Although leukemic cells from patients with acute myeloid leukemia display a seemingly unlimited proliferative capacity in vivo, their life span in liquid suspension culture is usually limited to a few cell divisions. One possible explanation for such limited in vitro proliferation is that certain growth factors sustaining leukemic cell division in the patient are absent from the in vitro culture environment. About 10 years ago, investigators in Dr R. C. Gallo's laboratory at the National Institutes of Health (NIH), in an attempt to identify such leukemia-specific growth factors, cultured fresh human leukemic cells with conditioned media derived from cultures of both human embryonic cells and mitogen-stimulated lymphocytes. Although the postulated leukemia-specific growth factor was never reproducibly identified, this experimental approach proved fruitful in a number of unexpected ways. The T cell mitogen interleukin 2 (TCGF) was first identified, and a factor-independent immortal cell line (HL-60) with distinct myeloid characteristics was developed. The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, can be induced to differentiate in vitro to a number of different cell types, and studies with this leukemic cell line have proved invaluable in a variety of different areas. These include: (a) providing insight into the control mechanisms of normal granulocyte/macrophage differentiation; (b) suggesting new therapeutic approaches for patients with leukemia; (c) providing a ready source of cDNA for cloning of important granulocyte and monocyte enzymes and monokines; (d) providing insight into possible mechanisms of rapid regulation of gene transcription; and (e) serving as an invaluable model for studying specific cellular oncogene expression in relationship to particular hematopoietic differentiation lineages. This review attempts to summarize the important characteristics of this unique cell line, with particular emphasis on HL-60 proliferation, differentiation, and cellular oncogene expression.

GROWTH AND PROLIFERATION

HL-60 promyelocytic leukemia cells continuously proliferate in suspension culture with a doubling time of ~36 to 48 hours. This characteristic alone makes HL-60 unusual among human myeloid leukemias. Most fresh myeloid leukemia cells when cultured in liquid suspension undergo a limited number of cell divisions prior to growth arrest and cell death, and relatively few human leukemic cell lines displaying distinct myeloid or myelomonocytic characteristics have been established. What are the characteristics of HL-60 cells that make them adapted to such continuous growth in liquid suspension? HL-60 cell surface expression of transferrin and insulin receptors appears critical to their proliferative capacity. HL-60 cells can proliferate in serum-free nutrient media (e.g., RPMI 1640) provided that it is supplemented with transferrin and insulin. The requirement for insulin and transferrin is absolute, as HL-60 proliferation immediately ceases if either of these compounds is removed from the serum-free culture media. Transferin requirement by HL-60 as well as by other cultured cells indicates that an active iron transport system is necessary for cell proliferation. HL-60 cells express the transferrin receptor on the cell surface, and this receptor expression markedly decreases as the cells are induced to differentiate terminally. Moreover, monoclonal antibodies to the transferrin receptor inhibit HL-60 cell proliferation. Similarly, insulin receptors are also displayed by HL-60 cells, and insulin receptor expression decreases with granulocytic differentiation of HL-60. The presence of the insulin and transferrin receptors on HL-60 cells cannot, however, in itself explain the unusual in vitro proliferative capacity of this cell line since a number of fresh leukemia cells also display these

From the Molecular Medicine Program, Fred Hutchinson Cancer Research Center, and the Department of Medicine, University of Washington Hospital, Seattle.

Submitted March 2, 1987; accepted June 19, 1987.

S.J.C. is a Scholar of the Leukemia Society of America.

Address reprint requests to Steven J. Collins, MD, Fred Hutchinson Cancer Research Center, AB-133, 1124 Columbia St, Seattle, WA 98104.

© 1987 by Grune & Stratton, Inc.
0006-4971/7005-0001$3.00/0
receptors but nevertheless are unable to grow continuously in culture.

The in vitro proliferation of normal myeloid progenitor cells is stimulated by the colony-stimulating factor (CSF) family of compounds. Certain CSFs also stimulate the proliferation of HL-60 cells.11-13 HL-60 cells have been postulated to produce certain CSF-like compounds that act as autostimulators of HL-60 growth, and several compounds exhibiting such "autocrine" activity have been partially purified from HL-60-conditioned media. Brennan et al described a 13,000 mol wt compound produced by HL-60 cells which stimulated HL-60 cell growth but which exhibited no CSF activity on normal mouse bone marrow cells.14 Perkins et al described a 25,000 mol wt HL-60 product with similar HL-60 autostimulatory activity. In addition, this compound exhibits CSF activity on normal bone marrow and shares antigenic determinants with certain CSFs.15 These HL-60 autostimulatory products have not been molecularly cloned, and it is presently unclear what their exact relationship is to each other or to the normal CSF molecules. One of the chromosome 5 GM-CSF alleles appears to be partially deleted in HL-60 cells,16 but there is no evidence that this gene is expressed in HL-60 cells, and the effect, if any, of this genomic rearrangement on the proliferation of HL-60 is presently unknown.

HL-60 cells also exhibit genetic abnormalities in specific cellular oncogenes (see below), and these abnormalities most likely also play a role in the unusual ability of these myeloid leukemic cells to proliferate continuously in vitro.

