Proliferation and Differentiation of Human Myeloid Leukemic Cells in Immunodeficient Mice: Electron Microscopy and Cytochemistry

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To study the influence of a biologic environment on cultured human leukemia cells, KG-1, KG-1a, and HL-60 cells were inoculated subcutaneously into newborn nude mice. The cells developed myelosarcomas at the site of inoculation and in lungs and kidneys. KG-1 and HL-60 myelosarcomas were successfully passaged through adult nude mice, whereas KG-1a tumors proliferated only after transplantation into newborn hosts. The human nature of the cells forming myelosarcomas in mice was assessed by chromosomal analyses and detection of cross-reactivity with an antibody to the human leukemia cell line K562. We undertook electron microscopic and cytochemical examinations of the cells proliferating in vitro and in the mice. The granules of KG-1 cells in vivo did not react for acid phosphatase, as observed in vitro, and the HL-60 cells proliferating in mice lost the perinuclear myeloperoxidase (MPO) demonstrated in cultured cells. Although the influence of an in vivo selection of cell subpopulations cannot be ruled out, the enzymatic changes are compatible with induced cell differentiation. Conclusive evidence of differentiation in vivo was observed in the KG-1a cell subline. The undifferentiated KG-1a blasts developed cytoplasmic granules and synthesized MPO during proliferation in vivo. These observations indicate that human leukemia cells from established cell lines proliferate in nude mice and may acquire new differentiated properties in response to the in vivo environment.

Established human myeloid leukemia cell lines have proved to be valuable tools for investigating the mechanisms of cell differentiation. Changes in cell morphology and function have been observed in cultured KG-1, and HL-60 myeloid leukemic cells. These findings are applicable to cell growth in vitro; however, the proliferation of human myeloid leukemia cells in animal hosts can provide an experimental system in which the cells are supported by and interact with a complex biologic framework. In such a system, it would be possible to define whether or not morphological and enzymatic myeloid cell markers develop in vivo spontaneously or under the action of agents that stimulated cell differentiation in vitro. Experimental tumors composed of human myelogenous leukemia cells, however, are difficult to generate and to maintain by mouse-to-mouse passages. To date, only the K562 cell line, inoculated into immunodeficient mice, has yielded well-defined myelosarcomas that have been maintained by long-term serial transplantation.

We have now succeeded in developing reproducible disseminated myelosarcomas composed of KG-1, KG-1a, and HL-60 human myeloid leukemia cells injected subcutaneously (s.c.) into newborn nude mice. Electron microscopy examinations, combined with peroxidase and lysosomal enzyme detection, demonstrated that KG-1 and HL-60 cells, proliferating in the mice, developed subtle changes compatible with altered differentiation. KG-1a cells displayed conclusive signs of myeloid differentiation that were not observed in vitro.

MATERIALS AND METHODS

Human Leukemia Cell Lines

The KG-1 cell line was cultured in α-medium with 20% inactivated fetal calf serum (IFCS). The KG-1 line and subline KG-1a were established by Koelle and Golde from a bone marrow sample of a patient with erythroleukemia that had developed into acute myeloid leukemia (AML). The cell line HL-60 (kindly provided by Dr. R.C. Gallo, NCI, Bethesda, MD) was developed from the peripheral leukocytes of a patient with acute promyelocytic leukemia (APML). HL-60 cells were cultured in RPMI 1640 containing 20% IFCS.

Mice

BALB/c nude mice, maintained in a pathogen-free enclosure, were used for transplantation of the human leukemia cells. The characteristics of this mutant strain of mice from our colony have been reported in detail. Nude mice were termed newborn (1–3 days old) or adult (4–5 wk old) according to their age at the time of heterotransplantation.

Heterotransplantation

A 50-μl aliquot containing 10³ viable cells from suspension culture was routinely transplanted s.c. into the dorsal region of newborn nude mice. All cell lines produced s.c. tumors and visceral dissemination.
tions. KG-1 and HL-60 subcutaneous tumors were excised and serially transplanted into adult nude mice.

**Histopathologic Procedures**

Mice were autopsied at the time of death or when showing signs of severe clinical disturbance. Slices of s.c. tumors and internal organs with neoplastic nodules were fixed in 10% formaldehyde (pH 7.0) and processed for light microscopy. Sections were stained with hematoxylin-eosin and Gomori's silver reticulin stain.

