ABSTRACT

The effects of L-ascorbic acid (LAA) on the in vitro growth of human leukemia colony-forming cells (L-CFC) were analyzed for all acute nonlymphocytic leukemia patients from whom bone marrow aspirates were received by this laboratory for cell culture study. Among 259 cases, 163 could be directly evaluated for LAA effect. L-CFC growth enhancement was noted in 53 (33%) and suppression in 28 (17%), with overall 50% of patients affected by LAA. Among 34 normal bone marrows tested, none were enhanced by LAA while 8 (24%) were suppressed. While caution is needed in interpreting L-CFC suppression by LAA, L-CFC enhancement is clearly significant. Two isomers of LAA, D-isoascorbic acid and D-ascorbic acid, which have weaker anti-scorbutic activity than that of LAA, also produced the L-CFC growth-enhancing effect, but to a lesser degree than that of LAA. A dose-response study also substantiated that D-ascorbic acid was definitely less effective than was LAA. Since D-ascorbic acid is the true optical isomer of LAA and has identical physicochemical properties as does LAA, this differential effect is clearly of biological nature. This study indicates that L-CFC growth suppression by LAA is observed in one-sixth of leukemic patients, L-CFC enhancement in one-third of patients, and that L-CFC growth enhancement is a clearly significant finding with a biological mechanism as the basis.

INTRODUCTION

The growth-modulating effects of LAA on human L-CFC have been reported from this laboratory on small numbers of patients (27, 30). The growth of leukemic cells is enhanced by LAA in one population of patients (27), and suppressed in another population (30). An interim analysis based on 97 patients tested for LAA effect confirmed this effect of LAA (26). A larger series of patients with acute nonlymphocytic leukemia has now been analyzed for this effect. In addition, normal bone marrow controls were analyzed in comparison for the significance of this effect on leukemia. Further, 2 isomers of LAA, DIAA and DAA, were tested in comparison with LAA in 8 patients, where LAA enhanced growth of leukemic cells. Both isomers have anti-scorbutic activity, but weaker than that of LAA (9, 12, 46). DAA in particular is the true optical mirror image of LAA with identical physicochemical properties as LAA, except for the light-rotating effect (43). Therefore, the distinction between biological versus physicochemical mechanism should be possible by the use of these isomers.

MATERIALS AND METHODS

Cells. All bone marrow aspirates from patients with acute nonlymphocytic leukemia received by this laboratory since 1976 were the subjects of this study. Two patients included only in the study of LAA isomers had blast cell proportion less than 30%, and should be classified as myelodysplastic syndrome (4). Normal marrows used for controls were obtained from hematologically normal patients with solid tumors undergoing bone marrow aspiration as a part of staging workup. No patient had received prior chemotherapy at the time of bone marrow aspiration for this study. Consent was obtained from all patients as designed and approved by the University of Kansas Human Subject Committee.

Chemicals. LAA, DIAA, and glutathione (reduced form) were purchased from Sigma Chemical Co., St. Louis, MO. A sample of DAA was obtained from Hoffmann-La Roche, Inc., Nutley, NJ (R021-5159, Lot 4182-121-15), and another sample from Dr. Seib of Kansas State University, Manhattan, KS. Both gave similar results in cell culture. Stock solutions of these chemicals were prepared by dissolving in distilled water. These were then filtered through Milipore membranes (Millipore-FG, 0.22 μm) and stored frozen (−20°C) in small aliquots until used.

