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Treatment of a Fatal Transplantable Erythroleukemia by Procedures That Lower Endogenous Erythropoietin

Azhar Hossain, Jung-Kon Kim, and W. David Hankins

Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205

The in vitro growth of primary erythroleukemia cells has been examined in the presence and absence of the hormone erythropoietin (EPO). Although these leukemic cells had previously been considered to be hormone-independent, addition of EPO was found to be essential for maximum growth in culture. Erythroid colonies that grew in the presence of EPO were leukemogenic when returned to mice. Influence of EPO on the in vivo growth of leukemic cells was indicated by our findings that (1) administration of the hormone caused a more severe leukemia and rapid death, and (2) transfusion of red blood cells, which lowers endogenous EPO, led to decreased spleen size and increased survival of leukemic mice. We suggest from our results that hormone-associated therapy might be efficacious in the treatment of this and, perhaps, other leukemias.

Key words: leukemia, antihormone therapy, hormone-associated therapy, erythropoietin

More than eight decades ago, Beatson noted that oophorectomy had beneficial effects for some women with breast cancer [1]. This was one of the first suggestions that some tumors are dependent on hormones for their growth. Subsequent studies demonstrated that approximately one-third of breast cancer patients showed a marked improvement upon administration of compounds that were antagonistic to or reduced the effectiveness of estrogen. In addition, the development of animal models for the induction, analysis, and treatment of mammary and prostatic cancers [2] led to successful antisteroid treatment of prostatic cancers. Yet, despite the initial excitement generated by these discoveries, hormone-associated therapy is today rarely considered as a primary treatment of other solid tumors or leukemias. Factors that may have contributed to the lack of widespread application of antihormone therapy include 1) the unequivocal success of chemotherapy, radiotherapy, and surgery in a few specific cancers, 2) lack of demonstration of efficacy of antihormone therapy in other animal cancers, and 3) a general opinion in the medical community that most tumors are autonomous and hormone-independent.

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Our laboratory has studied growth, differentiation, and hormone sensitivity of virus-transformed hemopoietic cells over the past several years. Unexpected observations, made during these studies, led us to propose a model of carcinogenesis that, if accurate, has conceptual and practical implications for the diagnosis and therapy of human cancer. Briefly, this model [3,4] holds that transforming events leading to cancer can provide a heritable growth/survival advantage to tumor cells without blocking their ability to differentiate or altering other fundamental cellular properties. The model also suggests that, excepting a growth advantage, the properties of tumors are just reflections of normal cell properties at or subsequent to the developmental stage at which the oncogenic transformation occurred. One prediction of this model is that most, if not all, tumor populations retain sensitivity to, and a requirement for, natural, physiologic growth factors for their survival and/or proliferation.

As one test of this prediction, we have assessed the erythropoietin (EPO) sensitivity of highly tumorigenic erythroleukemia cells. This report presents our preliminary results, which indicate that the leukemic erythroid progenitors do require EPO for growth in vitro and that survival of leukemic mice can be markedly extended by hormone-associated therapy in vivo.

MATERIALS AND METHODS

Derivation of Transplantable Erythroleukemia Cell Lines

Erythroleukemia cell lines were derived by the procedure of Oliff et al [5]. A total of eight transplantable lines were derived from eight individual leukemic mice. These lines have been passaged, approximately once each month, for the past 2 yr. At each passage, a single cell suspension was prepared from one leukemic spleen. Of this suspension, 1 million cells were inoculated intravenously into ten adult (6–8 wk old) mice. Within 2–4 wk, a fatal leukemia developed in the recipients. Chromosomal marker studies of Oliff et al [5] have indicated that transplantable erythroleukemia lines, derived by similar methodology, were clearly of donor origin.

In Vitro Culture of Erythroleukemia Cells

At random passages in vivo, the enlarged spleens containing leukemic cells were removed and single cell suspensions prepared. The cells were incubated in Iscove's modified Dulbecco's MEM containing 2% methylcellulose and 30% fetal calf serum and 0.021 mM β-mercaptoethanol, as described by Mager et al [6]. EPO, where indicated, was added to give a final concentration of 0.3 U/ml. Three different preparations of EPO were used in this study: sheep plasma EPO from Connaught Laboratories (Toronto, Ontario, Canada); human urinary EPO from the National Heart, Blood, and Lung Institute (Bethesda, MD); and murine EPO produced by murine erythroleukemia cells as previously described [7]. Methylcellulose cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Cultures were monitored on an inverted scope at a magnification of $\times$40.

