.1</td>
<td>89.6 ± 0.9</td>
<td>10.3 ± 0.5</td>
</tr>
</tbody>
</table>

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Fig. 1. Differentiation of HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (Θ, O) and Y2 (Δ, Δ) were either untreated (Θ, Δ) or treated (Θ, Δ) with 1 μM RA, and the ability to reduce NBT was assessed at the indicated times. All cultures contained 1.2% (v/v) DMSO, which was necessary for propagation of the cells. Cells were seeded at 2 × 10^5 cells/ml; differentiating cells reached a plateau of 5 to 6 × 10^7 cells/ml, while cells which did not differentiate reached a density of 1 to 2 × 10^6 cells/ml. Cells were not refed during these experiments. Each point, mean ± SE of 3 separate experiments, each done in duplicate.

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Table 2 Phosphoaminoacid distribution in HL-60/DMSO Y1 and Y2 leukemia cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>P-tyr</th>
<th>P-ser</th>
<th>P-thr</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60/DMSO Y1</td>
<td>−RA</td>
<td>1.0 ± 0.2</td>
<td>89.4 ± 1.1</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>0.1 ± 0.1</td>
<td>89.7 ± 1.0</td>
<td>10.2 ± 0.3</td>
</tr>
<tr>
<td>HL-60/DMSO Y2</td>
<td>−RA</td>
<td>1.1 ± 0.2</td>
<td>89.9 ± 1.0</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>0.1 ± 0.1</td>
<td>89.6 ± 0.9</td>
<td>10.3 ± 0.5</td>
</tr>
</tbody>
</table>

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Fig. 2. DMSO-induced differentiation of mature HL-60 leukemia cells. Cells were seeded at 2.0 × 10^5 cells/ml and maintained in the presence of DMSO (1.2%) for 6 days. Each point represents the mean ± SE of 3 separate experiments.
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Fig. 2. Tyrosine kinase and phosphotyrosine phosphatase activities in HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (Δ, C) and Y2 (A, Δ) were either untreated (Δ, O) or treated (Δ, A) with 1 μM RA, and the tyrosine kinase (A) and phosphotyrosine phosphatase (B) activities were determined. All cultures contained 1.2% (v/v) DMSO, which was necessary for cell propagation. Each point, mean ± SE of three experiments, each done in duplicate.

Fig. 3. Growth and differentiation of HL-60 leukemia cells treated with anthracyclines. HL-60 cells were exposed to either 25 nM ADR (Δ), 40 nM MCM (O), 50 nM ACM (Δ), or untreated (Δ), and cell numbers (A) and the ability to reduce NBT (B) were determined. Viability, as assessed by trypan blue exclusion, was greater than 80% in drug-treated cells, and greater than 95% in the untreated cells. Each point, mean ± SE of three experiments, each done in duplicate.

Table 3 Phosphoaminoacid distribution in HL-60 leukemia cells treated with anthracyclines

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-tyr</td>
</tr>
<tr>
<td>None</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>ACM (50 nm)</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>MCM (40 nm)</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>ADR (25 nm)</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

Fig. 4. Tyrosine kinase and phosphotyrosine phosphatase activities in HL-60 leukemia cells treated with anthracyclines. HL-60 cells were treated with 25 nM ADR (Δ), 40 nM MCM (O), 50 nm ACM (Δ), or untreated (Δ), and tyrosine kinase (A) and phosphotyrosine phosphatase (B) activities were measured. Each point, mean ± SE of three experiments, each done in duplicate.

Fig. 5. Growth inhibition of ADR-treated cells was nearly complete, but no significant changes in the P-tyr indices occurred (data not shown).

The purine antimetabolite 6-thioguanine produces cytoxicity in HL-60 cells, whereas this agent causes differentiation in a mutant subline (HL-60/HGPRT⁰) of HL-60 lacking HGPRT activity (15). Parental cells exhibited only growth inhibition when treated with 1 μM 6-thioguanine. HL-60/HGPRT⁰ cells were inhibited to the same extent by the 6-thiopurine only at the much higher concentration of 300 μM, and at this level 65% of the HL-60/HGPRT⁰ cells were functionally mature after 7 days (Fig. 5). Thus, using these two cell lines and a single agent (6-thioguanine), the effects of differentiation and cytotoxicity on P-tyr regulation can be dissected. Consistent with previous results, only the differentiating HL-60/HGPRT⁰ cells showed a decrease in P-tyr levels (Table 4). The tyrosine kinase activity of the HGPRT⁰ line was about 30% greater than that of parental cells, and upon treatment with 6-thioguanine this activity increased 2.3-fold. No change in P-tyr metabolism was seen when the parental line was exposed to the purine antimetabolite (Fig. 6). Basal protein phosphotyrosine phosphatase activity was similar in the untreated and mutant lines; in an analogous manner, this activity increased about 4-fold in the mutant and remained unchanged in the parental line following treatment with 6-thioguanine (Fig. 6).

