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MODULATION OF C-MYC EXPRESSION IN THE HL-60 CELL LINE

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A decrease in the expression of the myc proto-oncogene of HL-60 cells has been reported as an accompaniment of myeloid differentiation induced by either dimethylsulfoxide or retinoic acid. We report herein that several inhibitors of poly(ADP-ribose)-polymerase induced myeloid differentiation in HL-60 cultures. Studies on the expression of the c-myc gene in total cell RNA populations indicate that expression of this gene is inversely correlated with the state of differentiation, either myeloid or monocytic, of the cultured cells independent of the inducer and the rate of cell proliferation.

The HL-60 cell line was established from the peripheral circulating cells of a person suffering from acute promyelocytic leukemia (1). In culture, it is a heterogeneous population of cells consisting of approximately 90 percent myeloblasts and promyelocytes and 10 percent differentiated myeloid or monocytic cells (2). This cell line is capable of undergoing morphological and functional differentiation in response to the addition of a number of chemicals to the culture medium, e.g., methotrexate, 5-azacytidine, hexamethylene bisacetamide, DMSO, and TPA (3,4,5).

Recent studies by Favera et al. (6) and Collins and Groudine (7) have demonstrated that the c-myc proto-oncogene, while retaining the normal DNA structure as demonstrated by DNA restriction mapping, is amplified in the HL-60 cell line. Myc hybridizing sequences in the poly A-containing RNA were also demonstrated (8,9). Myeloid differentiation induced by either DMSO or retinoic acid decreased the level of myc RNA below the detection

Abbreviations used: DMSO, dimethylsulfoxide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; (PADPR) - polymerase, poly(ADP-ribose)-polymerase; SDS , sodium dodecyl sulfate; NBT , nitroblue tetrazolium; EDTA , ethylenediaminetetraacetic acid.

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level of these studies (8,9). No data were presented in these studies concerning the capability of either inducers of monocytic differentiation or inhibitors of cell proliferation to affect myc RNA levels. HL-60 cultures were investigated to determine if other chemicals that reportedly modify cellular phenotypes induce HL-60 differentiation and to identify those conditions that are associated with altered c-myc expression.

Methods

Cell culture -- HL-60 cells were kindly supplied by Dr. R. K. Boutwell. Cells were kept in continuous culture in RPMI-1640 supplemented with 10 mM HEPES and 10% heat-inactivated fetal calf serum in a humidified 5% CO_2 atmosphere. The doubling time under these conditions was found to be 30 hours. For studies concerning differentiation, cultures were initiated at 2 x 10^5 cells/ml on day 0 with the chemical to be studied present in the culture medium. Cultures were harvested on day 5. Cytospin cell preparations were used for Wright's stain (10), NBT reduction (3), and a combined esterase stain (11). Cell number was determined with a hemocytometer. Differential counts were performed on a minimum of three hundred cells. Lysozyme was measured by the method of Kryostek and Sachs (12); chicken egg white lysozyme obtained from Sigma was used as the standard for these assays.

RNA isolation -- Total cell RNA was isolated by the method of Chirgwin et al. (13). Briefly, the cells were washed and pelleted; the pellet was homogenized with a Brinkman Polytron in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sodium N-laurylsarcosinate, and 0.1 M β-mercaptoethanol. This solution was layered over 1 ml of 5.7 M CsCl, 0.1 M EDTA (pH 7) and centrifuged at 32,000 RPM in an SW 60 Ti rotor for 25 hours. The RNA pellet was resuspended by heating to 65°C for 5 minutes in 1 mM EDTA (pH 7), 1% SDS. RNA concentrations were determined spectrophotometrically assuming 1 A_{260} = 32 μg RNA.

