Correlation between the Induction of Leukemic Cell Differentiation by Various Retinoids and Modulation of Protein Kinases

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ABSTRACT

During retinoic acid induced differentiation of the human promyelocytic leukemia cell line HL60 and the human myeloblast cell line RDFD along the myeloid pathway, there is marked modulation of both the cyclic adenosine 3':5'-monophosphate dependent protein kinase and the phospholipid-sensitive calcium dependent protein kinase. In order to further assess whether these kinases are intimately associated with the differentiation process, we have correlated the modulation of these enzymes and phosphorylations of their substrates with the extent of differentiation induced by various retinoid derivatives. We observed that there was a direct relationship between the degree of differentiation of the two leukemic cell lines and the elevation of the cyclic adenosine 3':5'-monophosphate dependent protein kinase and phospholipid sensitive calcium dependent protein kinase activities. In addition, the increased phosphorylation of the various substrates of these enzymes also correlates with the degree of differentiation. These observations support the hypothesis that modulation of these protein kinases and phosphorylation of their substrates are necessary steps in the differentiation process.

INTRODUCTION

Numerous myeloid leukemic cell lines have been established recently which can be induced to differentiate in vitro utilizing a variety of agents (1–6). The human promyelocytic cell line HL60 has been studied extensively, since these cells undergo both functional and morphological maturation upon differentiation and are bipotent in that they can differentiate along either the myeloid pathway or the monocyte-macrophage pathway depending on the inducer utilized (7, 8).

During the differentiation of the HL60 cells along the myeloid pathway, there is marked modulation of the cAMP-dPK3 and cAMP binding proteins (9, 10). Recently, the enzyme PL-Ca-PK has been found in leukemic cells, including HL60 cells (11). Studies with purified enzyme have shown that PL-Ca-PK has substrate specificity distinct from that seen with other protein kinases to date (12, 13). Stimulation of the PL-Ca-PK and the protamine kinase has been noted to occur early during the initial period of the differentiation process in HL60 utilizing various inducers (14, 15).

In order to further assess whether these kinases are intimately associated with the differentiation process, we have correlated the modulation of these enzymes and phosphorylations of their substrates with the extent of differentiation induced in HL60 cells and in the human myeloblast cell line RDFD by various retinoid derivatives.

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The abbreviations used are: PL-Ca-PK, phospholipid-sensitive Ca2+-dependent protein kinase; cAMP, cyclic adenosine 3':5'-monophosphate; cAMP-dPK, cyclic adenosine 3':5'-monophosphate-dependent protein kinase; NBT, nitroblue tetrazolium; MOPS, 3-(N-morpholino)propane sulfonic acid; DTT, dithiothreitol; RA, retinoic acid.

MATERIALS AND METHODS

Materials. RPMI 1640 and fetal bovine serum were obtained from Grand Island Biological Co. (Grand Island, NY), and RA, 12-O-tetradecanoylphorbol-13-acetate, histone $\beta_b$, protamine sulfate, and ATP were obtained from the Sigma Chemical Co. (St. Louis, MO). Histone HLY was obtained from Worthington Biochemicals (Freehold, N.J.). [γ-32P]ATP was obtained from New England Nuclear (Boston, MA). The retinoid derivatives depicted in Fig. 1, with the exception of all trans-retinoic acid, were kindly supplied by Hoffmann-La Roche. The OKM-1 monoclonal antibody was obtained from Ortho Pharmaceutical Corp. (Raritan, N.J). Fluorescein-labeled goat antitoxin antibody was obtained from Miles Pharmaceuticals (Springfield, VA). The HL60 human promyelocytic leukemia cell line was a gift of Dr. Robert Gallo (National Cancer Institute, Bethesda, MD). The RDFD human myeloblast leukemia cell line was a gift of Dr. Daniel Wright (Walter Reed Research Institute, Washington, DC).