To ensure that the observed effects on P-tyr metabolism were not unique to the HL-60 leukemia, WEHI-3B D⁺ murine myelomonocytic leukemia cells were also used. This leukemic cell line, developed in a BALB/c mouse (23), can be induced to differentiate into mature granulocytes in vitro by the anthracyclines ACM and MCM and, in contrast to HL-60, ADR as well (24). WEHI-3B D⁺ cells grow with a doubling time of 9 h and treatment with the anthracyclines leads to a profound inhibition
of cellular replication (Fig. 7). Consistent with the relatively rapid rate of growth of these cells, the anthracycline antibiotics induced maximal differentiation in 3 days (compared to 6 days for HL-60) (Fig. 7); 55 to 67% of WEHI-3B D+ cells exposed to the anthracyclines were able to reduce NBT. Untreated WEHI-3B D+ cells contained 1.2% of their phosphoaminoacids as P-tyr residues; granulocytic differentiation caused a reduction in P-tyr to 0.1 to 0.2% (Table 5). Basal tyrosine kinase activity of these murine cells was about 25% of that of untreated HL-60 and this enzymic activity increased by 3- to 4-fold with cellular differentiation. Untreated WEHI-3B D+ cells possessed approximately 70% of the protein phosphotyrosine phosphatase activity of HL-60 cells; this enzymatic activity rose about 70% of the protein phosphotyrosine phosphatase activity of HL-60 and this enzymic activity increased by 3- to 4-fold with cell numbers (A) and the ability to reduce NBT (B) were assessed. Each point, mean ± SE of three experiments, each done in duplicate.

Table 4 Phosphoaminoacid distribution in HL-60 and HL-60/HGPRT- leukemia cells treated with 6-thioguanine

<table>
<thead>
<tr>
<th>Cell line (GMP)</th>
<th>Concentration of 6-thioguanine (µM)</th>
<th>% of distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-tyr</td>
</tr>
<tr>
<td>HL-60</td>
<td>0</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>HL-60</td>
<td>1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>HL-60/HGPRT-</td>
<td>0</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>HL-60/HGPRT-</td>
<td>300</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

DISCUSSION

Initial studies by our laboratory on the regulation of P-tyr metabolism in HL-60 leukemia cells undergoing differentiation induced by DMSO or 12-O-tetradecanoylphorbol-13-acetate indicated that cellular P-tyr residues decreased when cells acquired the differentiated phenotype, whereas tyrosine kinase and protein phosphotyrosine phosphatase activities both increased sharply (13, 14). Corroborating these findings in part are the reports of Glazer et al. (25) and Chapekar et al. (26) that induction of tyrosine kinase activity accompanied the differentiation of HL-60 cells initiated by the combination of α-interferon and tumor necrosis factor or the mixture of retinoic acid and the calcium ionophore A23187. The experiments described in this paper extend these findings to demonstrate that analogous changes in P-tyr regulation are produced by other inducers of granulocytic maturation. The results demonstrate that the observed effects are (a) associated with the differentiation process, (b) not directly coupled to the cessation of proliferation, and (c) not unique to HL-60 cellular differentiation.

Since the phenomenon of tyrosine phosphorylation appears to be linked to cellular proliferation, and since terminal differentiation involves the programmed shutdown of replication, the decrease in total P-tyr residues that occurs with maturation is not surprising. However, it is significant that only termination of replication through differentiation and not through the action of cytotoxic agents leads to decreases in protein P-tyr levels. This finding suggests that the observed changes in tyrosine phosphorylation represent programmed physiological events that occur in response to the maturation process rather than to a series of secondary responses to decreased replication.

The increase in both tyrosine kinase and phosphotyrosine phosphatase activities that occur in concert with the fall in protein P-tyr residues implies that the phosphotyrosine phosphatase activity more than compensates for the rise in tyrosine...
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occurs during the maturation process. A variety of potential substrates has been described that may be involved (27–32). Ultimately, an understanding of the role of tyrosine phosphorylation will depend upon the characterization of these substrates and the functional changes caused by their phosphorylation.

The concurrent increase in both tyrosine kinase and phosphotyrosine phosphatase activities would seem to be inefficient from the standpoint of cellular energy balance. It is conceivable that the elevated tyrosine kinase activity is required for initiation of the differentiated phenotype, as part of a signal transducing mechanism. It is unlikely that elevated levels of this enzyme are required for the maintenance of the mature state, since functionally mature granulocytes isolated from the peripheral blood of normal human donors possess relatively low levels of tyrosine kinase activity (14). Also possible is that the biochemical insult which results in the transformation of the HL-60 cell caused the development of a functional state with a relatively high ratio of tyrosine kinase activity to phosphotyrosine phosphatase activity, with physiological differentiation serving to correct this defect by creating a reversal of this ratio. HL-60 cells, by virtue of their molecular defect, may be unable to respond to physiological signals for differentiation with a decrease in tyrosine kinase activity but are able to compensate by a large increase in phosphotyrosine phosphatase activity.

The results are consistent with the concept that tyrosine phosphorylation is an important intracellular mediator of proliferation and differentiation. Furthermore, the findings imply that the development of effective inhibitors of tyrosine kinase or of agents which activate or increase the production of phosphotyrosine phosphatase activity may be useful for the treatment of the leukemias.

REFERENCES

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