Cell Culture Assay. A detailed description of the cell culture method used in this study was reported previously (33). Briefly, the cell culture system consisted of 2 layers of 0.3% agar in a 35-mm plastic Petri dish perforated at the bottom by 5 small holes. Cells obtained by bone marrow aspiration were incorporated into the top agar layer. Both layers contained a growth medium consisting of 70% Alpha medium free of LAA (Grand Island Biological Co., Grand Island, NY), 15% fetal calf serum (Flow Laboratories, Rockville, MD) and 15% leukocyte-conditioned medium. The latter was prepared by incubation of normal human peripheral leukocytes with phytotrehammagglutinin (Wellcome Research Laboratories, Beckenham, England). Cultures were incubated for 2 weeks or longer at 37°C in an atmosphere continuously flushed with 7% CO2. Throughout the incubation period, each culture dish was removed from the incubator once daily and fed from the top with 0.5 ml of growth medium, with or without one of the ascorbic acid isomers, either LAA, DIAA, or DAA. LAA is known to have extremely short half-life in culture (14, 29) and therefore ascorbic acid isomers were added to culture every day with daily feeding when these were under study. Unless otherwise specified, glutathione was also added at 0.3 μM whenever an ascorbic acid isomer was added. Glutathione was shown to potentiate the effect of LAA, but was ineffective by itself (27, 30, 32). The fed medium diffused through the agar layers and drained out of the dish through the holes on the bottom. Colonies of 50 or more cells were counted, usually at 2 to 3 weeks of culture using an inverted microscope, but the culture period can be extended for long-term observation. It has been previously substantiated that colonies grown in this culture system from bone marrow of leukemic patients are leukemic in origin (25, 27, 30, 33), while normal bone marrow grows normal myeloid colonies, GM-CFC (21). The standard cell number plated per dish was 5 x 10⁵, but often this was appropriately reduced in patients with high plating efficiencies.

Experimental Design. Whenever an LAA isomer was tested, 8 or 10 dishes of cultures were set up with a bone marrow aspirate under study. These were randomly divided into 2 groups of 4 or 5 dishes, to eliminate possible introduction of bias. Both groups received daily feeding of the same growth medium, but one group had LAA added to this medium and the other did not. Student's t test was used for testing significant differences between 2 groups of cultures.
RESULTS

The total number of leukemic cases for which bone marrows were received for cell culture studies is 259. Five specimens had bacterial or fungal contamination. Cell culture was tried on 254 specimens, of which 169 (67%) grew 10 or more colonies per 5 x 10^5 cells. LAA effects were not tested in 6 specimens. Of 163 cases tested, 53 (33%) had L-CFC growth significantly enhanced and 28 (17%) were suppressed by LAA. Thus, about 50% of patients were affected by LAA one way or the other (Chart 1). Among 34 normal bone marrow specimens, each of which were tested simultaneously with one or more leukemic specimens, none were enhanced by LAA while 8 (24%) were suppressed (Chart 1). The growth-enhancing effect of LAA in a leukemic case can be visually appreciated (Fig. 1). The effects of DIAA and DAA were compared to that of LAA on 8 cases in whom LAA had shown growth enhancement (Chart 2). Both these isomers were less effective than LAA. On the average, DIAA was 30% and DAA was 20% as effective as LAA in growth enhancement. A dose-response study was performed comparing LAA and DAA (Chart 3). Over a wide range of concentration DAA was shown to be consistently less effective than LAA.

DISCUSSION

This study confirms our preliminary observations (27, 30) that the growth of L-CFC in vitro can be modulated by LAA in approximately 50% of patients with acute nonlymphocytic leukemia. Since this study involves a large number of patients, the relative proportions of patients in whom LAA enhances the growth of L-CFC (33%) and LAA suppresses it (17%) can be visually appreciated (Fig. 1). The effects of DIAA and DAA were compared to that of LAA on 8 cases in whom LAA had shown growth enhancement (Chart 2). Both these isomers were less effective than LAA. On the average, DIAA was 30% and DAA was 20% as effective as LAA in growth enhancement. A dose-response study was performed comparing LAA and DAA (Chart 3). Over a wide range of concentration DAA was shown to be consistently less effective than LAA.
taken with reasonable confidence. One point which was not apparent in our last interim analysis (26) is that the growth enhancement is more common than the growth suppression. Since this study involves a large number of normal bone marrow controls simultaneously tested with leukemia, there is another new issue evolving. A good proportion (24%) of normal controls is also suppressed, whereas none of 34 is enhanced. Because of this finding, leukemic cell suppression will have to be examined carefully, although some leukemic samples exhibiting complete or near complete suppression should be a really significant suppression. On the other hand, all the leukemic cell enhancement can be regarded as highly significant.