DIFFERENTIATION

The characteristic of HL-60 cells that has attracted the most research interest is their capacity to differentiate in vitro to a variety of different cell types of the myelomonocytic lineage. Various agents induce HL-60 cells to differentiate to four general types of cells: (a) granulocytes, (b) monocytes, (c) macrophagelike cells, and (d) eosinophils. These designations are somewhat arbitrary, and certain HL-60-inducing agents give rise to cells with characteristics that overlap these categories. Moreover, as emphasized later, the induced HL-60 cells are usually deficient in certain characteristics of their normal cell counterpart. Nevertheless, these categories provide a convenient outline for discussing the numerous and complex phenotypic changes induced in this leukemic cell line by a wide variety of agents. These categories are discussed separately below.

Granulocytes. The HL-60 cells are predominantly promyelocytes but in most cell cultures, particularly passage 60 or less, <5% of the cells exhibit spontaneous differentiation to morphologically mature cells including myelocytes, metamyelocytes, banded and segmented neutrophils. The addition of several agents including retinoic acid markedly increases this spontaneous differentiation, with most cells acquiring morphological, functional, enzymatic, and surface membrane antigen characteristics of mature granulocytes (Table 1). A variety of different compounds induces such granulocytic differentiation of HL-60 cells (Table 2). Many of these compounds also induce erythroid differentiation in Friend mouse erythroleukemia cells.19,20 By several criteria, this granulocytic differentiation of HL-60 cells is somewhat incomplete and defective. For example, most granulocyte-induced cultures consist predominantly of metamyelocytes and banded neutrophils rather than fully differentiated multilobulated polys. In addition, the granulocyte-induced HL-60 cells lack lactoferrin, suggesting that they are deficient in secondary granules.21,22 The LDH isoenzyme profile of granulocyte-induced HL-60 differs quantitatively from the LDH isoenzymes of normal granulocytes consistent with incomplete differentiation.23 Deficiencies have been described in the myeloperoxidase/peroxide/halide killing sys-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Uninduced</th>
<th>Granulocyte</th>
<th>Monocyte</th>
<th>Macrophagelike</th>
<th>Eosinophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>+3,4</td>
<td>22,147</td>
<td>22,42</td>
<td>81</td>
<td>98,97</td>
</tr>
<tr>
<td>Eosinophil peroxidase</td>
<td>-</td>
<td>97</td>
<td></td>
<td></td>
<td>98,97</td>
</tr>
<tr>
<td>ASD chloroacetate esterase</td>
<td>+4</td>
<td>42,51</td>
<td>81</td>
<td>81</td>
<td>97</td>
</tr>
<tr>
<td>Nonspecific esterase</td>
<td>-4,81</td>
<td>81</td>
<td>81</td>
<td>81</td>
<td>97</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+58</td>
<td>148</td>
<td>148</td>
<td>148</td>
<td>97</td>
</tr>
<tr>
<td>Biebrich scarlet</td>
<td>-96</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>Plastic adherence</td>
<td>-81,156</td>
<td>81,156</td>
<td>81,156</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>-74,150</td>
<td>74,150</td>
<td>149</td>
<td>149</td>
<td>152</td>
</tr>
<tr>
<td>Chemotactic receptors</td>
<td>+150,151</td>
<td>150,151</td>
<td>150,151</td>
<td>149</td>
<td>152</td>
</tr>
<tr>
<td>Complement receptors</td>
<td>+57,74</td>
<td>57,74</td>
<td>57</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>Fc receptors</td>
<td>+67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>-81,61</td>
<td>81,61</td>
<td>81,61</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>NBT reduction</td>
<td>-74</td>
<td>74</td>
<td>74</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>-17,47,74</td>
<td>17,47,74</td>
<td>17,47,74</td>
<td>17,47,74</td>
<td>17,47,74</td>
</tr>
<tr>
<td>Microbicidal</td>
<td>-21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Monocyte/macrophage surface antigens</td>
<td>-42,154</td>
<td>42,154</td>
<td>42,154</td>
<td>42,154</td>
<td>42,154</td>
</tr>
<tr>
<td>Granulocyte surface antigens</td>
<td>-88,153</td>
<td>88,153</td>
<td>88</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>Insulin receptors</td>
<td>+10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Transferrin receptors</td>
<td>+7,8</td>
<td>7,8</td>
<td>7,8</td>
<td>7,8</td>
<td>7,8</td>
</tr>
</tbody>
</table>