Immunofluorescence

Spectrin immunofluorescence assays were performed as previously described [8]. Colonies picked from culture dishes were suspended in phosphate-buffered saline containing 20% fetal calf serum and spun onto glass slides using a Shandon cytocentrifuge. The antisera against spectrin was a generous gift from Dr. John Portis (Rocky

Mountain Laboratories, Hamilton, MT). The ability of this antiserum to recognize spectrin was confirmed by immunoprecipitation and PAGE, as described by Koury et al [9].

Hypertransfusion

Transfusion of mice was as described by Oliff et al [5]. Briefly, each transfused mouse received weekly intraperitoneal injections (1 ml) of a suspension prepared to contain 80% packed red blood cells from syngeneic exbreeder mice.

RESULTS

Stimulation of Erythroleukemia Cell Growth In Vitro by EPO

To test whether erythroleukemia cells retained erythropoietin sensitivity, we cultured primary spleen cells from leukemic mice with and without the hormone. It should be noted that, under the culture conditions employed herein, virtually no erythroid cell growth is observed in cultures of normal spleen cells even in the presence of EPO. As for the leukemic spleen, very few cells were observed without EPO, whereas in the presence of EPO numerous large, tightly packed colonies appeared. Although some small colonies were occasionally present in cultures without added EPO, Figure 1 is representative of the hormone's effects on leukemia cell proliferation in vitro. Since the EPO preparation employed for Figure 1 was quite impure (specific activity 15 U/mg), we cannot be sure that the stimulatory agent is authentic EPO. However, similar results were obtained when we used 0.3 U/ml of partially purified human urinary EPO or partially purified murine EPO produced by erythroleukemia cells [7]. Nonetheless, it is still possible that the stimulatory effects are due to a contaminant in the EPO preparation.

To optimize the conditions for culture of these cells, a dose-response experiment was performed; the results are shown in Figure 2. Spleen cells were cultured in the methylcellulose colony assay as described for Figure 1. Although the number of colonies definitely increased with increasing EPO, precise quantitation was difficult owing to the fact that the colonies were suspended at different viewing levels throughout the methylcellulose. Therefore, total nucleated cell counts were determined following recovery of the cells from methylcellulose by centrifugation. A plateau was observed at ~ 0.1–0.3 U/ml. Consequently, an EPO concentration of 0.3 U/ml was chosen for subsequent experiments.

Characterization of Leukemic Cells That Grew in the Presence of EPO in Culture

The colonies that developed in the EPO-treated cultures were removed with a micropipette, pooled, and centrifuged onto glass slides using a cytocentrifuge. Slides were stained with Wright stain or benzidine stain or were assayed for the presence of spectrin by indirect immunofluorescence (Table I). The vast majority of the cultured cells exhibited blastic morphology resembling proerythroblasts and were positive for spectrin. Nevertheless, very little evidence of terminal differentiation was observed; virtually all cells grown in the presence of EPO were benzidine-negative.

Next, EPO-induced colonies were pooled and inoculated into adult syngeneic mice, and the recipients were monitored for 3 mo. Adults were chosen because newborn mice are sensitive to induction of erythroleukemia by virus that may be

