Growth Suppression of Human Leukemic Cells \textit{in Vitro} by L-Ascorbic Acid

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\textbf{ABSTRACT}

The suppressive effect of L-ascorbic acid on the growth of bone marrow cells from patients with acute nonlymphocytic leukemia was studied using a modified agar culture method featuring daily feeding to allow the growth of leukemic cell colonies. In seven of 28 patients (25\%), the numbers of leukemic cell colonies grown in culture were reduced to 21\% of control by the addition of L-ascorbic acid (0.3 mM) to the culture medium. Glutathione did not suppress leukemic cell colonies although it has a similar oxidation-reduction potential to that of L-ascorbic acid. The addition of L-ascorbic acid reduced the pH of the medium. However, a comparable reduction of pH by the addition of HCl did not suppress leukemic cell colonies. In simultaneous cultures for leukemic and normal marrow cells, the suppression of leukemic cell colony was noted with a concentration of L-ascorbic acid as low as 0.1 mM (a concentration achievable \textit{in vivo}), but normal myeloid colonies were not suppressed until the concentration of L-ascorbic acid reached an extremely high level (1 mM). In conclusion, growth of leukemic cells in culture was suppressed by L-ascorbic acid in a substantial proportion of patients with acute nonlymphocytic leukemia. This suppression was a specific effect of L-ascorbic acid and was not due to its oxidation-reduction potential or pH change. Leukemic cells were selectively affected at an L-ascorbic acid concentration attainable \textit{in vivo} while normal hemopoietic cells were not suppressed.

\textbf{INTRODUCTION}

L-Ascorbic acid is not generally considered to be cytotoxic. Although there are reports concerning its toxicity in several tumor cell lines of animal origin (1, 10, 12), a direct cytotoxicity to human tumor cells has not been demonstrated. We here report that in a substantial proportion of patients with acute nonlymphocytic leukemia, the growth of leukemic cells in culture is suppressed by L-ascorbic acid.

It is generally known to be difficult to obtain \textit{in vitro} growth of leukemic cell colonies with freshly aspirated bone marrow cells from patients with acute nonlymphocytic leukemia (15, 24). However, we have recently developed a culture method for the growth of human leukemic colonies (21). This is a modification of the agar culture method for normal myeloid colonies (commonly known as CFU-C) (2, 22) featuring daily feeding with new culture medium. The feeding technique was adopted from another culture method we developed previously for mouse myeloma (18) in which it was found to be important to feed the cultures daily with new culture medium containing L-ascorbic acid (19). In our recent study on acute nonlymphocytic leukemia (21), the daily feeding was also found to be very important for the growth of leukemic colonies in all the 8 patients studied. However, the addition of L-ascorbic acid to the feeding enhanced colony growth in only 2 of 8 patients. For the rest of the patients, although the feeding was needed, L-ascorbic acid did not enhance the colony growth (21).

We now have 28 patients with acute nonlymphocytic leukemia whose leukemic cells can be grown as colonies in the modified culture with feeding. Eight (29\%) show a requirement for L-ascorbic acid in addition to the feeding. Among the rest, we have identified another subpopulation of 7 patients (25\%) in whom the growth of leukemic colonies by the feeding is suppressed by the addition of L-ascorbic acid, and this phenomenon is the subject of our report.

\textbf{MATERIALS AND METHODS}

\textbf{Patients.} The clinical characteristics of the 7 leukemic patients in this study are shown in Table 1. Normal marrows used for controls were obtained from hematologically normal patients with solid tumors who were undergoing bone marrow aspiration as a part of staging workup. No patient had received prior treatment 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.

\textbf{Culture Assay.} The details of the culture method have been published (21). Briefly, the culture system consists of 2 layers of 0.3% agar in a 35-mm plastic Petri dish perforated at the bottom with 6 small holes. Bone marrow cells were placed in the upper of the 2 agar layers suspended in a medium free of L-ascorbic acid (Grand Island Biological Co., Grand Island, N. Y.)-containing 15% fetal calf serum and 15% leukocyte-conditioned medium prepared as described previously (21). Cultures were incubated at 37\°C with 7% CO2 for 2 to 3 weeks. Throughout this period, each culture dish was taken out of incubator once daily to be fed from the top with 0.5 ml of L-ascorbic acid-free medium containing 15% each of fetal calf serum and leukocyte-conditioned medium with or without freshly prepared L-ascorbic acid, GSH, or HCl. In the previous study on the growth of mouse myeloma cells in culture, GSH was shown to enhance the effect of L-ascorbic acid (19), and both GSH and L-ascorbic acid were used in some experiments in this study. When the effect of L-ascorbic acid was under study, it had to be supplied to the cultures daily throughout the entire growth period because it has very short half-life in culture (4, 16). Likewise, GSH and HCl were also added daily when these were under study in order to make all the culture systems

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\textbf{3 The abbreviation used is: GSH, glutathione.}
comparable. The fed medium passed through the agar layers and drained from the dish through holes at the bottom. Colonies were counted with an inverted microscope scoring only those with 50 or more cells.