Cell Culture. The HL60 and RDFD leukemia cell lines were Mycoplasma free and were maintained in RPMI 1640 media at 10% heat inactivated fetal bovine serum in a 5% CO2 humidified atmosphere at 37°C. In cellular differentiation experiments, RA or the various analogues of RA were added following a 48-h subculture; these inducers were dissolved in ethanol or dimethyl sulfoxide such that the final concentration of solvent (0.1%) did not affect the growth or differentiation of either myeloid cell line. The cells were exposed to the inducer for 96 h. The extent of myeloid differentiation was assessed by NBT reduction determined by the method of Neuberger et al. (16), and the appearance of the OKM-1 antigen was determined by the method of Muro et al. (17). Viability was determined by trypan blue exclusion and was always greater than 90%. Values for the dose effective in achieving a half maximal response in each of the assays were determined from dose-response plots, as shown in Fig. 2. All retinoids were assayed in at least 3 independent experiments at each concentration. Variations in the percentage of differentiated cells among the various experiments for each retinoid were less than 10%.

Subcellular Fractions. Cells were homogenized in a buffer consisting of 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.0, 120 mM sucrose, 5 mM MgSO4, 0.4 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml) and were homogenized in a glass Teflon homogenizer with greater than 90% of the cells disrupted. The homogenate was centrifuged at 3 x 106 g to give a cytosolic fraction. Protein concentrations were determined using the method of Bradford (18).

Enzyme Assays. The cAMP-dPK activity was assayed in a reaction mixture of 120 μl containing 40 mM MOPS, pH 7.5, 1 mM DTT, 50 μM [γ-32P]ATP (1 μCi), 7.5 mM MgSO4, 200 μg histone HLY, and 5–20 μg of cellular cytosolic protein in the presence and absence of 10 μM cAMP, while the protamine kinase assay consisted of 40 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 8, 1 mM DTT, 20 μM [γ-32P]ATP (1 μCi), 5 mM MgSO4 and protamine sulfate (5.3 μg/ml). The PL-Ca-PK activity was assayed in a 120-μl reaction mixture containing 40 mM MOPS, pH 7.5, 1 mM DTT, 20 μM [γ-32P]ATP (1 μCi), 4 mM MgSO4, 0.5 mM CaCl2, and 24 μg histone f1, and in the presence and absence of phosphatidylserine (25 μg/ml). All assays were run in duplicate, and from each tube duplicate 50-μl aliquots were quantitated. Under these conditions all assays were linear with time and enzyme concentration. Aliquots were placed on 2-cm squares of Whatman P81, placed into cold 1% sodium pyrophosphate, and washed three times in the same solution. All discs were then rinsed in 95% ethanol and 95% ethanol-chloroform (3:1, v/v). The paper discs were dried and counted by liquid scintillation. One enzyme unit is defined as 1 nmol [32P] transferred per min.

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LEUKEMIC CELL DIFFERENTIATION AND PROTEIN KINASES

RETINOID

All-trans Retinoic Acid
(Retinol)

CheMICAL STRUCTURE

Ro 10-1670, trimethylmethoxyphenol (TMMP) analog of retinoic acid

Ro 12-0955, chloroethylmethoxyphenol (CDMMP) analog of retinoic acid ethyl ester

Ro 13-6298, retinoid
N-4-hydroxyphenol retinamide

Ro 10-0733

Ro 11-9588

Fig. 1. Structures of retinoids.

In Vivo Phosphorylation. The phosphorylation of cytosolic proteins was determined in assays containing 50 mM MOPS, pH 7.5, 5 mM MgSO4, 1 mM DTT, 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 20 μM [γ-32P]ATP (5–10 μCi), and 100 μg protein (total volume, 100 μl). To assay for the ability of cAMP to modulate phosphorylation it is necessary to remove endogenous cAMP from the cytosolic fraction. This is accomplished by adding 1 mg of acid washed heat-activated charcoal per mg of cytosol fraction protein and centrifuging at 5000 × g to precipitate the charcoal. Assays were carried out with no addition or in the presence of 20 μM cAMP, 100 μM free Ca2+, phosphatidylserine (25 μg/ml), or the combination of 100 μM free Ca2+ and phosphatidylserine (25 μg/ml). Reactions were started by adding enzyme, and after 2 min at 30°C reactions were stopped by adding 100 μl of a buffer consisting of 2% sodium dodecyl sulfate, 100 mM Tris-HCl, pH 6.8, 20% glycerol, and 2% 2-mercaptoethanol. The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis upon 6–17% gradient gels using the system of Laemmli (19). Phosphorylated proteins were visualized by autoradiography using Kodak AR film.