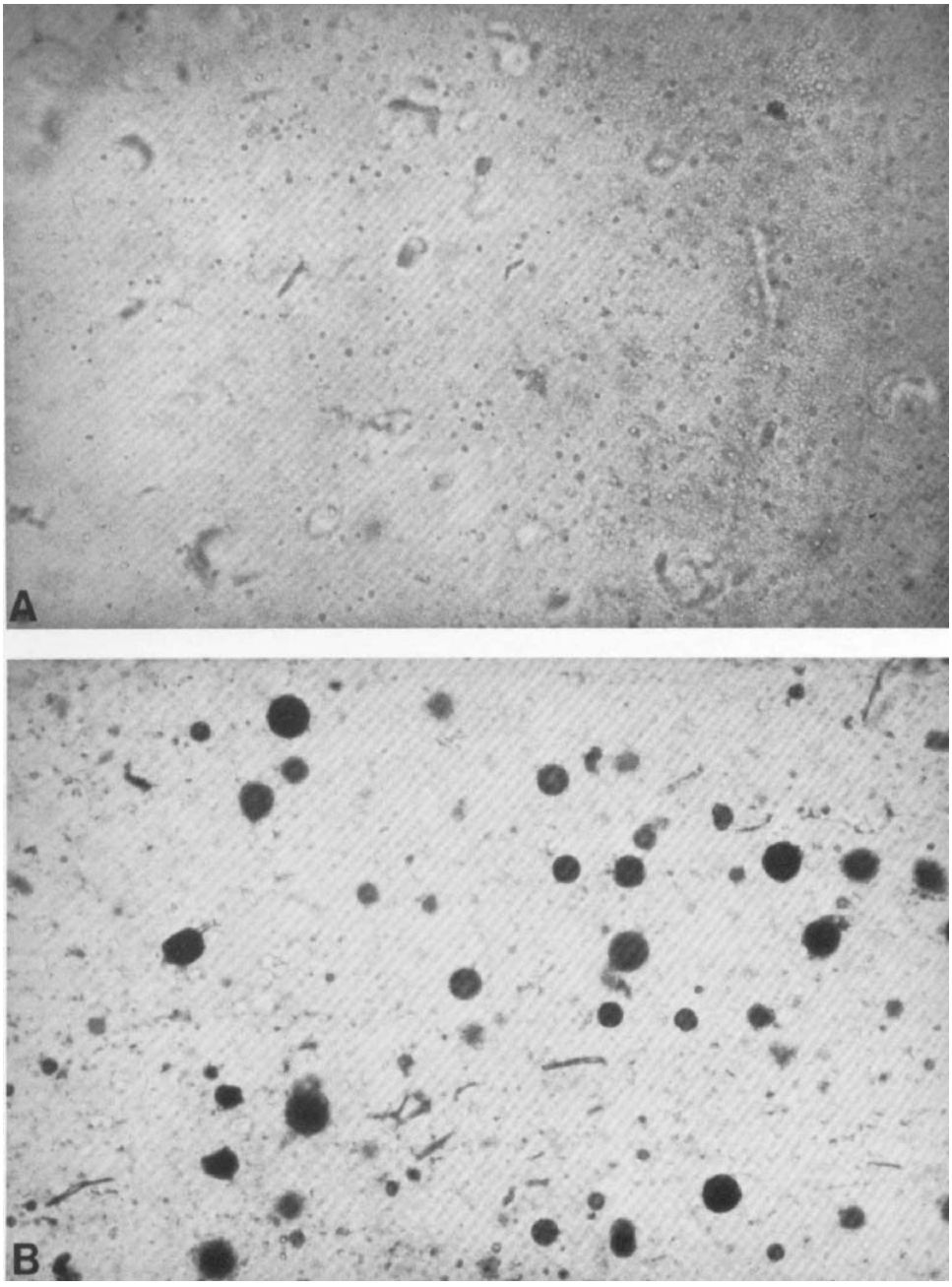

Fig. 1. Effect of EPO on leukemia cell growth in vitro. Spleen cells from passage 5 of transplantable erythroleukemia line 19 were seeded at a concentration of 1 million cells/ml in methylcellulose as described in Materials and Methods. Connaught step III erythropoietin was added to half the cultures (B) and the remainder (A) received an equivalent amount of Hanks' balanced salt solution. Photographs were made in situ using a Leitz inverted microscope at a magnification of ×25.

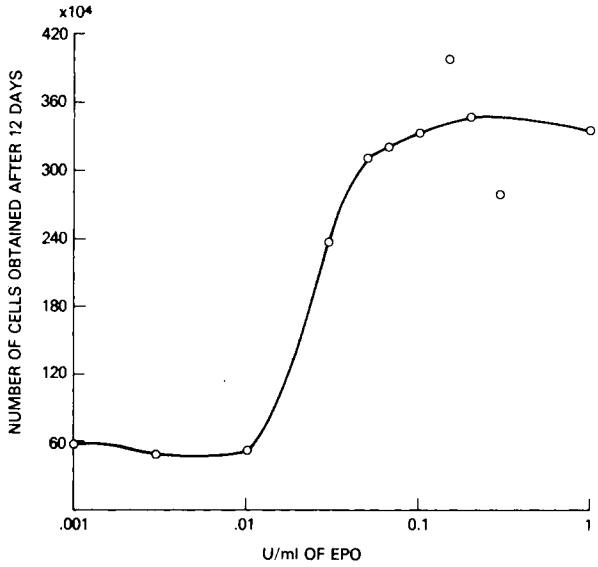

Fig. 2. Effect of different doses of EPO on leukemia cell growth in culture. Cultures were prepared as in Figure 1 except that the final concentration of EPO was varied as indicated. On day 12, cells were collected by centrifugation, and the number of nucleated cells per plate was determined.