Arrows indicate a quantitative increase (↑) or decrease (↓) from uninduced HL-60 levels. Blank spaces correspond to characteristics absent from the literature. (+) indicates present, (−) indicates absent, ± indicates discrepant or equivocal literature findings.
Differentiation is initiated at the cell surface is provided by differentiation inducers. Indeed, most fresh leukemic cells in cells appear to be quite exceptional among acute nonlympho-

anisms (Table 1), suggests that these granulocyte inducers, particularly retinoic acid, may be of therapeutic benefit in patients with myelogenous leukemia. Unfortunately, HL-60 cells appear to be quite exceptional among acute nonlymphocytic leukemias (ANL) in their response to granulocytic differentiation inducers. Indeed, most fresh leukemic cells in short-term culture in the presence of granulocyte-inducing agents do not exhibit differentiation.36 Nevertheless, a subset of ANL patients exists, primarily those classified as acute promyelocytic leukemia, whose leukemic cells clearly exhibit induced granulocytic differentiation in vitro.36 Moreover, there are several anecdotal reports of patients with promyelocytic leukemia and with myelodysplastic syndrome who have apparently benefited from systemic therapy with retinoic acid.36-32

The mechanism of action of the HL-60 granulocyte inducers is unknown. At least a 12-hour exposure to DMSO or retinoic acid is necessary before any differentiation occurs and continuous exposure to these compounds is required to induce maximum differentiation.18,33 Although retinoic acid is believed to exert its biologic effect in many cells by interacting with a specific cytoplasmic retinoic-acid binding protein (RABP).34,35 Our own observations and those of other researchers36 indicate that HL-60 cells lack such a binding protein. Evidence that retinoic acid-induced HL-60 differentiation is initiated at the cell surface is provided by the observation that granulocytic differentiation occurs in HL-60 cells when cultured with retinoic acid immobilized on a solid substrate.37 HL-60 cell lines resistant to the differentiation-inducing effects of retinoic acid exhibit an apparent high-mol-wt acidic surface membrane glycoprotein that is absent from the wild-type HL-60 cells.38,39 This aberrant surface membrane glycoprotein is hyposialylated, but its relationship to the retinoic acid resistance is presently uncertain.40

Monocytes. Different compounds induce HL-60 to differentiate to cells exhibiting distinct "monocytic" characteristics. These cells morphologically can be confused with immature HL-60 granulocytes, and they share many important functional characteristics with HL-60 cells treated with granulocytic inducers (Table 1). They can be readily distinguished from these latter cells, however by different markers, including positive staining for the monococyte/macrophage specific enzyme α-naphthyl acetate esterase (nonspecific esterase-NSE),41 by the presence of monocytic cell membrane antigens,42,44 and by their capacity for mediating antibody-dependent cytotoxicity (ADCC).44-46

A variety of different naturally occurring compounds induces monocytic differentiation of HL-60. These include vitamin D3 as well as various products found in mitogen-stimulated lymphocyte-conditioned media (LCM).45-51 1,25 dihydroxyvitamin D3 induces monocytic differentiation of HL-60 cells at concentrations as low as 10-24 mol/L.52,53 and specific high-affinity cytosol receptors for vitamin D3 have been observed in HL-60.46 This compound also induced monocytic/macrophage differentiation in normal human bone marrow cells.49 Vitamin D3 could have therapeutic potential but, as with retinoic acid, HL-60 cells appear to be unusual among ANL cells in their differentiative response to this agent. Other naturally occurring compounds of interest that induce monocytic differentiation of HL-60 cells include various products produced by mitogen-stimulated mononuclear cell cultures.51 An HL-60 monocyte-inducing compound with mol wt of ~40,000 termed differentiation-inducing factor (DIF) has been isolated from LCM and from CM from the T cell leukemia cell line HUT-102.52,53 Another mitogen-stimulated lymphocyte product, γ-interferon, (IFN-γ) induces monocytic differentiation of HL-60.54,55,56 whereas IFN-α and IFN-β alone do not exhibit this inducing capacity. Synergism among different naturally occurring compounds has been demonstrated in the induction of monocytic differentiation of HL-60. For example, retinoic acid in relatively small concentrations synergistically acts with DIF,55 IFN-γ56 and IFN-α55,56 to induce monocytic differentiation of HL-60. This latter finding is interesting since retinoic acid by itself is a granulocytic inducer of HL-60. In addition, lymphotoxin and tumor necrosis factor (cachectin), which are also products of mitogen-stimulated mononuclear cells, also act in synergy to promote IFN-γ-γ-induced differentiation of HL-60.59

Therapeutically, monocytic differentiation of HL-60, unlike granulocytic differentiation, is not necessarily accompanied by the loss of proliferative capacity of the induced cells (ie, monocytic HL-60 differentiation is not necessarily terminal differentiation). For example, recombinant IFN-γ alone as well as IFN-γ plus retinoic acid induces monocytic
characteristics of HL-60 cells, but they have little effect on inhibiting HL-60 cell proliferation.\textsuperscript{45,46} Moreover, Elias et al. identified a factor in mitogen-stimulated LCM that markedly enhances thymidine incorporation of HL-60 cells.\textsuperscript{48} An early stimulatory effect of tumor necrosis factor on HL-60 thymidine incorporation has also been observed.\textsuperscript{59} Thus, monocytic differentiation of HL-60 may in certain cases be preceded by enhanced cell proliferation, and HL-60 cells induced to acquire particular monocytic characteristics have not necessarily lost the capacity to proliferate.