**Electron Microscopic Studies**

Small (1 cu mm) blocks of s.c. and visceral tumors were collected 15–20 days after transplantation. The tissues were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 hr, rinsed 3 times in buffer, and postfixed in 2% aqueous osmium tetroxide. After dehydration, specimens were embedded in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate for electron microscopic examination. Blocks of tumors, fixed for only 1 hr in glutaraldehyde-sodium cacodylate mixture, were incubated in myeloperoxidase (MPO) and acid phosphatase media. After incubation, the blocks were processed as described above.

Aliquots of 10^7/ml malignant hematopoietic cells proliferating for 7 days in culture were fixed with equal volumes of glutaraldehyde in 0.05 M sodium cacodylate buffer for 1 hr. Then, the cells were postfixed in osmium tetroxide and embedded or were incubated for MPO and acid phosphatase. Thin sections were examined in a transmission electron microscope and representative cells of each sample were photographed.

**Cytogenetic Studies**

To confirm the human origin of the leukemia cells, chromosome analyses were performed using G-banding techniques; 20–100 metaphases were examined from each sample of cells prior to transplantation and in cells recovered from s.c. and visceral tumors. The cells were subcultured in tissue culture media (EM15A).

**Antigenicity of Heterotransplanted Leukemia Cells**

A heterologous antibody raised to K562 cells that cross-reacts with other granulocytic cell lines was used to assess the presence of human leukemia antigen receptors on the transplanted cells. Antibody-dependent, complement-mediated cytotoxicity (ADCMC) of goat immune gamma (γ) globulin (IγG) to K562 cells was determined on cells from s.c. KG-1, KG-1a, and HL-60 tumors cultured for 4 days. In addition, sections of tumors were fixed in 10% formaldehyde in methanol (to inhibit endogenous peroxidase activity) containing 0.3% hydrogen peroxide to ascertain immunoperoxidase activity. The IγG was absorbed with nude mouse lymphohematopoietic cells.

**Hematologic Studies**

Total and differential mouse peripheral leukocytic counts were determined with a Particle Data Counter. Differential bone marrow cell counts were made on smears stained by the May-Grünwald-Giemsa technique. These studies were aimed at determining a possible involvement of the hematopoietic tissues of the host by the leukemia cells.

**RESULTS**

**General Findings**

A lag phase occurred immediately after s.c. inoculation of the human myeloid leukemia cells into newborn nude mice and was followed by the appearance of a local nodule that increased in volume during the ensuing days (Fig. 1). The KG-1 and KG-1a leukemia cell lines developed s.c. myelosarcomas in 31 of 39 and 34 of 35 newborn mice injected, respectively (Table 1). The HL-60 cells proliferated in only two of the injected newborn mice, but these two developed multiple s.c. myelosarcomas rather than a single tumor. In a number of mice (see Table 1), the growth of KG-1, KG-1a and HL-60 s.c. myelosarcomas was associated with the development of similar neoplastic nodules in lungs and kidneys. The visceral nodules found 20 days after inoculation of the cells were not numerous (usually 3–5 myelosarcomas in each lung and 2–3 in each kidney); however, these nodules grew steadily and, 50–70 days after transplantation, appeared as large confluent neoplastic masses. This pattern of disseminated neoplastic growth was consistently observed only after transplantation of cell lines into newborn nude mice. KG-1 and HL-60 myelosarcomas passed to adult nude mice yielded s.c. tumors that did not disseminate. The percentage of myelosarcomas produced in adult mice, as compared to the first inoculations in newborns, decreased for KG-1 cells but dramatically increased for HL-60 cells (Table 2). On the contrary, KG-1a myelosarcomas did not proliferate after transplantation into adult mice and, therefore, were passaged through newborn nude mice.

**Electron Microscopy of Cultured and Transplanted Cells**

**KG-1 cell line.** Ultrastructural features of cultured KG-1 cells are similar to atypical promyelocytes (Fig. 2). Round, indented, or occasionally, lobulated nuclei contained abundant euchromatin and large, often marginated, nucleoli. Cytoplasmic features included a well developed Golgi and considerable rough endoplasmic reticulum (RER), often in lamellar arrays. Mitochondria had a round shape with irregular disposition of the cristae.

![Graph](https://www.bloodjournal.org/content/1016.2007129187/Fig.1) Growth rates of s.c. myelosarcomas formed by human leukemic cells in nude mice. (A) First inoculation. (B) Third mouse-to-mouse passage.
Granules were generally round and electron-dense; however, a small number were less dense and had a particulate or honeycomb configuration. Smooth, membrane-bound multivesicular bodies (MVB) were numerous and sometimes contained electron-dense material. Fusion or contact between MVB or MVB and granules was observed. The electron-dense and some less dense granules contained MPO and/or acid phosphatase (Fig. 2, insets B and C), but MVB did not demonstrate either enzyme (Table 3).

