Early Decline in c-myb Oncogene Expression in the Differentiation of Human Myeloblastic Leukemia (ML-1) Cells Induced with 12-O-Tetradecanoylphorbol-13-acetate

Ruth W. Craig and Alexander Bloch

ABSTRACT

The relationship of oncogene expression to proliferation and differentiation has been examined in a line of human myeloblastic leukemia (ML-1) cells. Proliferating leukemic cells were found to express a 4.3-kilobase cellular homologue (c-myb) of the transforming sequence of avian myeloblastosis virus. A rapid decline in the expression of this transcript was seen in cells induced to differentiate with 12-O-tetradecanoylphorbol-13-acetate. The level of c-myb RNA was decreased by >50% as early as 3 hr after 12-O-tetradecanoylphorbol-13-acetate exposure, and at 8 to 72 hr the reduction was ≥4-fold. Subsequent to the decrease in oncogene expression at 3 hr, DNA synthesis began to decline; by 24 hr, cell proliferation had ceased. At this time, monocyte- and macrophage-like cells were beginning to emerge. These findings demonstrate that c-myb is expressed during ML-1 cell proliferation and declines prior to the loss of DNA synthesis that accompanies the differentiation process.

INTRODUCTION

Oncogenic retroviruses have been demonstrated to cause cell transformation in vitro and tumor formation in vivo through the expression of specific sequences known as oncogenes (3). Cellular homologues (c-onc) of these viral genes may participate in maintaining the proliferative cycle in normal tissues, as well as in cancers of nonviral origin. If this is the case, decreased expression of specific c-onc genes might be expected to translate into decreased cell proliferation.

We have examined oncogene expression in relation to cell proliferation and differentiation, using a line of human myeloblastic leukemia (ML-1) cells. These cells differentiate to nondividing monocyte- and macrophage-like cells upon treatment with TPA and other agents, such as antineoplastic drugs and conditioned medium factors (20-22). The expression of c-myb, the cellular homologue of the transforming sequence (v-myb) of avian myeloblastosis virus, was assayed during the growth and TPA-induced differentiation of this cell line. The c-myb gene was selected for study because avian myeloblastosis virus produces myeloblastic leukemia in chickens (13), and the ML-1 cells are myeloblastic in nature (20). Our findings demonstrate that decreased c-myb expression is an early event in the loss of proliferation that accompanies TPA-induced ML-1 cell differentiation.

MATERIALS AND METHODS

Materials. The ML-1 cell line was generously provided by Dr. J. Minowada, of the Hines Veteran’s Administration Medical Center, Hines, IL. Recombinant plasmids containing the v-myb (10) and v-myc (27) sequences were kindly supplied by Dr. J. M. Bishop of the University of California at San Francisco. RPMI 1640 medium and FBS were purchased from Grand Island Biological Co., Grand Island, NY. TPA was obtained from Chemicals for Cancer Research, Inc., St. Louis, MO, and stored as described previously (20). [α-32P]TTP (800 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL. [methyl-3H]Thymidine (30 to 38 Ci/mmol) and [5-3H]Juridine (14 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Chemical and Radiosotope Division, Irvine, CA. DNase I and DNA polymerase I were supplied by Worthington Biochemical Corp., Freehold, NJ, and Boehringer Mannheim, Inc., West Germany, respectively. Oligodeoxynucleotidylic acid-cellobiose was obtained from Collaborative Research, Lexington, MA, and nitrocellulose membrane filters were from Schleicher and Schuell, Keene, NH.

Cell Culture. ML-1 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 7.5% heat-inactivated FBS, as described in a prior report (20). For isolation of RNA and other assays, cells were harvested from logographically growing cultures, and incula of 3 x 10⁶ viable cells/ml were incubated at 37°C for 1 to 72 hr in the presence or absence of 5 x 10⁻⁹ M TPA, using the same medium containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cell growth and viability were assayed by hemocytometer using trypan blue dye exclusion.

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Any adherent cells (generally ≤30% in TPA-treated cultures) were harvested using the method of Schon-Hegrad and Holt (19). A modification of this method, in which washed cells were agitated at 37°C in 0.02% EDTA, was used to reduce cell adhesion for cell count determinations.