RNA dot blot and hybridization -- Serial dilutions of total cell RNA were spotted directly onto dried nitrocellulose that had been previously soaked in 20 x SSC (SSC = 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0) overnight (modified dot blot) (14). The filter was briefly rinsed in 20 x SSC and baked in a vacuum oven at 80°C for 2 1/2 hours. Prehybridization was performed for 12 hours at 42°C in a buffer composed of 10 x Denhardt's buffer (Denhardt's buffer is 0.02% of bovine serum albumin, ficoll, and polyvinylpyrollidone), 5 x SSPE (SSPE = 0.18 M NaCl, 10 mM NaP_i, 1 mM EDTA pH 7.4), 200 μg/ml salmon sperm DNA, 50% deionized formamide, and 0.1% SDS. Hybridization was carried out for 26 hours at 42°C in the same buffer with the exception that it contained 5 x Denhardt's buffer. The probe used was a PBR322-derived plasmid containing a 1.5 kilobasepair Pst 1 fragment consisting of the majority of the v-myc sequence (15) (obtained from Dr. H. Temin). This was nick translated with an Amersham Nick Translation Kit and 0.5 mCi α^{32}P-dCTP (New England Nuclear, 800 Ci/mmole) to an activity of 10^8 CPM/μg. The filters were washed twice in the following buffers containing 0.1% SDS: 2 x SSC at room temperature, 0.1 x SSC at room temperature, 1 x SSC at 45°C, and 0.1 x SSC at 45°C. The washing procedure took approximately 4 hours. Autoradiography was performed at -70°C with a Dupont Cronex Quanta II intensifying screen and KODAK X-OMAT AR film.

Results

We have assayed a number of chemicals for their ability to induce differentiation and inhibit cell proliferation in HL-60 cultures. Table I summarizes the results with the chemicals, DMSO, cycloleucine, sodium butyrate, dexamethasone, hydroxyurea, TPA, and mezerein, which have been previously reported to affect growth and differentiation of HL-60 cells. The results are in excellent agreement with the literature values (3,4,5). Dexamethasone and hydroxyurea, at the concentrations tested, neither induced morphological differentiation nor altered either the NBT reduction or combined esterase stains (referenced to control cultures).

Several chemicals that had not yet been tested in this system, including inhibitors of cellular phosphodiesterase (papaverine, and theophylline) and inhibitors of (PADPR)-polymerase, were assayed for their capability to induce differentiation and inhibit cell proliferation. Both of the (PADPR)-polymerase inhibitors tested, 3-aminobenzamide and theophylline, were capable of inducing morphological myeloid differentiation. This result was confirmed by both the NBT reduction stain (Table II) and the presence of naphthol AS-D chloroacetate esterase activity (data not shown). Addition of (PADPR)-polymerase inhibitors to the culture medium also resulted in the formation of a small percentage (less than 1 percent) of large atypical cells with multilobed nuclei that resembled megakaryocyte precursors. Because of the naphthol AS-D chloroacetate esterase activity of these cells, it was felt that they were of the myeloid lineage. Differentiation induced by the (PADPR)-polymerase inhibitors did not proceed to the morphologically most differentiated myeloid forms; the majority of the differentiated cells accumulated at the myelocyte stage. Both papaverine, a phosphodiesterase inhibitor, and 3-aminobenzoic acid, an analog of 3-aminobenzamide that does not inhibit (PADPR)-polymerase, inhibited cell proliferation but were unable to induce either morphological or functional differentiation. These results are summarized in Table II.

TABLE I

The effects of various chemicals on differentiation and cell proliferation in HL-60 cultures

Chemical (Concentration)	Number of cells (% of control culture)	Percent of Cells in Culture				% of cells NBT positive	Lysozyme Activity units/10^6 cells (% control)
		myeloblast/ promyelocyte	myelocyte	meta- myelocyte	band/ granulocyte		
Control		90	8	2	0	5	1.3 (---)
Dexamethasone (1 μM)	89	86	11	3	0	7	.9 (69)
Hydroxyurea (79 μM)	25	82	14	4	0	12	6.0 (462)
Cycloleucine (20 mM)	30	66	26	8	0	28	4.4 (338)
Sodium butyrate (0.6 mM)	49	40	47	11	2	50	3.3 (254)
DMSO (180 mM)	40	20	47	30	3	88	2.7 (208)
TPA (60 nM)	10	98% of cells are monocytes by morphology and esterase stain					3.3 (254)
Mezerein (60 nM)	17	98% of cells are monocytes by morphology and esterase stain					3.2 (246)

Cells were cultured and analyzed as described in Methods.