Although the normal control data already indicate that LAA growth enhancement is a significant finding, additional evidence in this study using ascorbic acid isomers demonstrates that this enhancing effect is truly biological in nature. DAA, unlike DIAA, is the true optical mirror image, or optical enantiomorph, of LAA (43). DAA and LAA should have identical physicochemical properties except for the rotation of polarized light. Therefore these 2 compounds cannot give different experimental results, unless the mechanism involved in the experiment is biological in nature. It is not surprising that some growth-enhancing activities are noted with DIAA and DAA, although weaker than with LAA. In fact this is additional supportive evidence that the growth-enhancing activity is biological, because both DIAA and DAA have antiscorbutic activity in vivo weaker than that of LAA (9, 12, 46). Moreover, a similar result obtained in an animal system (32) is a further reassurance for this finding in the human cell system.

Now, on the basis of this study, we conclude that there are 3 populations of acute nonlymphocytic leukemia in terms of LAA effect: one enhanced by LAA, one suppressed by LAA, and one unaffected by LAA. A question comes up as to why the acute nonlymphocytic leukemia cells from one patient are enhanced and cells from another patient are suppressed. No ready answer is available, although there are analogies to other tumors. It is universally known in medical oncology that breast cancer can regress following either removal of the ovaries or supplementation of an estrogen (40), and that an adenocarcinoma of breast can be suppressed, whereas an adenocarcinoma of prostate can be stimulated to grow by the same androgenic hormone (41).

Reviewing the literature, there are only few studies on the effect of LAA on tumor cells. These are mostly on cell lines as opposed to fresh specimens from patients, and most of them demonstrated suppression (3, 5, 7, 19, 35, 38), with only one exception (6). This is not surprising, because LAA in cell culture medium has very short half-life even at 4°C (14, 23). One can assume that there was very low or no LAA in medium which was used to establish these cell lines. Therefore there must have been selection against LAA-dependent clones.

The biochemical mechanism for these LAA growth-modulating effects is speculative at the moment. In studies on the LAA-suppressive effect in cell lines, H2O2 has been shown to be involved in the mechanism (18, 19, 35). Lipid peroxide formation of microsome due to LAA (44) may affect cell viability through release of lysosomal enzymes (45).

One of the most important questions is whether this in vitro finding of leukemic cell suppression by LAA deprivation can be reproduced in vivo. Using this culture system and another similar system, it has been shown that there is a good correlation between in vitro cytotoxicity of chemotherapeutic drugs on malignant cells obtained freshly from the patients and the clinical response of the same patients to the same drugs (28, 29, 31, 34, 39, 42). Leukemic cells which require an exogenous source of L-asparagine in vitro have also been shown to be sensitive to depletion of this amino acid in vivo (8). The cells of similar embryological origin have been shown to be sensitive to LAA deficiency, both in vitro and in vivo; odontoblasts regress to cuboidal or squamous form in scorbutic guinea pigs (15), and chick chondrocytes disintegrate unless cultivated in medium supplemented with LAA (20). There are in vivo studies that guinea pig leukemia (24) and solid tumor (22) growth is suppressed by LAA depletion. Therefore it is conceivable that leukemic cells sensitive to LAA depletion in vitro may also be suppressed in vivo by LAA deficiency. It is feasible to test this possibility because LAA plasma concentration can be reduced to a near zero level in 1 month and maintained at this level for 2 to 3 months in humans on scorbutic diets before any significant clinical evidence of scurvy develops (1, 2, 10, 13, 16, 17, 36, 37). The use of LAA oxidase (11) can be explored if more rapid reduction of LAA is desirable.

ACKNOWLEDGMENTS

I thank the staff of the Hematology Section of University of Kansas Medical Center, Farber Cancer Research Center, Wifflord Hall Medical Center, and member institutes of Southwest Oncology Group for providing leukemic bone marrows; Dr. W. E. Scott of Hoffmann-La Roche Co. and Dr. P. Seib of Kansas State University for samples of o-ascorbic acid; Dr. B. F. Kinkler for advice; and L. Payne, J. Meyer, S. O’Brien, and B. Bergmen for excellent technical assistance.