TABLE I. Characteristics of Cells That Grew in Culture in the Presence of Erythropoietin

Line	Morphology	Hemoglobin-positive	Spectrin-positive	Tumorigenic deaths/recipients
EL-16	Erythroid	No	Yes	10/10
EL-17	Erythroid	No	Yes	10/10
EL-18	Erythroid	No	Yes	8/8
EL-19	Erythroid	No	Yes	8/8
EL-20	Erythroid	No	Yes	10/10

produced by the leukemic cells. In control experiments, 15 daily injections of conditioned medium from erythroleukemia cultures did not induce erythroleukemia in adult mice within 3 mo. In contrast, inoculation of pooled colonies from the EPO-treated cultures produced a fatal leukemia within 3 months after inoculation. This tumorigenicity was a characteristic of all the colonies tested (Table I). These results indicate that the cells stimulated to proliferate and form colonies were erythroid and demonstrate that the EPO-stimulated erythroblasts were malignant in that they induced a fatal leukemia.

In Vivo Influence of EPO on Erythroleukemia Development

The apparent requirement for EPO for in vitro growth of the erythroleukemia cells led us to test the possibility that tumor growth in vivo is also modulated by EPO (Fig. 3). Tumor cells were inoculated and recipient mice were hypertransfused at weekly intervals throughout the course of the leukemia development. As can be seen from a comparison of groups A and B, the hypertransfusion led to a significant extension ($P = 0.001$) in the survival times of the leukemic mice. Thus mice that

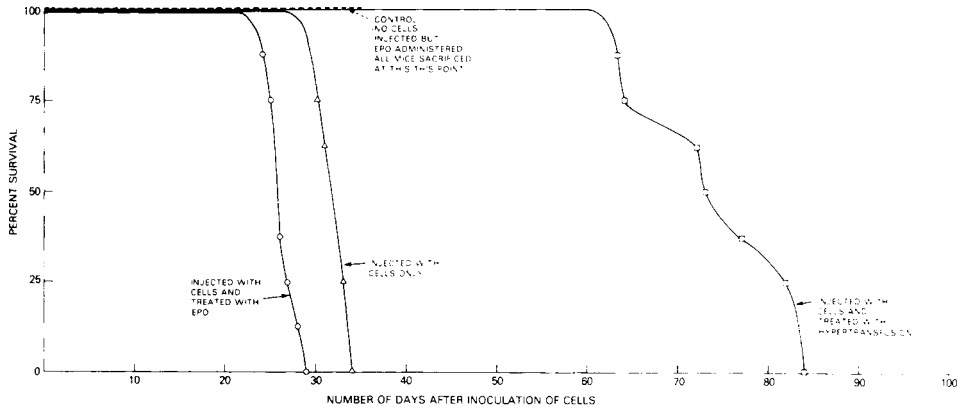

Fig. 3. Effect of hypertransfusion or EPO administration on the survival of mice with erythroleukemia. One million cells from transplantable erythroleukemia line EL-19 were injected intravenously into each 50 adult mice. The mice were divided into four groups. Group A (△) received no further treatment. Group B (□) received weekly transfusions of 1 ml per mouse of reconstituted blood containing 80% red blood cells. Group C (○) was transfused as B, but each mouse also received daily intraperitoneal injections of 0.2 unit of Connaught step III erythropoietin. All mice were monitored and the spleens palpated each day. Group D (---) was a control for group C. These mice received daily injections of EPO but had not been inoculated with leukemic cells. None of the mice in group D died during the monitoring period.

were hypertransfused lived two to three times longer than those that did not receive red blood cell transfusions.

The increased survival times were most likely mediated through a reduction of endogenous EPO as a result of the transfusion. However, since leukemic mice were severely anemic, we considered the possibility that hypertransfusion had simply corrected the anemia and thereby increased survival. Two experimental findings argue against this contention. First, palpation of recipient mice 25 days after inoculation revealed that spleens from transfused mice were considerably smaller than those of untreated mice. This provides circumstantial evidence that the leukemic cells grow more slowly in the transfused mice. An illustration of this difference in spleen is shown in Figure 4. Second, simultaneous administration of EPO plus hypertransfusion led to more rapid leukemia and survival times similar to those of untreated recipients (groups B and C were significantly different at $P = 0.001$). That is, the transfusions were of little survival value when mice were simultaneously administered exogenous EPO.