The KG-1 cells from s.c. myelosarcomas in nude mice maintained the general morphological characteristics seen in cultured cells, with some exceptions. Frequent whorl-like arrangements of fibrils and/or membrane cisternae with numerous attached and free ribosome-like bodies were observed (Fig. 3). Cytoplasmic granules in vivo were more diverse than in cultured cells. Most were round and electron-dense, some were elongated, and a few were large basophilic-type granules. Occasionally, several electron-dense granules appeared within large autophagic vacuoles, possibly representing early stages of Auer rod formation. KG-1 cells in vivo contained fewer MVB than cultured cells. MPO reaction was strongly positive in the electron-dense granules and revealed the presence of nonreactive cores that were not easily detected in routinely stained sections (Fig. 3, inset B). Large granules with light matrix had either a slightly positive or a negative MPO reaction (Fig. 3, inset C). Acid phosphatase reaction was negative in all granules, as well as in the MVB. MPO reaction was also negative in the MVB.

**KG-1a cell line.** KG-1a cells proliferating in vitro were morphologically undifferentiated (Fig. 4) with large, round nuclei containing mainly euchromatin and large nucleoli. The cytoplasm was poorly developed, containing round or elongated mitochondria with electron-dense matrixes and irregular cristae, sparse short strands of RER, and an undeveloped Golgi apparatus. MVB containing numerous small vesicles and, in some instances, minute amounts of a dense amorphous material, were observed in the cytoplasm of KG-1a cells in culture (Fig. 4, inset B). MVB in KG-1a cells in vitro were less numerous than in the cells of the parent line KG-1 maintained in similar conditions. Granules were not observed in the cytoplasm of cultured KG-1a cells, and reactions for MPO and acid phosphatase were consistently negative.

KG-1a cells from s.c. myelosarcomas in mice contained undifferentiated nuclei similar to those seen in vitro. The cytoplasm, however, showed definite signs of differentiation. The RER was more developed with rigid and irregularly dilated cisternae (Fig. 5). MVB were also present in these cells (Fig. 5 inset B). A definitive diagnostic feature of differentiation in KG-1a cells was the presence of homogeneous, small, round, electron-dense cytoplasmic granules, appearing to be similar to a variant of primary promyelocytic granules. These granules were MPO-positive, revealing the myeloid origin of the cells (Fig. 5, inset C), and were acid phosphatase-negative. The MVB were negative for both enzymes.

**HL-60 cell line.** Cultured HL-60 cells had round, indented, or occasionally segmented nuclei containing mainly euchromatin and one or two large nucleoli (Fig. 6A). Narrow RER cisternae, containing scarce particulate material, were numerous in the cytoplasm, along with many free ribosomes. Large numbers of granules were detected, some of which displayed small, electron-dense cores, whereas others had a diffusely light or dense matrix.

The MPO reaction demonstrated enzymatic activity in the granules, in the RER cisternae, and in the nuclear envelope (Fig. 6A, inset). A few small, granulocytic-type cells with dense multilobulated nuclei and heterogeneous cytoplasmic granules were observed in vitro (Fig. 6B).

HL-60 cells forming myelosarcomas showed morphological features of both myeloblasts and promyelocytes (Fig. 7). The RER cisternae were markedly dilated by a light particulate material (Fig. 7, inset B), whereas numerous cytoplasmic granules with an electron-dense central area surrounded by a clear halo and a single membrane were seen. Other larger granules

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**Table 1. Incidence and Distribution of Tumors Formed by Human Myeloid Leukemia Cells Inoculated Into Newborn Nude Mice**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Nature</th>
<th>Number of Mice Inoculated</th>
<th>Number of Mice With Sarcomas in s.c. Tissue</th>
<th>Lungs</th>
<th>Kidneys</th>
</tr>
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<tbody>
<tr>
<td>KG-1</td>
<td>Myelogenous (AML)</td>
<td>39</td>
<td>31</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>KG-1a</td>
<td>Myelogenous (AML)</td>
<td>35</td>
<td>34</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>HL-60</td>
<td>Promyelocytic (APML)</td>
<td>21</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

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**Table 2. Incidence of Takes of s.c. Human Myelosarcomas in Nude Mice Through Serial Passages**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG-1</td>
<td>31(29)*</td>
<td>37(23)</td>
<td>31(10)</td>
<td>15(7)</td>
<td>4(3)</td>
</tr>
<tr>
<td>KG-1a</td>
<td>31(30)</td>
<td>15(5)</td>
<td>10(5)</td>
<td>5(5)</td>
<td>5(3)</td>
</tr>
<tr>
<td>HL-60</td>
<td>16(16)</td>
<td>14(13)</td>
<td>14(14)</td>
<td>10(9)</td>
<td>10(10)</td>
</tr>
</tbody>
</table>

*KG-1 and HL-60 myelosarcomas were developed in newborns and transplanted into adult nude mice.