**Macrophagelike.** The primary inducer of macrophagelike HL-60 cells is the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). This TPA-induced macrophagelike phenotype is frequently combined with the “monocytic” phenotype in discussions of HL-60 differentiation; indeed, these induced cell types both share many of the same enzymatic, histochemical, and functional characteristics (Table 1). Several important phenotypic characteristics clearly distinguish the HL-60 macrophagelike differentiation from HL-60 monocytic differentiation, however. First, TPA-treated HL-60 cells are markedly more adherent than HL-60 monocytes. TPA-treated cells exhibit intense adherence to plastic with prominent pseudopodia formation and also markedly adhere to each other.\textsuperscript{61,62} Second, several specific surface antigens present on normal and HL-60 monocytes are not induced in TPA-treated HL-60 cells.\textsuperscript{42,43,50,65} Third, no induction of chemotactic peptide receptors or of NBT reduction activity occurs in TPA-treated HL-60,\textsuperscript{56} whereas these characteristics are readily demonstrated in HL-60 monocytes.\textsuperscript{52,56} In contrast to monocytic induction, TPA induction of HL-60 is associated with a rapid profound loss in proliferative capacity. Few HL-60 cells are able to enter S-phase after exposure to TPA.\textsuperscript{61,62} Moreover, TPA-induced HL-60 differentiation can occur in the absence of cell division or new DNA synthesis.\textsuperscript{53,54} Fifth, in contrast to the HL-60 granulocyte and monocyte inducers, prolonged exposure to TPA is not necessary to induce macrophagelike differentiation. Significant macrophagelike differentiation occurs in HL-60 cells after TPA exposure as short as 20 minutes.\textsuperscript{61} Finally, unlike the granulocytic and monocytic HL-60 inducers, TPA induction is not confined to a small subset of fresh nonlymphocytic leukemia cells. Indeed, most acute myeloid leukemia cells as well as chronic myelogenous leukemia (CML) blast crisis cells exhibit this macrophagelike differentiation when exposed to TPA.\textsuperscript{61,62}

There is a curious dose dependency of the TPA-induced phenotypic changes in HL-60 cells. At low doses of TPA (3 × 10^{-10} mol/L), HL-60 colony formation in soft agar in the absence of colony-stimulating factor (CSF) is enhanced ~twofold (S. Collins, unpublished observations). Such colony formation is suppressed at TPA concentrations of ≥10^{-9} mol/L. This dose-dependent TPA stimulation is similar to that reported on the KG-1 myeloid leukemia cell line\textsuperscript{60} as well as on fresh human myeloid leukemia cells\textsuperscript{61} and on mouse bone marrow colony-forming cells.\textsuperscript{72} At slightly higher concentrations (4 × 10^{-10} mol/L), TPA has induced granulocytic differentiation of HL-60 in liquid suspension culture.\textsuperscript{73} Concentrations >5 × 10^{-10} mol/L induced the macrophagelike phenotype. Thus, at low concentrations of TPA, HL-60 proliferation is somewhat enhanced, at slightly higher concentrations granulocytic maturation occurs and, at still higher concentrations, the macrophagelike HL-60 phenotype is induced.

TPA exerts different phenotypic changes on HL-60 cells depending on the differentiative state of these cells. In HL-60 cells induced to granulocytes or monocytes TPA induces respiratory burst activity with the conversion of oxygen to superoxide anion.\textsuperscript{52,54} This reaction forms the basis for the nitroblue tetrazolium (NBT) test, which is commonly used to quantitate HL-60 differentiation since undifferentiated HL-60 cells do not exhibit this particular response to TPA.\textsuperscript{74,75} Similarly, TPA rapidly induces chemiluminescence in HL-60 cells induced to granulocytes but not in undifferentiated HL-60.\textsuperscript{76}

In contrast to the granulocytic and monocytic HL-60 inducers considerable information is available related to the mechanism of action of TPA. HL-60 variant subclones have been isolated that are resistant to DMSO but that differentiate with TPA, suggesting that different pathways are involved in DMSO- and TPA-induced HL-60 differentiation.\textsuperscript{77} TPA may exert many of its effects through protein kinase C, a calcium- and phospholipid-dependent enzyme that is widely distributed in numerous tissues and which plays a crucial role in transducing various extracellular signals across the cell membrane.\textsuperscript{78,79} HL-60 cells harbor specific receptors for TPA,\textsuperscript{80,81} and considerable evidence in HL-60 as well as in other cells shows that the TPA receptor is indeed protein kinase C.\textsuperscript{82-84} Normally, protein kinase C is transiently activated by diacylglycerol, but TPA can substitute for endogenous diacylglycerol, resulting in prolonged activation of the enzyme.\textsuperscript{85} This activation of protein kinase C in HL-60 cells could result in the phosphorylation of specific substrate proteins leading to the macrophagelike phenotypic changes induced by TPA. What are the possible substrates for activated protein kinase C in TPA-stimulated HL-60 cells? Following 1-hour treatment with TPA, increased phosphorylation of at least 14 proteins has been noted in HL-60.\textsuperscript{86} Specifically, rapid phosphorylation of the HL-60 transferrin receptor occurs, associated with a decline in surface transferrin-receptor number.\textsuperscript{87} In addition, phosphorylation of two cytosol proteins (17 and 27 kd in size) occurs in HL-60 cells within 15 minutes after exposure to TPA.\textsuperscript{88} Activation of topoisomerase II by protein kinase C has been observed in vitro and this, together with the observation that TPA-induced HL-60 differentiation is partially blocked by inhibitors of topoisomerase II, suggests that this DNA-modulating enzyme could be an important target for activated protein kinase C in TPA-treated HL-60 cells.\textsuperscript{89}