RESULTS

The ability of the eight retinoids listed in Fig. 1 to induce differentiation of HL60 and RDFD cells over a wide concentration range was determined. The dose response curves are shown in Fig. 2. Values for the dose effective in achieving a half maximal response in inducing differentiation in the HL60 and RDFD cells are given in Table 1. All-trans-retinoic acid displays the greatest potency in HL60, as noted previously by Breitman (20) as well as in the RDFD cells. However, the dose required for half maximal stimulation was 10-fold greater than that noted by Breitman (20). In general, all retinoids which were active in inducing differentiation of HL60 were all also effective in inducing differentiation of RDFD and with similar potency, with the exception of N-4 hydroxyphenol retinamide, which had a lower 50% effective dose (dose effective in achieving 50% OKM-1 positive cells) in the RDFD cells and the RO10-1670 which had a lower 50% effective dose in the HL60 cells.

Marked stimulation of the PL-Ca-PK, cAMP-dPK, and protamine kinase occur early during HL60-induced differentiation. The three retinoid compounds, RA, RO-10-1670, and RO-12-0955, which produce maximal, moderate, and minimal differentiation of HL60 and RDFD cells, respectively, were selected, and their effects on the activities of the kinases were determined.

RA is the most potent inducer of HL60 and RDFD differentiation, as demonstrated by the percentage of cells acquiring NBT positivity and acquisition of the OKM-1 antigen; this inducer also has the most dramatic effect in stimulating PL-Ca-PK and protamine kinase activities in the two cell types (Table 2). 10-1670, a significantly less potent inducer of differentiation, as indicated by the decreased percentage of cells acquiring the makers of the mature phenotype, produces a

| Table 1 Activity of retinoid derivatives: differentiation of HL60 and RDFD leukemia cells |
|-----------------|-----------------|-----------------|
|                  | ED50* (m)       | ED50* (m)       |
| HL60             | RDFD            |
| All trans-retinoic acid | 9 × 10^-8     | 1 × 10^-7       |
| RO 10-1670       | 1 × 10^-6       | >1 × 10^-4      |
| RO-12-0955       | >1 × 10^-4      | >1 × 10^-4      |
| Retinol          | >1 × 10^-4      | >1 × 10^-4      |
| RO 13-6298       | 6 × 10^-7       | 7 × 10^-7       |
| N-4-hydroxyphenol retinamide | >1 × 10^-4 | 5 × 10^-7 |
| RO 10-0733       | >1 × 10^-4      | >1 × 10^-4      |
| RO 11-9588       | 7 × 10^-4       | 8 × 10^-4       |

* ED50, 50% effective dose (dose effective in achieving 50% OKM-1 positive cells). Structures of retinoids are shown in Fig. 1. Methods for determinations are described in "Materials and Methods."
LEUKEMIC CELL DIFFERENTIATION AND PROTEIN KINASES

Table 2 Effect of retinoids on phospholipid-sensitive Ca**-dependent protein kinase activity and cellular differentiation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Kinase activity (relative activity)*</th>
<th>Differentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PS histone</td>
<td>+PS Protamine OKM-1 NBT</td>
</tr>
<tr>
<td>HL60</td>
<td>None</td>
<td>1^b (97 ± 42^c)</td>
<td>1 (188 ± 26)</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td>1.13 ± 0.05</td>
<td>4.22 ± 1.14</td>
</tr>
<tr>
<td>10-1670</td>
<td></td>
<td>0.96 ± 0.08</td>
<td>2.39 ± 0.70</td>
</tr>
<tr>
<td>12-0955</td>
<td></td>
<td>0.98 ± 0.18</td>
<td>1.71 ± 0.42</td>
</tr>
<tr>
<td>RDFD</td>
<td>None</td>
<td>1 (70 ± 8)</td>
<td>1 (120 ± 22)</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td>1.19 ± 0.11</td>
<td>2.56 ± 0.17</td>
</tr>
<tr>
<td>10-1670</td>
<td></td>
<td>1.26 ± 0.13</td>
<td>1.73 ± 0.17</td>
</tr>
<tr>
<td>12-0955</td>
<td></td>
<td>1.21 ± 0.10</td>
<td>1.35 ± 0.06</td>
</tr>
</tbody>
</table>

* Specific activity (pmol of [32P] transferred/min/mg of protein).
* Mean of three determinations ± SEM.
* Numbers in parentheses, actual enzyme activities.