REFERENCES

BIological effect of ascorbic acid on Leukemic cells

20. Levenson, G. E. Behavior in culture of three types of chondrocytes, and their
19. Koch, C. J., and Biaglow, J. E. Toxicity, radiation sensitivity modification, and
metabolic effects of dehydroascorbate and ascorbate in mammalian cells. J.
20. Leversent, G. E. Behavior in culture of three types of chondrocytes, and their
23. Mohberg, J., and Johnson, M. J. Stability of vitamins in a chemically defined
25. Park, C. H. Growth modulation of human leukemic colony forming cells in vitro
by L-ascorbic acid. In: F. L. Meyskens and K. Prasad (eds.), Modulation and
26. Park, C. H. Cell-cycle manipulation of human leukemic precursor cells with
27. Park, C. H. Growth modulation of human leukemic colony forming cells in vitro
by L-ascorbic acid. In: F. L. Meyskens and K. Prasad (eds.), Modulation and
28. Park, C. H., Amare, M., and Hoogstraten, B. Analysis of the growth enhancing
Chemotherapy sensitivity assessment of leukemic colony-forming cells with in
vitro simultaneous exposure to multiple drugs: clinical correlations in acute
31. Park, C. H., Amare, M., Savin, M. A., and Hoogstraten, B. Growth suppression of
1065, 1980.
Maloney, T. R. In vitro drug sensitivity of leukemic colony-forming cells in acute
nonlymphocytic leukemia: clinical correlation update with various drug expo
sure methods. In: S. E. Salmon and J. M. Trent (eds.), Human Tumor Cloning,
requirement for colony formation by mouse plasmacytoma cells. Science
34. Park, C. H., Savin, M. A., Hoogstraten, B., Amare, M., and Hatheway, P.
Improved growth of in vitro colonies in human acute leukemia with the feeding
35. Park, C. H., Wiernik, P. H., Morrison, F. S., Amare, M., Van Sloten, K., and
Maloney, T. R. Clinical correlations of leukemic clonogenic cell chemosensitivity
assessed by in vitro continuous exposure to drugs. Cancer Res., 43: 2346–
2349, 1983.
36. Peters, R. A., for the Accessory Factors Committee. Vitamin-C requirement of
38. Prasad, K. N., Sinha, P. K., Ramajum, M., and Sakamoto, A. Sodium
ascorbate potentiates the growth inhibitory effect of certain agents on neuro-
S., and Moon, T. E. Quantitation of differential sensitivity of human tumor stem
40. Stoll, B. A. Breast cancer—endocrine therapy. In: Endocrine Therapy in
41. Stoll, B. A. Prostatic cancer—endocrine therapy. In: Endocrine Therapy in
42. Von Hoff, D. D., Casper, J., Bradey, E., Sandbach, J., Jones, D., and Makuch,
R. Association between human tumor colony-forming assay results and response of an individual patient’s tumor to chemotherapy. Am. J. Med., 70:
1027–1032, 1981.
43. West, E. S., Todd, W. R., Mason, H. S., and VanBruggen, J. T. Steroisomerism
44. Wills, E. D. Lipid peroxide formation in microsomes. General considerations.
45. Wills, E. D., and Wilkinson, A. E. Release of enzymes from lysosomes by
irradiation and the relation of lipid peroxide formation to enzyme release.
46. Yourga, F. J., Esselen, W. B., Jr., and Fellers, C. R. Some antioxidant
properties of D-isoascorbic acid and its sodium salt. Food Res., 9: 188–196,
1944.

Fig. 1. Growth-enhancing effect of LAA on bone marrow cells from a patient with acute myelocytic leukemia. Two dishes of culture were set up simultaneously in
identical fashion. Both received daily feeding, but one culture (A) with LAA and the other (B) without. The pictures were taken on Day 30 of culture. The best colonies
were chosen from each of 2 groups (A1, B1) at lower magnification 20) and close-up views (x 200) were taken (A2, B2). Parts of holes made at the bottom of culture
dish with 18-gauge needle are shown as black shadows on the right side of pictures (A1, B1). The counted numbers of colonies were 1440 ± 40 and 60 ± 13 for A and
B groups, respectively, per 5 x 10⁶ cells.

CANCER RESEARCH VOL. 45 AUGUST 1985
3972

Downloaded from cancerres.aacrjournals.org on May 31, 2017. © 1985 American Association for Cancer Research.
Biological Nature of the Effect of Ascorbic Acids on the Growth of Human Leukemic Cells

Chan H. Park

*Cancer Res* 1985;45:3969-3973.