TABLE II

The effects of cAMP inducers and (PADPR)-polymerase inhibition on differentiation and cell proliferation in HL-60 cultures

Chemical (Concentration)	Number of cells (% of control culture)	Percent of Cells in Culture				% of cells NBT positive	Lysozyme Activity units/10^6 cells (% control)
		myeloblast/ promyelocyte	myelocyte	meta- myelocyte	band/ granulocyte		
3-Aminobenzoic acid (10 mM)	44	89	10	1	0	8	1.3 (100)
Papaverine (133 μM)	30	84	15	1	0	10	3.5 (269)
Theophylline (5 mM)	12	49	38	12	1	52	6.5 (500)
3-Aminobenzamide acid (10 mM)	20	42	45	10	3	50	4.1 (315)

Cells were cultured and analyzed as described in Methods.

Kryostek and Sachs (12) and Kasukabe et al. (16) have reported that cellular lysozyme activity was a measure of differentiation of specific mouse myeloid leukemia cell lines. We attempted to measure cellular lysozyme by the technique of Kryostek and Sachs (12) in order to develop an objective, quantitative assessment of differentiation in HL-60. Although chemicals that induced morphological differentiation increased cellular lysozyme, no correlation existed between the extent of differentiation and the level of lysozyme activity. A correlation did, however, exist between cellular lysozyme activity and the degree of inhibition of cell proliferation (see Tables I and II).

HL-60 cultures treated with the chemicals listed in Tables I and II were used as the source of RNA for dot blot analysis. Figure 1 demonstrates the results of a "dot blot". From the intensity of the spots on the autoradiogram, it is apparent that with this assay papaverine-,

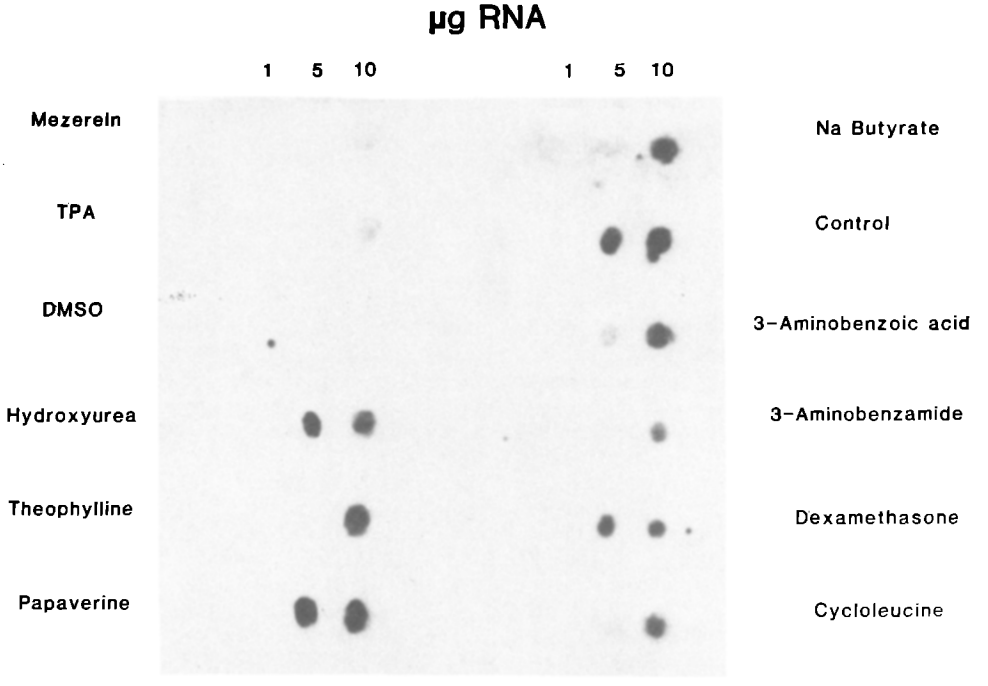

Figure 1. The relative expression of c-myc in HL-60 cultures. Cell culture, RNA preparation, blotting and hybridization were performed as described in Methods. All chemicals were used at concentrations indicated in Tables I and II.