HL-60 variants stably resistant to TPA-induced differentiation have been derived by culturing the cells in gradually increasing concentrations of TPA.\textsuperscript{90,91} These TPA-resistant HL-60 cells generally exhibit the same number of TPA receptors exhibited by wild-type HL-60.\textsuperscript{90,91} Moreover, similar cellular proteins are rapidly phosphorylated after TPA exposure in both the TPA-sensitive and TPA-resistant HL-60 cells, but the quantitative level of this rapid phosphorylation is significantly less in the TPA-resistant v the TPA-sensitive cells.\textsuperscript{92} These observations suggest that a block
exists in the enzymatic activity of protein kinase C after its interaction with TPA in the TPA-resistant cells. This block may be related to the aberrant translocation of this enzyme from cytosol to membrane fractions that has been observed in the TPA-resistant HL-60 cells as compared with the TPA-sensitive HL-60 cells.93

The TPA-induced binding to, and activation of, protein kinase C appears to be important in the TPA-induced differentiation of HL-60 cells. Several observations suggest, however, that activation of protein kinase C cannot by itself account for this macrophagelike differentiation. For example, the synthetic diacylglycerol 1-oleoyo-2-acetylglycerol (OAG) binds to and activates protein kinase C and induces the rapid phosphorylation of HL-60 cellular proteins.94 This compound, however, does not induce HL-60 differentiation.94 Similarly, the macrocyclic lactone bryostatin binds to and activates protein kinase C in HL-60 but actually inhibits TPA-induced HL-60 differentiation in a dose-dependent manner.94 These observations suggest that a TPA effect other than, or in addition to, activation of protein kinase C must account for the macrophagelike differentiation of HL-60. The nature of this additional TPA action remains to be defined.

**Eosinophils.** Several observers have noted cells positive for the eosinophil specific Luxol-fast-blue stain in a small percentage (4% to 7%) of HL-60 colonies cloned in semisolid agar culture.125 The proportion of colonies containing such cells increased with the addition of crude preparations of GM-CSF. Preparations containing EO-CSF, which stimulates the formation of normal eosinophil colonies, had no effect on the incidence of such colonies.12 Similarly, purified recombinant GM-CSF induces a small but significant increase in the number of Biebrich scarlet cells in HL-60 liquid suspension cultures.13 Moreover, variant HL-60 subclones exhibiting virtually 100% staining with Biebrich scarlet as well as other eosinophil characteristics have been isolated and stably maintained in continuous suspension culture.96 Fischkoff et al noted that the proportion of cells exhibiting eosinophilic characteristics in the wild type HL-60 line could be markedly increased by culturing the cells in slightly alkaline media (pH 7.6 to 7.8).97 These induced cells have been extensively characterized and demonstrate multiple eosinophilic granule proteins, including the eosinophil major basic protein (MBP), the Charcot-Leyden Crystal (CLC) protein, eosinophil peroxidase, acid phosphatase, and arylsulfatase.97,98 Parental, uninduced HL-60 cells also exhibit the eosinophil MBP. The amount of this protein markedly diminishes when the culture is treated with granulocytic inducers such as DMSO.97

Some of the HL-60 eosinophilic characteristics are aberrant as compared with those of normal eosinophils. This is particularly true with regard to granule formation. For instance, the HL-60 eosinophilic granules are chloroacetate esterase and PAS positive, whereas normal eosinophilic granules are negative for both.97 HL-60 eosinophils express significantly less CLC and arylsulfatase protein than do normal eosinophils.98 Such abnormalities are similar to those observed in the dysplastic eosinophils present in the bone marrow of patients with acute myelomonocytic leukemia. This latter syndrome is associated with karyotypic abnormalities involving the long arm of chromosome 16.99,100 and similar karyotypic abnormalities in this chromosome region have been noted in HL-60 metaphases.97

**HL-60 Oncogene Structure and Expression**

HL-60 cells provide an invaluable model system for studying the relationship of oncogene structure and expression to normal and leukemic cell proliferation and differentiation. Several mutations involving specific cellular oncogenes have been noted in HL-60, reinforcing the concept that the development of malignancy is a multistage phenomenon, that cancer results not from a single mutation but from a series of mutations. In addition, studies on HL-60 have provided important insight into the relationship of specific oncogene expression to differentiation along a specific hematopoietic lineage.

HL-60 cells exhibit specific genomic mutations involving the cellular oncogenes c-myc, N-ras and p53. These are discussed separately.