*KG-1a myelosarcomas were developed and transplanted only into newborn nude mice.

Mouse-to-mouse passages were performed 30 days apart.

*The first figure corresponds to number of mice transplanted and the second indicates number of takes.
were homogeneously dense or light. The MPO reaction demonstrated the presence of enzyme only in the granules (Fig. 7, inset C).

Nuclear-Cytoplasmic Asynchrony

The three cell lines showed no correspondence between nuclear and cytoplasmic differentiation. KG-1 cells in culture had immature nuclei associated with the production of MPO-positive and acid phosphatase-positive cytoplasmic granules, whereas granules of cells proliferating in the mice contained only MPO. Although this enzymatic change may reflect a certain degree of granular evolution in vivo, the nuclei remained immature.

Asynchrony was particularly marked in KG-1a cells proliferating in the mice; the nuclei had immature features, as those observed in vitro, but the cells developed cytoplasmic granules and synthesized MPO, both of which were consistently absent in cultured cells.

HL-60 cells displayed asynchrony in the cytoplasm itself. Thus, while there were no nuclear changes, the cells contained either numerous free ribosomes in vitro or dilated RER in vivo. These structural characteristics are commonly found in myeloblasts; however, under both experimental conditions, HL-60 cells had active granule formation and synthesis of MPO, typical of the promyelocytic stage.

Human Characteristics of Transplanted Cells

All myeloid cells retained the original human characteristics observed in vitro through transplantation and mouse-to-mouse passages. This was demonstrated by the permanence of the human karyotype in cells obtained from multiple passages. Comparative karyotypic studies were not made, as the purpose of these tests was to assess the permanency of the human chromosomal pattern observed in the cell lines in vitro. Also, cells from the myelosarcomas that developed in mice maintained the antigenic determinants and cross-

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Table 3. Summary of Ultrastructural Characteristics of KG-1, KG-1a, and HL-60 Cells In Vitro and In Vivo

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Granules</th>
<th>Granular Enzymes</th>
<th>Perinuclear RER Enzymes</th>
<th>MVB</th>
<th>Whorl-Like RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MPO</td>
<td>MVB</td>
</tr>
<tr>
<td>In vivo</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>AcP</td>
<td></td>
</tr>
<tr>
<td>KG-1a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>MPO</td>
<td>MVB</td>
</tr>
<tr>
<td>In vivo</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>AcP</td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>MPO</td>
<td>MVB</td>
</tr>
<tr>
<td>In vivo</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>AcP</td>
<td></td>
</tr>
</tbody>
</table>
reacted with an antibody to myeloid K562 cells. Tissue sections processed by the immunoperoxidase technique gave a strong brown-orange reaction when stained with benzidine, indicating that the transplanted cells had maintained this antigen.

**Hematologic Findings**

Atypical cells, occasionally found in smears of the peripheral blood of the mice after s.c. transplantation of KG-1, KG-1a, and HL-60 cells, were an expression of the release and dissemination of neoplastic cells from the tumors developed in the s.c. and visceral tissues. However, the total and percent numbers of normal mouse blood cells remained unchanged. The cellular composition of bone marrow imprints from tumor-bearing mice was also normal.

**DISCUSSION**

The results of these studies demonstrate that human myeloid leukemia cells have tumorigenic properties in vivo. Thus, KG-1, KG-1a, and HL-60 human leukemia
cells inoculated into newborn nude mice yielded local s.c. and disseminated tumors without involvement of the hematopoietic tissues of the hosts. The myelogenous leukemic cells proliferating in mice preserved the properties defined in vitro, such as their basic morphological features, the human karyotype, and the ability to cross-react with an antibody against K562 cells.