hydroxyurea- and dexamethasone-treated and control cultures were expressing myc hybridizable sequences at the same level in the total cell RNA populations. Differentiation along either the myeloid path induced by either DMSO, cycloleucine, sodium butyrate, theophylline, or 3-aminobenzamide or the monocytic line induced by either TPA or mezerein resulted in a decrease in the level of myc RNA. Within the set of chemicals that induced myeloid differentiation, the relative potency of the inducing agent (i.e., the ability of the chemical to reduce the proportion of the cell population composed of either myeloblasts or promyelocytes) correlated with the percentage reduction of myc RNA. Sodium butyrate, cycloleucine, theophylline and 3-aminobenzamide reduced myc RNA to approximately half that in the control culture. TPA and mezerein reduced myc RNA to a barely detectable level, and cells treated with DMSO had no detectable myc RNA. It should be emphasized that neither papaverine nor hydroxyurea, both potent inhibitors of cell proliferation, had any detectable effect on the level of myc RNA. 3-Aminobenzoic acid appears to be an exception; this chemical did not induce differentiation by any of the criteria measured, yet it did reduce the level of myc RNA from that in the control cultures.

Discussion

Inhibitors of (PADPR)-polymerase have previously been shown to be capable of modulating gene expression in primary hepatocyte cultures (17) and to induce differentiation in Friend erythroleukemia cells (18). We have demonstrated that the (PADPR)-polymerase inhibitors, 3-aminobenzamide and theophylline, induced myeloid differentation in HL-60. This finding is consistent with the hypothesis of Kanai et al. (19) concerning a possible mode of action of DMSO in HL-60. Following the addition of DMSO to HL-60 cultures, PADPR-polymerase activity decreases prior to any indication of morphological myeloid differentiation (19). It is hypothesized that at least some of the actions of DMSO and the (PADPR)-polymerase inhibitors, in

the above systems, are a result of a decrease in (PADPR)-polymerase activity and subsequent alterations of chromatin structure and activity (19).

In contrast to the (PADPR)-polymerase inhibitors, the phosphodiesterase inhibitor papaverine, which induces differentiation in a murine neuroblastoma line (20) but differs from theophylline in that it does not inhibit (PADPR)-polymerase, has no capability, at the concentrations tested, to induce differentiation of HL-60. Papaverine was an effective inhibitor of HL-60 proliferation. These results appear to contradict those reported by Chaplinski and Niedel (21), who report that cAMP is an inducer of myeloid differentiation in HL-60. However, theophylline and dibutryl-cAMP, the reported inducers of cellular cAMP and HL-60 differentiation have alternative modes of action ((PADPR)-polymerase inhibitor and butyrate-like effects, respectively) which may be the means by which these chemicals induce differentiation in HL-60. This hypothesis is supported by the lack of morphological differentiation following the addition of other inducers of cellular cAMP reported herein and by Chaplinski and Niedel (21).

Westin et al. (8,9) have reported a decrease in the level of myc RNA in the poly-A containing RNA of HL-60 cells treated with either DMSO or retinoic acid. We studied a wider range of chemicals with varied modes of action and effects on HL-60. Inducers of both myeloid and monocytic differentiation were found to decrease the level of myc RNA in total cell RNA. The cytostatic agents, hydroxyurea and papaverine, which block progression through the cell cycle at different points have no detectable effect on this parameter. The expression of c-myc in HL-60 thus appears to be an intrinsic property of the undifferentiated HL-60 cell unrelated to its active proliferation. In this respect, the myc proto-oncogene in HL-60 resembles the proto-oncogenes already studied in the developing mouse embryo, where the active expression of specific proto-oncogenes has been shown to correlate with distinct developmental stages of the liver (22).

In accordance with this conclusion, myc RNA has been demonstrated in normal human myeloblasts but to be absent from mature circulating granulocytes (9).

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