**c-myc amplification.** The human c-myc gene is defined by virtue of its homology to v-myc, the transforming gene of the avian myelocytoma virus, which induces myeloid tumors in chicks. The protein product of this cellular oncogene is localized in the nucleus.101 and enhanced expression of the c-myc gene is rapidly induced following mitogenic stimulation of resting lymphocytes or fibroblasts.102 In addition, this oncogene acts in concert with other oncogenes, particularly those in the ras gene family, to malignantly transform primary rodent fibroblast cultures.103 HL-60 cells exhibit a 15- to 30-fold genomic amplification of c-myc as compared with normal cells.104,105 This observation was the first reported example of amplification of a cellular oncogene in any human malignancy. This c-myc amplification was also present in the fresh, uncultured leukemic cells of the patient from whom the HL-60 cell line was derived, indicating that this amplification did not arise during prolonged in vitro culture.105 This c-myc amplification exhibited by HL-60 appears to be unusual among human hematopoietic malignancies. In a survey of >100 fresh human leukemias and lymphomas, Rothberg et al noted only one other hematopoietic malignancy (a Burkitt lymphoma) that exhibited genomic amplification of c-myc.106

In correlation with this genomic c-myc amplification, HL-60 cells exhibit high levels of steady state c-myc RNA. These levels significantly decrease as HL-60 cells are induced to differentiate terminally with granulocytic inducers.107 In addition, monocytic and macrophagelike differentiation of HL-60 is associated with a marked decrease in c-myc RNA levels.108,109 This decreased c-myc expression in differentiating HL-60 cells appears to mimic that which occurs in normal hematopoiesis since a similar decrease in c-myc RNA has been noted in normal bone marrow progenitor cells undergoing terminal differentiation in liquid suspension culture.110

Decreased c-myc transcription rather than enhanced c-myc mRNA degradation appears to account for the diminished steady-state c-myc RNA in differentiated HL-60.111-113
In retinoic acid-treated HL-60 this transcriptional downregulation occurs as a block in elongation of the myc RNA transcript between exons 1 and 2 rather than as decreased transcript initiation. This novel mechanism of transcriptional downregulation might be particularly adapted to genes in which transcription is rapidly regulated and HL-60 cells provide the prototype model for studying this phenomenon.

N-ras. HL-60 genomic DNA is capable of malignant transforming NIH3T3 cells in transfection assays, and the HL-60 gene that mediates this transformation has been identified as the N-ras gene, a member of the ras family of oncogenes which also includes Kirsten and Harvey ras. Acquisition of NIH3T3-transforming ability frequently is associated with specific point mutations within the ras oncogenes, and a point mutation involving the second nucleotide of N-ras codon 61 has been documented in HL-60. Other acute myeloid leukemia cells exhibit NIH3T3-transforming activity and N-ras point mutations. In contrast to HL-60, these mutations usually involve N-ras codons 12 and 13.

The normal cell function of the N-ras cellular oncogene is presently unknown. Why acute myeloid leukemia cells preferentially exhibit mutations in this particular ras gene rather than in other members of the ras gene family is also unclear. In contrast to c-myc gene expression, expression of the N-ras gene does not appear to change significantly as HL-60 cells are induced to differentiate. An approximately twofold decrease in N-ras RNA was noted in DMSO-differentiated HL-60, whereas no change in N-ras expression was noted in TPA-treated HL-60 cells.

p53. The p53 protein is frequently found at elevated levels in various types of tumor cells. This protein shares some characteristics with the c-myc product. Both are localized to the nucleus, and p53 can substitute for myc in the myc–ras cooperative transformation of primary rodent fibroblast cultures. The p53 locus is on the short arm of chromosome 17, and this gene is extensively deleted in HL-60 associated with absent p53 expression. Moreover, the opposite p53 allele is absent, presumably secondary to the monosomy 17 that has been demonstrated in HL-60 metaphases. The selective advantage if any of this p53 deletion is unknown. Because c-myc and p53 may share similar physiologic functions, it has been suggested that loss of p53 in progenitors of HL-60 may have led to the subsequent selection of cells harboring amplification of c-myc.

Although the expression of cellular oncogenes was initially presumed to be associated with actively proliferating cells, some recent rather surprising observations indicate that expression of some cellular oncogenes is enhanced in certain terminally differentiated cells. Examples of this in HL-60 involved the fos, fms, src, and fgr oncogenes.

c-fos. The c-fos gene is the homolog of v-fos, the transforming gene of the FBJ-murine sarcoma virus, which induces osteosarcomas in mice. The c-fos gene product is localized in the nucleus. Mitogen stimulation of resting fibroblasts induces the rapid (within 5 to 10 minutes) onset of c-fos mRNA and protein product, suggesting that c-fos may play a role in rendering resting cells competent to divide. It is somewhat paradoxical that a similar rapid induction of c-fos is noted when HL-60 cells are treated with TPA, since this compound also induces a rapid, pronounced inhibition of HL-60 proliferation. Enhanced c-fos expression has also been noted during the monocytic differentiation of the mouse WEHI-13B cell line, as well as in normal monocytes and macrophages. Moreover, HL-60 cells induced to monocytes with vitamin D3 also exhibit enhanced fos expression, although such fos expression occurs more gradually without the rapid initial c-fos expression noted in TPA-treated HL-60. The role that c-fos plays in monocyte/macrophage differentiation is presently unclear. c-fos expression does not appear to be absolutely essential for monocyte differentiation since vitamin D3 has been noted to induce monocytic differentiation of some HL-60 variant subclones without inducing enhanced c-fos expression.