DISCUSSION

In the present study, we investigated the effects of a hormone, erythropoietin, on transformed erythroid cells that produce a rapid and fatal leukemia when inoculated into syngeneic mice. We found that addition of EPO to cultures of the leukemic cells produced dramatic effects on their proliferation. In fact, little if any growth occurred in the absence of added hormone. This observation of EPO sensitivity in vitro led us to examine the influence of EPO on leukemia cell growth in vivo. Considerable

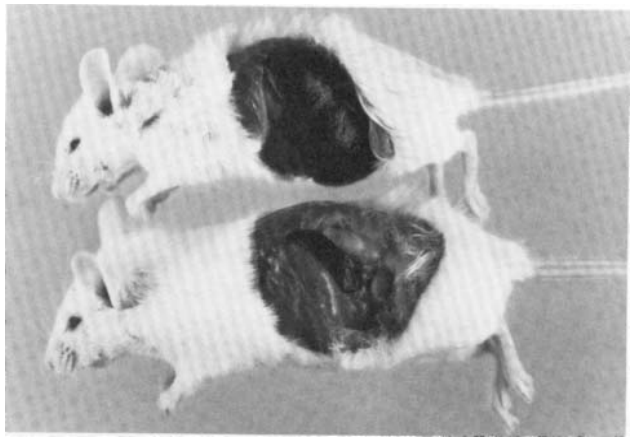

Fig. 4. Effect of transfusion of spleen size in erythroleukemic mice. On day 25 of the experiment described in the legend to Figure 3, one mouse from group A (top, untreated) and one from group B (bottom, hypertransfused) were sacrificed, and the spleens and livers were exposed and photographed.

evidence was obtained that EPO dramatically stimulated the proliferation or viability of erythroleukemia cells in the animal as well as in culture.

Such growth-promoting effects of EPO on leukemic cells are interesting for three reasons. First, EPO is a hormone usually associated with an induction of terminal erythroid differentiation. Although EPO in this study caused extensive proliferation, the stimulated erythroid cells did not exhibit hemoglobin synthesis or nuclear condensation, which are characteristic of terminal red blood cell development. Although these results do not argue against a differentiative role for EPO, they do emphasize a possible role for the hormone in stimulating proliferation, and perhaps ensuring viability, of erythroid progenitors.

Second, these results provide an example of erythroleukemia cells that have become tumorigenic without losing their sensitivity to a growth factor that presumably regulates the growth of the antecedent normal erythroid progenitors. Several investigators have suggested that tumor cells lose their requirement for natural growth factors. Shrader and Crapper [10], for example, recently associated the abrogation of requirement for exogenous lymphoid growth factors with a truly malignant phenotype. In earlier reports [3,4], we proposed a model of carcinogenesis that suggests that normal cells can become transformed by acquisition of a heritable growth advantage without an arrest in differentiation or abrogation of growth factor requirements. Most of the evidence cited in support of the model was derived from observations that hemopoietic cells, transformed in vitro or in vivo by any of a number of retroviruses, were able to continue terminal maturation and retained sensitivity to physiologic hormones [11,12]. However, in that these primary transformants did not

induce tumors upon inoculation into mice, it could be argued that they were not truly malignant cells but had only received a growth stimulation. In the present study, however, the erythroleukemia cells stimulated to proliferate in vitro induced a fatal leukemia in 100% of the mice receiving these cells. Therefore, the present experiments provide additional support for the notion that truly malignant cells retain sensitivity to physiologic growth factors.

Third, the present experiments suggest that at least one type of murine leukemia is treatable by procedures that lower an endogenous hormone. It should be noted that our encouraging results were obtained under experimental conditions (eg, number of cells inoculated, transfusion schedule, etc) that were arbitrarily chosen. It can be anticipated that even longer survival times could be achieved by optimization of the treatment regimen. Our current extension of these studies includes preparation of hormone-related therapeutic reagents such as 1) neutralizing monoclonal antibodies against EPO and 2) competitive analogs of EPO.

The experiments in this study were designed to test the hypothesis [3,4] that apparently autonomous leukemic cells retain hormone sensitivity and therefore respond to antihormone therapy. As an extension of this hypothesis, it is possible that other mouse and human leukemias also retain sensitivity to physiologic growth factors and are treatable on that basis. Furthermore, in addition to breast and prostatic cancers, solid tumors of the lung, colon, liver, brain, and other tissues may retain sensitivity to external growth factors. Accordingly, experiments are in progress in our laboratory to assess the hormone sensitivities of such tumors in primary cultures. In this regard, it is noteworthy that bombesin, a newly identified growth factor, not only stimulated normal human epithelial cells [3] but also increased the colony-forming efficiency of cells derived from small cell carcinomas of the lung [4].

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