To assess the stability and/or reversibility of the TPA effect, cells were centrifuged from the drug-containing medium and washed with drug-free medium. The cell pellet was incubated at 37°C for 30 min in two 25-ml aliquots of drug-free medium and then was resuspended in fresh medium.

Assay of DNA and RNA Synthesis. DNA and RNA synthesis were evaluated by measuring the incorporation of radiolabeled thymidine or uridine into acid-insoluble material using established methods (26). After 3 hr exposure to the labeled precursors (0.032 µCi/ml), cells were washed with phosphate-buffered 0.9% NaCl solution and extracted with ice-cold 5% trichloroacetic acid. Acid-precipitable material from 10⁶ viable cell equivalents was collected on glass microfiber (GF/C) discs, which were successively rinsed with 5% trichloroacetic acid, absolute ethanol, and acetone. After drying, radioactivity was quantitated by liquid scintillation counting. This procedure was found to remove ≥88% of the unincorporated precursors, and quenching by the disc was found to be minimal (about 10%). Precursor incorporation in untreated ML-1 cells was linear with time and cell concentration.

Assessment of Morphological Differentiation. Morphological differentiation was monitored by examining fixed slide preparations, which

Received August 15, 1983; accepted October 25, 1983.
were stained as described previously (20). Differentiation was scored using established criteria (11), macrophage-like cells manifesting a re- niform nucleus and decreased nucleocyttoplasmic ratio (20), monocyte-like cells exhibiting a more centrally located maturing nucleus and less cytoplasmic enlargement than did macrophage-like cells, and intermediate-stage (promonocyte-like) cells typically displaying an immature nu- cleus in the presence of moderate cytoplasmic enlargement.

**Assay of Oncogene Expression.** Labeling of isolated (23) plasmid DNA was accomplished by standard nick translation procedures (16), using \([\alpha-^{32}P]TTP\) (0.1 Ci/0.1 ml reaction mixture). The labeled DNA was separated by column chromatography with SP-Sephadex using an elu- tion buffer of 0.01 m sodium acetate (pH 5.0) containing 0.3 m NaCl. The PVM2 recombinant probe containing the v-myb sequence (10) was used either before or after isolation of a v-myb-containing fragment obtained by restriction enzyme cleavage of the intact plasmid with Kpnl.

RNA was precipitated with ethanol and quantitated spectrophotometri- cally. Gel electrophoresis was carried out in 1.5% agarose containing 10 m m sodium phosphate (pH 6.5) and 2.2 m formaldehyde. Samples were electrophoresed for 450 V-hr using 10 m sodium phosphate (pH 6.5) as the running buffer. Transcript sizes were estimated by comparison with rRNA markers, which were detected by ethidium bromide staining.

RNA blotting analysis was accomplished using modifications of estab- lished methods (25). RNA samples, denatured by incubation at 50°C for 15 min in 12 m sodium phosphate buffer (pH 7.4) containing 60% deionized formamide and 1.8 m formaldehyde, were either dot-blotted onto nitrocellulose filters presoaked in 1.5 m NaCl-0.15 m trisodium citrate or subjected to electrophoresis and transferred to nitrocellulose filters.

Prehybridization of the filters was carried out at 62–65°C for approxi- mately 3 hr using a buffer consisting of 0.75 m NaCl-0.075 m trisodium citrate, 0.1% SDS, 0.1% sodium pyrophosphate, denatured salmon sperm DNA (150 /¿g/ml), and 10x Denhardt’s solution (0.2% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll). The filters were annealed with radiolabeled DNA probes under the same conditions, except that a concentration of 1x Denhardt’s solution was used in the hybridization buffer. After hybridization, the filters were rinsed at 62–65°C at least 6 times with 2x Benton-Davis solution (50 m m Tris-HCl, pH 7.9, containing 1 m NaCl, 0.1% SDS, 0.1% sodium pyrophosphate, and 1 x Denhardt’s solution); then washed three times for 30 min in 0.3 m NaCl-0.03 m trisodium citrate containing 0.1% SDS and 0.1% sodium pyrophos- phate. The filters were exposed to XAR-5 film at -70°C for up to 2 weeks using Optex High Plus intensifying screens. Relative changes in onco- gene expression were quantitated by densitometric scanning of the autoradiographs.