c-fms. The c-fms gene is the cellular homolog of the transforming gene of the McDonough strain of the feline sarcoma virus. The c-fms gene product appears to be identical to the cell membrane receptor for the monocyte/macrophage colony-stimulating factor (CSF-1). HL-60 cells induced to differentiate to monocytes with vitamin D3 or to macrophagelike cells with TPA express enhanced c-fms RNA levels as compared with uninduced HL-60 cells. The kinetics of this enhanced expression is considerably slower than induced c-fos expression, with c-fms transcripts initially noted ~6 hours after inducer treatment and peaking at 24 to 48 hours following induction. By means of immunologic reagents CSF-1 receptors have been detected on monocyte-induced HL-60 cells, but their numbers appear relatively small when compared with normal monocytes/macrophages. c-fms is the cellular homolog of the transforming gene of the Rous sarcoma virus and codes for a cell membrane-associated protein (pp60\textsuperscript{c-src}) with tyrosine kinase activity. The levels of pp60\textsuperscript{c-src} kinase activity are increased in HL-60 cells induced to macrophages, monocytes, or granulocytes with TPA, vitamin D3, and DMSO, respectively. Normal nonleukemic monocytes also exhibit relatively high levels of pp60\textsuperscript{c-src} activity. Terminally differentiated rat CNS neurons similarly exhibit relatively high levels of c-src. The physiologic function of c-src in mature myeloid cells and neurons is presently unknown, but the high level of expression of this cellular oncogene in these terminally differentiated cell types indicates that its function is probably unrelated to active cell proliferation.

c-src. The c-src gene is the cellular homolog of the transforming gene of the Rous sarcoma virus and codes for a cell membrane-associated protein (pp60\textsuperscript{src}) with tyrosine kinase activity. The levels of pp60\textsuperscript{src} kinase activity are increased in HL-60 cells induced to macrophages, monocytes, or granulocytes with TPA, vitamin D3, and DMSO, respectively. Normal nonleukemic monocytes also exhibit relatively high levels of pp60\textsuperscript{src} activity. Terminally differentiated rat CNS neurons similarly exhibit relatively high levels of c-src. The physiologic function of c-src in mature myeloid cells and neurons is presently unknown, but the high level of expression of this cellular oncogene in these terminally differentiated cell types indicates that its function is probably unrelated to active cell proliferation.

c-fgr. c-fgr is the cellular homolog of the transforming gene of the Gardner-Rasheed feline sarcoma virus. This gene is a member of the src family of oncogenes and codes for a cell membrane-associated protein with tyrosine kinase activity. This gene is expressed in peripheral blood leukocytes with preferential expression in mature monocytes and granulocytes and little expression in B and T lymphocytes. Induction of HL-60 granulocytic differentiation is associated with a marked increase in the level of expression of c-fgr (K.
robbins, personal communication, july, 1986). as with c-src, the physiologic function of c-fgr in the terminally differentiated hl-60 cells is presently unknown.

summary and future studies

how closely does the in vitro hl-60--induced differentiation mimic normal granulocyte/macrophage differentiation? as emphasized above, differences exist between the induced hl-60 cells and normal granulocytes and monocytes. whether this reflects an intrinsic defect in the karyotypically abnormal hl-60 leukemic cells or represents the failure of such in vitro manipulations to mimic the normal in vivo hematopoietic environment is unknown. a related question is whether the bipotential (granulocyte/macrophage) nature of the hl-60 promyelocytes has its counterpart in normal promyelocytes. granulocytes and monocytes clearly share a common stem cell, as evidenced by the frequent observation of mixed granulocyte/macrophage colonies in cfu-c assays. this stem cell, however, is generally believed to be more immature than the normally granulated, peroxidase-positive promyelocyte, whose progeny are usually assumed to be granulocytes. the behavior of hl-60 promyelocytes suggests that the normal promyelocyte may also be capable of differentiating to monocytes. in this regard, it is noteworthy that studies in which hl-60 cells are treated sequentially with granulocytic (retinoic acid) and monocytic (vitamin d3) inducers indicate that early events in the induction of hl-60 promyelocytes to granulocytes or monocytes are shared by cells of both lineages.138 nevertheless, the caveat remains that the leukemic hl-60 promyelocyte is aberrant and does not truly reflect the differentiative potential of normal promyelocytes.