Table 3 Effect of retinoids on cyclic AMP dependent protein kinase activity

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Kinase activity (pmol of [32P] transferred/min/mg of protein)</th>
<th>Differentiation of NBT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−cAMP</td>
<td>+cAMP</td>
</tr>
<tr>
<td>HL60</td>
<td>None</td>
<td>90 ± 22^g</td>
<td>421 ± 59</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td>140 ± 63</td>
<td>685 ± 88</td>
</tr>
<tr>
<td>10-1670</td>
<td></td>
<td>156 ± 72</td>
<td>558 ± 57</td>
</tr>
<tr>
<td>12-0955</td>
<td></td>
<td>141 ± 47</td>
<td>459 ± 20.5</td>
</tr>
<tr>
<td>RDFD</td>
<td>None</td>
<td>75 ± 24</td>
<td>433 ± 53</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td>102 ± 36</td>
<td>673 ± 65</td>
</tr>
<tr>
<td>10-1670</td>
<td></td>
<td>103 ± 38</td>
<td>505 ± 42</td>
</tr>
<tr>
<td>12-0955</td>
<td></td>
<td>91 ± 26</td>
<td>491 ± 44</td>
</tr>
</tbody>
</table>

* Average ± SEM of three separate determinations.

In order to further document a correlation between the degree of differentiation and the extent of protein kinase elevation, HL60 cells were exposed to varying concentrations of RA (1 μM, 250 nM, 100 nM, and 50 nM), N-4-hydroxyphenyl retinamide (1 μM, 500 nM, 250 nM, and 100 nM), 13-6298 (1 μM, 500 nM, and 250 nM), and 12-0955 (1 μM) for 96 h. The extent of differentiation and the extent of protein kinase elevation, PL-Ca-PK or protamine kinase activities, were determined as described in "Materials and Methods." All retinoids are utilized at final concentrations of 1 μM.

In addition, the effects of these inducers on cAMP-dPK activity were also investigated. There was a correlation between the degree of differentiation of the HL60 and RDFD cells and the elevation of cAMP-dPK activity (Table 3). RA, the most potent inducer of differentiation, results in a 1.6-fold stimulation of cAMP-dPK activity (Fig. 3). There was an excellent correlation between the extent of differentiation and elevation of protein kinase activities, independent of the retinoid utilized. All of the correlation coefficients were highly significant (P < 0.01).

If elevation of these kinases activities is an essential component of the differentiation process, then increased phosphorylation of the various substrates of these enzymes should also be observed and should correlate with degree of differentiation. Modulation independent phosphorylations of proteins of molecular weight 19,000, 34,000, 54,000, and 65,000 are enhanced upon differentiation in both HL60 and RDFD cells. Cyclic AMP-dependent phosphorylated protein bands appear at M, 43,000, 50,000, 59,000, 120,000, 175,000, and 185,000 in both the HL60 and RDFD cells (Fig. 4). In addition, cAMP-dependent bands appear at M, 89,000 and M, 48,000 in HL60, while similar bands appear at M, 220,000 and M, 20,000 in RDFD. The intensities of all these bands are dependent on the inducer utilized and correlate directly with the degree of differentiation.

A similar situation is noted when one examines the phospholipid-dependent phosphorylations (Fig. 5). Phospholipid dependent phosphorylations occur at M, 41,000, 45,000, 49,000, and 52,000 in both HL60 and RDFD, and the intensities of these bands again correlate with the ability of the inducer to induce differentiation of the cells and stimulate PL-Ca-PK activity. The phosphorylation of M, 45,000, 49,000, and 52,000 are also rapidly induced in vivo (not shown).

DISCUSSION

The primary events in myeloid differentiation still remain unknown. The availability of numerous cell lines in which features of differentiation can be induced has provided an opportunity to further delineate and investigate the initial events in this process. Numerous cell lines have been studied, and although a single unifying mechanism underlying differentiation has not been established, various investigations have indicated that cyclic nucleotides and cyclic nucleotide-dependent phosphorylations may indeed be involved. We have utilized the variation in the degree of differentiation induced by different retinoids to provide further support that cAMP dependent and independent phosphorylations are intimately involved in the differentiation process.