**RESULTS**

A dot-blot hybridization analysis of the relative levels of c-myb RNA in control and TPA-treated ML-1 cells is shown in Fig. 1a. In untreated ML-1 cells, RNA sequences homologous to the v-myb probe were readily detectable. The expression of these sequences did not change substantially from 8 to 72 hr of incubation, the variation between independent RNA preparations being <23%. In contrast, sequences homologous to the v-myb probe were appreciably decreased in cells treated with TPA, this reduction being observed in cells exposed for 8, 15, or 72 hr. Decreased levels of c-myb sequences were seen in samples of both total and poly(A)-selected RNA. Some variation in the extent of hybridization was noted in different RNA preparations from TPA-treated cells. An analysis of the size of the c-myb transcript using gel blot hybridization is shown in Fig. 1b. In untreated ML-1 cells, a RNA species of approximately 4.3 kilobases was detected in total and poly(A)-enriched (Fig. 1c) RNA preparations, in agreement with reports of a transcript of similar size (4 to 4.5 kilobases) in other hematopoietic tissues (5, 29). A decrease in the level of this transcript was seen in cells exposed to TPA, validating the dot- blot hybridization analysis. As determined by quantitative densi- tometry, a small decrease (approximately 16%) was noted as early as 1 hr. A >50% reduction was consistently observed at 3 hr, with a 4- to 5-fold decrease being seen at 8 to 72 hr.

The reduction in c-myb expression in TPA-treated cells did not appear to result from nonspecific inhibition of RNA transcription. As measured by [3H]uridine incorporation, RNA synthesis was not decreased at 3 hr (when a >50% decrease in c-myb expression was evident) and declined by only about 6% at time points up to 11 hr. As measured by the orcinol reaction (28), no change in cellular RNA content was noted at the 24-hr time point, a 1.7- fold increase being observed in TPA-treated samples at 72 hr.

The expression of another cellular oncogene, c-myc, was not substantially affected by TPA treatment. Using the intact plasmid as a probe, this oncogene was only very faintly detectable; with the Psfl v-myb-containing fragment as a probe, expression was detectable only after prolonged exposure of the autoradiographs. Under these conditions, c-myc expression was decreased by only about 15.5% in TPA-treated cells at time points up to 15 hr. In addition, several of the in vitro RNA translation products from TPA-treated samples were found to be increased above control levels.

The changes in c-myb expression and ML-1 cell proliferation that occurred during TPA treatment are shown in Chart 1. Decreased oncogene expression was seen before a significant decline in cell proliferation was noted. Within 3 hr after exposure of the leukemic cells to TPA, the level of c-myb RNA had decreased by more than 50% (Chart 1c), although at this time incorporation of [3H]thymidine into acid-insoluble material had not declined appreciably (Chart 1b). By 8 hr, c-myb expression in TPA-treated cells had reached a nadir (75% reduction), and incorporation of [3H]thymidine had begun to decline. By 24 hr, incorporation of [3H]thymidine had decreased to nearly unde- tectable levels, and cell proliferation had ceased (Chart 1a). As determined by flow cytometry (8, 24), entry of cells into S phase had also come to a halt, with only about 3.1% of cells remaining in S phase at 24 hr, as compared to 42.9% in controls.

The time course of accrual of differentiation-associated char-acteristics in TPA-treated cells is shown in Chart 1. At 10 hr, TPA caused only a minor increase in ML-1 cell differentiation. At 24 hr, about 27% of the treated cell population had entered the differentiation sequence, with 10% of the cells exhibiting mono- cyte-like or macrophage-like characteristics (as compared to

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3 R. W. Craig, O. S. Frankfurt, H. Sakagami, and A. Bloch. Macromolecule and cell cycle effects of different classes of agents inducing the maturation of human myeloblastic leukemia (ML-1) cells, manuscript in preparation.