the synergistic effect of various naturally occurring compounds, including retinoic acid, tumor necrosis factor, dfis, and the interferons on hl-60 cells may reflect some of the normal control mechanisms involved in hematopoiesis. although the usual in vitro hematopoietic stem cell agar assays generally exploit the effect of single compounds such as csf or erythropoietin on stem cell proliferation and differentiation, the situation in vivo is undoubtedly more complex. normal hematopoietic stem cell proliferation and differentiation probably results from a complex array of synergistic inhibitory and stimulatory signals provided by lymphokines, monokines, vitamins, corticosteroids, interferons, and the various csfs. hl-60 cells provide a convenient system for assessing the synergistic effect of such biologic response modifiers on granulocyte/macrophage/macrophage differentiation.

what is the relation between tpa--induced hl-60 and normal monocyte/macrophage differentiation? tpa is a plant derivative that appears to mimic the effect of naturally occurring compounds such as diacylglycerol in activating the protein kinase c family of enzymes. conceivably, tpa is not so selective as such endogenous activators of these enzymes and might simultaneously activate a number of different cellular enzymes and targets. this "sledgehammer" effect might account for the unusual macrophage-like phenotype exhibited by tpa-treated hl-60 cells. tpa is a very active membrane stimulator and induces oxidase activity, chemiluminescence, and degranulation in both normal and induced hl-60 monocytes and macrophages. such membrane effects may also be mediated through protein kinase c.139,140 tpa may simultaneously induce both differentiation and membrane activation of hl-60 cells to account for the unusual phenotype of tpa-treated hl-60.

the recent isolation and analysis of protein kinase c cDNA clones indicates that at least three distinct types of this enzyme are present in mammalian species and that expression of these different enzymes varies from tissue to tissue.141-143 it will be of interest to determine the distribution of these different protein kinase c enzymes in uninduced v induced hl-60 cells. conceivably, these various protein kinases may act on different substrates, may have different affinities for tpa, and may be differentially distributed in uninduced v induced hl-60. this might account for the different phenotypic changes induced in hl-60 by different concentrations of tpa and might also explain the different effects induced by tpa depending on the state of differentiation of the hl-60 cells.

the pronounced antiproliferative effect of tpa on hl-60 is of particular interest and could have therapeutic relevance, since a similar tpa--induced effect is also noted in most fresh acute myelogenous leukemia cells. the adverse effects (including tumor promotion) exerted by tpa on other cell types, however, rules out its current use as a therapeutic agent.144 future studies attempting to define the specific hl-60 tpa targets that trigger this growth inhibition should provide a greater understanding of the antiproliferative effects of tpa and may lead to more selective therapeutic agents for myeloid leukemia.

the hl-60 cell line, because of its distinct monocyte/granulocyte characteristics, will continue to provide a ready source of specific human mRNA for use in cloning important granulocyte and monocyte enzymes and monokines. examples of such monocyte/granulocyte specific clones derived from hl-60 to date include the gene for tumor necrosis factor (cachectin) cloned from a tpa-treated hl-60 cDNA library145 and the candidate gene that is defective in chronic granulomatous disease, cloned from a granulocyte-induced hl-60 cDNA library.146

hl-60 cells exhibit structural abnormalities of at least three different cellular oncogenes (c-myc, n-ras, and p53) and one hematopoietic growth factor (gm-csf). how such mutations are related to the continuous proliferation and block in differentiation exhibited by hl-60 is an area of active investigation. of particular experimental interest is the relationship between the hl-60 c-myc amplification and this block in differentiation. does the induction of decreased levels of c-myc RNA per se lead to differentiation of hl-60 cells or are other direct or indirect effects of differentiation inducers on other hl-60 genes required: an "antisense" 15-base pair (bp) oligonucleotide complementary to the translation initiation region of c-myc was recently noted to induce differentiation of hl-60 cells (j. holt, personal communication, december, 1986). the control "sense" oligomer was ineffective in inducing such differentiation. these
observations are preliminary but suggest that the c-myc product is critical to maintaining HL-60 cells in a relatively undifferentiated proliferative state and that inhibition of this product alone may result in the terminal differentiation of HL60 cells. The normal physiologic function of most of the cellular oncogenes remains to be defined. Particularly perplexing are those oncogenes such as fgr, fos, and src whose expression is enhanced in certain terminally differentiated rather than actively proliferating cells. How closely HL-60 leukemic cell differentiation mimics the differentiation of normal myeloid progenitor cells is presently unclear, and significant differences between the mechanisms of HL-60 and normal myeloid progenitor cell differentiation may exist. Nevertheless, the specific changes in c-myc, fos, fgr, and src cellular oncogene expression that characterize HL-60 cell differentiation also appear to occur during normal myeloid cell differentiation. Thus, HL-60 cells should continue to provide an excellent model system for studying both the regulation of expression of these cellular oncogenes and the physiologic role these particular genes play in the terminal differentiation of normal monocytes, macrophages, and granulocytes.

REFERENCES


105. Dalla Favera R, Wong-Staal F, Gallo R: Onc gene amplifi-
cation in promyelocytic cell line HL-60 and primary leukemia cells of the same patient. Nature 299:61, 1982
140. Virgilio F, Lew D, Pozzan T: Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca$^{2+}$ levels. Nature 310:691, 1984
necrosis factor: Precursor structure, expression and homology to lymphotoxin. Nature 312:724, 1984
The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression

SJ Collins