Strickland et al. (21) have demonstrated that the addition of
compounds that elevate intracellular cAMP concentrations to retinoic acid treated teratocarcinoma cells results in acquisition of a new phenotype characterized by synthesis of plasminogen activator, laminin, and type IV collagen. Mouse epithelial cells in culture are also induced to differentiate by exogenous dibutyryl cyclic AMP and prostaglandin E\textsubscript{3}, as well as other compounds known to increase intracellular levels of cyclic AMP (22). The addition of dibutyryl cAMP to mouse neuroblastoma results in morphological maturation and acquisition of enzymatic and electrical activities associated with the mature state (23). Exposure of cultured human small cell lung carcinoma cells to dibutyryl cAMP also results in marked alteration in the proliferation rate and a marked increase in 3,4-dihydroxy-L-phenylalanine decarboxylase activity (24). Similar induction of morphological and biochemical maturation has been observed upon addition of cAMP derivatives to Chinese hamster ovary cells (25). Finally, several investigators have demonstrated that agents that are known to increase intracellular cAMP levels induce the differentiation of HL60 cells (20, 26, 27). In addition, these agents interact with retinoids in a synergistic fashion in the induction of HL60 differentiation (27, 28).

Numerous investigators have shown that modulation of cyclic AMP-dPK by means of agents that increase intracellular cyclic AMP levels is an integral part of the cyclic AMP responsive differentiation system. Mutants of S49 cells the growth of which is no longer regulated by cAMP have been found to lack detectable cAMP-dPK (29). Gottesman et al. (30) have isolated numerous mutants of Chinese hamster cells which were no longer responsive to growth inhibition induced by cAMP elevating agents. These mutants possessed protein kinases with increased $K_\text{m}$ for cAMP or kinases which were no longer stimulated by cAMP. Exposure of HL60 cells to myeloid pathway inducers, i.e., RA and dimethylformamide, results in the elevation of Type I cAMP-dPK with phosphorylation of specific substrates early during the differentiation pathway (9). Elias and Stewart (10) have demonstrated accumulation of the Type I isozyme in non-histone chromosomal proteins during HL60 differentiation.

Our current studies have supported the hypothesis that cAMP-dPK is intimately involved in the differentiation of leukemic cells. Utilizing various retinoid derivatives which induce different degrees of differentiation of HL60 and RDFD cells, as indicated by NBT reduction and acquisition of the OKM-1 antigen, we have shown that there is a direct correlation between the degree of induction of differentiation in the HL60 and RDFD cells, elevation of cAMP-dPK activity, and cAMP dependent phosphorylation of specific substrates.

In addition to cAMP-dPK, the PL-Ca-PK and protamine kinase also appear to play a role in the differentiation process, since marked stimulation of the activities of both enzymes and phosphorylation of their various substrates correlate with the degree of differentiation along the myeloid pathway achieved by the various retinoid inducers. Our observation that there is a marked stimulation of cytosolic PL-Ca-PK activity during retinoid induced differentiation of HL60 cells is in agreement with the findings of Kraft et al. (31), who also noted stimulation of PL-Ca-PK during retinoid induced differentiation of embryonal carcinoma cells. These results are in opposition to the observations of Taffet et al. (32), who noted that the addition of retinol to the RAW204 macrophage line resulted in inhibi-
tion of PL-Ca-PK activity. PL-Ca-PK activity and specific endogenous substrate proteins have been demonstrated in chronic myelocytic leukemia, acute myelocytic leukemia, and acute monocytic leukemia cells isolated from patients (11). 12-O-Tetradecanoylphorbol-13-acetate, which appears to specifically bind and activate PL-Ca-PK, induces HL60 to differentiate along the monocyte-macrophage pathway (7, 33, 34, 35). In this situation there is a rapid association of PL-Ca-PK with particulate fractions and a loss of cytosolic kinase activity. In addition, diacylglycerol, which markedly increase the apparent affinity of the PL-Ca-PK for calcium as well as for phospholipid and renders the enzyme fully active, also induces differentiation of HL60 along the monocyte-macrophage pathway (13, 36).

The exact role of these kinases in leukemic cell differentiation remains to be discerned. A specific delineation of their function awaits the isolation of their phosphorylated substrates and delineation of their role in the differentiation process. These studies are under way.

REFERENCES


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