4 R. W. Craig and A. Bloch, unpublished data.

TPA-treated cultures (Chart 2); at 72 hr nearly 80% of the population displayed maturing phenotype. A comparison of these results with the data in Chart 1 indicates that the decline in c-myb expression largely precedes the acquisition of differentiated phenotype.

**DISCUSSION**

Since sequences homologous to v-myb are highly conserved in the genome of vertebrates (2), this gene is thought to code for a vital cellular function. This study examined the temporal relationship between changes in c-myb expression, DNA synthesis, and differentiation in ML-1 cells. A considerable decline in c-myb expression was observed at 3 hr, followed by a decrease in DNA synthesis between 3 and 24 hr. An increase in morphological differentiation became manifest at about 24 to 48 hr. This sequence of events demonstrates that the c-myb gene is expressed during ML-1 leukemic myeloblast proliferation and that its decline precedes the loss of proliferation that accompanies differentiation.

Gonda et al. (5) and Chen (4) have postulated that c-myb expression may be involved in cell proliferation in immature hematopoietic tissues, since the expression of this gene is restricted to such tissue types in chickens. The demonstrated association between decreased c-myb expression and cessation of myeloblastic ML-1 cell proliferation tends to support this hypothesis. A correlation between oncogene expression and proliferation is further suggested by reports demonstrating elevated c-myb expression in some neoplastic cells (15, 17).

The expression of another cellular oncogene (c-ras) has been studied (6) in regenerating rat liver, where an increase in c-ras expression occurs at the time of activation of DNA synthesis associated with regenerative growth. Conversely, the decrease in c-myb expression in ML-1 cells occurs at the time of inactivation of DNA synthesis, during the onset of differentiation. Thus, these reports link oncogene expression to cell growth processes.

An association between oncogene expression and differentiation has been provided by several investigators. Westin et al. (29, 30) have observed decreases in the expression of c-myb and c-myc in human promyeloctytic leukemia (HL-60) cells induced to differentiate with dimethyl sulfoxide or retinoic acid. Muller et al. (14) have demonstrated that fluctuations in the expression of c-abl and c-fos occur during mouse prenatal and/or early postnatal development. A correlation between v-onc gene expression and differentiation has been observed in avian erythroblastosis virus-transformed cells (7), where continuous differentiation...
expression of a viral gene is "necessary to maintain the undifferen-
tiated state," inhibition of the viral product allowing cells to
undergo partial differentiation. A similar relationship may exist in
ML-1 cells, where the c-myb gene is expressed during myelo-
blast proliferation, this expression declining prior to the loss of
proliferation that accompanies the differentiation process.

The facts that the c-myb transcript is quite labile and that its
decline is followed by decreased ML-1 cell entry into S phase
represent a parallel to the lability of the mediators of prolifera-
tion proposed to control transit from G, to S phase (18). This coinci-
dence may indicate the existence of a functional relationship
between the endogenous mediators of cell proliferation and the
product of an oncogenic sequence.

ACKNOWLEDGMENTS

We thank Dr. J. M. Bishop for providing the recombinant plasmids, Dr. F. G.
Berger for many helpful discussions, Dr. W. A. Held for in vitro translation, and Dr.
O. Frankfurter for flow cytometric determinations. We are grateful to R. J. Maua and
R. A. Hromchak for their excellent technical assistance.

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Fig. 1. Expression of c-myb in control and in TPA-treated ML-1 cells. RNA was isolated from ML-1 cells cultured in the presence or absence of $5 \times 10^{-10}$ M TPA for 1 to 72 hr and either (a) dot-blotted onto nitrocellulose filters or (b and c) transferred to filters after agarose gel electrophoresis. Filters were hybridized with the labeled intact probe containing the v-myb sequence (a and c) or a KpnI myo-containing fragment of the plasmid (b). a, dot-blot hybridization of 20 μg total RNA or 5 μg poly(A)-selected RNA. The 15-hr control sample did not differ substantially from the other controls; b, gel blot hybridization of 20 μg total RNA; c, gel blot hybridization of 5 μg poly(A)-selected RNA. Pairs of samples (control and treated) from different time points were isolated from separate cell inocula. C, control; T, TPA treated.
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\textit{Cancer Res} 1984;44:442-446.

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