The ultrastructural and ultracytochemical comparative studies demonstrated nuclear-cytoplasmic asynchrony in the human myelogenous leukemic cells proliferating both in vitro and in vivo; however, some signs of differentiation were observed in the cells composing myelosarcomas in mice. Thus, the granules of myeloid KG-1 cells in vivo were more homogeneous than in vitro and maintained a strong MPO reaction, whereas the acid phosphatase content demonstrated in cultured cells was absent. In general, acid phosphatase is observed in granules of myeloid cells at earlier stages of differentiation. The KG-1 cells in the myelosarcomas also showed elongated bodies comparable to atypical Auer rods and fibrillar and ribosome-like arrange-
DIFFERENTIATION OF HUMAN LEUKEMIC CELLS

Fig. 7. (A) HL-60 cells in vivo with numerous cytoplasmic granules and dilated RER (x10,500). (B) Highly developed Golgi apparatus, dilated RER, and large, dense granules indicative of differentiated cytoplasm of many HL-60 cells in vivo (x18,750). (C) Cytoplasmic granules of HL-60 cells in vivo with a strong MPO reaction (x12,000).

ments, similar to the cytoplasmic inclusions seen in human acute granulocytic leukemia (AGL). The nature of the MVB found in KG-1 cells (and KG-1a cells as well) is still unknown; however, since the acid phosphatase reaction was consistently negative, the MVB do not appear to be derived from lysosomes. MVB in hematopoietic cells may be associated with Golgi complex and, presumably, may be involved in secretory mechanisms.

Promyelocytic HL-60 cells forming myelosarcomas demonstrated a strongly positive MPO reaction that was restricted to the granules; in cultured cells, however, the enzymatic reaction had a less mature pattern, involving the nuclear envelope and RER cisternae as well as the granules. Also, HL-60 myelosarcoma cells contained dilated RER cisternae filled with protein-like material, which indicated that secretion took place in vivo, whereas cultured cells had morphological features similar to those originally described for this cell line and displayed flat RER cisternae.

It is possible that some of the subtle changes observed in KG-1 and HL-60 cells could be the result of an in vivo selection of subpopulations of the transplanted cells. However, cytoplasmic changes were consistently observed in repeated experiments, indicating that, if selection occurred, it was deterministic and not random.

Among all the cell lines tested, the most significant changes were observed in the KG-1a cell subline. The KG-1a cells in vitro displayed a blastic morphology with scarce organelles and consistently lacked cytoplasmic granules. In contrast, KG-1a cells forming myelosarcomas in mice had strands of moderately dilated SER and synthesized peroxidase, which appeared packaged into specific cytoplasmic granules. Thus, KG-1a cells evolved from an agranular, blastic stage in vitro to cells containing morphological and enzymatic markers proper to differentiation along the myeloid series. Such changes as took place during the spontaneous proliferation of the KG-1a cells in the tissues of the animal hosts were not attained in vitro, even after inductive treatment with human colony-stimulating factor (CSF) or phorbol diesters, as previously reported.

Nuclear and cytoplasmic alterations in HL-60 cells have been induced in vitro by the addition of dimethyl sulfoxide (DMSO) and phorbol diester to the culture medium. Also, colony formation of KG-1 cells in semisolid cultured medium was stimulated by CSF, whereas KG-1a cells showed little or no response to in vitro treatment with CSF. Despite the different properties shown in vitro, the three cell lines consistently exhibited cytoplasmic changes after transplantation into nude mice. It appears that the host environment was not only favorable for preserving the basic features of the human myeloid cells, but also induced alterations consistent with some degree of maturation.

Mouse tissues are known to be capable of synthesizing natural biologic factors that affect differentiation of mouse leukemic cells in culture. Those factors are T lymphocyte-independent; thus, the athymic condition of the host would not impair their production.

These studies show that human leukemic cells can proliferate in nude mice while undergoing changes compatible with differentiation. Persistent tumorigenicity may be a manifestation of the heterogeneous
characteristics of the population of leukemic cells. Thus, not all cells of a given culture aliquot or tumor sample are identical in their degree of differentiation. Furthermore, terminal stages of cell maturation were not observed in the myelosarcomas. It will be of importance to determine whether the induction of further cell differentiation by biologic and/or chemical agents effectively modifies neoplastic growth.

As we have reported elsewhere,21,26 a possible advantage of the human myeloid leukemic cells—mouse models is their suitability for in vivo therapeutic tests. In addition, the disseminated growth pattern of the KG-1, KG-1a, and HL-60 myelosarcomas will make it possible to investigate whether different tissue microenvironments may influence the characteristics and proliferation of the human malignant myeloid cells in mice.

REFERENCES

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