It was shown in our previous study (21) that leukemic marrow cells gave rise to no or negligibly low numbers of colonies unless the cultures were fed daily and that the colonies which

Table 1
Clinical data of 7 leukemic patients

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Blast cells (%)</th>
<th>Chemotherapy</th>
<th>Complete remission</th>
<th>Survival (mos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>F</td>
<td>AML</td>
<td>95</td>
<td>HU, AC</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>M</td>
<td>AML</td>
<td>80</td>
<td>None</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>F</td>
<td>AML</td>
<td>30</td>
<td>None</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>F</td>
<td>EL</td>
<td>20</td>
<td>None</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>74</td>
<td>M</td>
<td>AML</td>
<td>83</td>
<td>TG, VR, PR</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>F</td>
<td>AML</td>
<td>60</td>
<td>AD, VR, AC, PR</td>
<td>Yes 14+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>M</td>
<td>AMML</td>
<td>70</td>
<td>RZ, VR, AC, PR</td>
<td>No 8+</td>
<td></td>
</tr>
</tbody>
</table>

* Blast cell percentage of bone marrow aspirate used for culture study.
* Complete remission as defined by the disappearance of all evidence of disease with normal marrow (blast, <5%) and normal peripheral smear.
* AML, acute myeloblastic leukemia; EL, erythroleukemia; AMML, acute myelomonocytic leukemia.
* HU, hydroxyurea; AC, 1-β-D-arabinofuranosylcytosine; None, no chemotherapy given; TG, 6-thioguanine; VR, vincristine; PR, prednisone; AD, adriamycin; RZ, Rubidazone.
* Abnormal karyotype (47 XYG +) identified in the bone marrow.
* She appeared to have oligoleukemia initially but developed a picture of full-blown acute leukemia 4 months later.
* The blast cells were once cleared from peripheral blood but not reduced below 5% in bone marrow.

It was shown in our previous study (21) that leukemic marrow cells gave rise to no or negligibly low numbers of colonies unless the cultures were fed daily and that the colonies which grew only with feeding were leukemic in origin as substantiated by the morphological, cytochemical, and chromosomal studies. On the other hand, normal bone marrow yielded the same number of myeloid colonies with or without feeding. To ensure that the colonies seen in the feeding culture were leukemic colonies and not normal myeloid colonies, simultaneous control cultures without feeding were performed on all 7 leukemic marrows. The number of colonies in these controls were only 5.2 ± 2% (S.E.) of the total number of colonies counted in the matched feeding culture. Therefore, even if all these colonies in the control cultures were normal myeloid colonies and all of these also grew in the feeding cultures, they represented a negligibly small fraction of the total number of colonies in the feeding culture and would not materially alter the results of the study. In addition, the colonies of 2 patients (Cases 1 and 2) were picked up and subjected to morphological and cytochemical studies as described (21). This directly confirmed leukemic origin of colonies of the patients in this study.

RESULTS

The suppression of leukemic cell growth in the presence of L-ascorbic acid and GSH resulted in a reduction in the number of leukemic cell colonies to an average of 21 ± 9.2% (S.E.) of the controls as shown in Chart 1. This effect was reproduced in 2 cases tested. The colony suppression was consistently
there do not appear to be any outstanding features which can
detect any characteristic feature for these patients. One patient
distinguish this subpopulation of patients from the rest. The
characteristics of these patients are shown in Table 1, but
with a high proportion of blasts in bone marrow has been
number of patients studied thus far may still be too few to
therapy. However, a smoldering course in the acute leukemia
effect.

Our findings indicate that L-ascorbic acid suppresses the in
vitro growth of leukemic cells in a substantial proportion (25%) of
patients with acute nonlymphocytic leukemia. The clinical
characteristics of these patients are shown in Table 1, but
there do not appear to be any outstanding features which can
distinguish this subpopulation of patients from the rest. The
number of patients studied thus far may still be too few to
detect any characteristic feature for these patients. One patient
(Case 2) had an unequivocal picture of acute myelocytic
leukemia with 80% blasts in bone marrow and an abnormal
karyotype but had an unusually long survival without chemo-
therapy. However, a smoldering course in the acute leukemia
with a high proportion of blasts in bone marrow has long been
recognized (11). Another patient (Case 3) had low blast cell
percentage and appeared to be oligoleukemic (9) at the time
of initial culture study, but she developed a full-blown acute
leukemia shortly. Also, it is known that the cell culture pattern
of many with oligoleukemia is identical with that of typical acute
leukemia (25), and the normal myeloid colony formation is not
likely to occur unless the blast cell proportion is reduced below
20% (3). The blast cell proportion was low in another patient
(Case 4), but she also had marked dyserythropoiesis, this
being compatible with erythroleukemia (5). Response to the
chemotherapy and survival are also difficult to assess. Chem-
otherapy was not given to 3 patients, and in one patient (Case
6), the chemotherapy was not intended for complete remission
because of the number of clinical reasons such as advanced
age, the possible smoldering course, etc.

It is most interesting to note that this suppressive effect on
leukemic cells is specific to L-ascorbic acid. This suggests the
possibility of a specific metabolic pathway being involved in
this effect. There are possible mechanisms which could explain
this effect. The lack of catalase may lead to cellular damage by
the accumulation of H₂O₂ due to L-ascorbic acid (1, 10, 12).
The lipid peroxide formed in mitochondria with the help of L-
ascorbic acid (17, 26, 29) may cause injury to the lysosomal
membrane with the release of enzymes (27) which in turn can
lead to the damage of the cells (8). The increased level of
cyclic adenosine 3':5'-monophosphate induced by L-ascorbic
acid (13) may inhibit cell growth (6, 13).

By far, the most important point in this study is that a
profound suppression of leukemic cells can be achieved "without
any damage to normal myeloid precursor cells" using the
appropriate concentrations of L-ascorbic acid. This is because
the major limiting factor in the drug treatment of cancer is the
toxicity to normal tissue, especially to hemopoietic tissue.
Among the 3 concentrations of L-ascorbic acid tested (Chart
3), the 2 lower ones (0.1 and 0.3 mM) are considered most
appropriate covering the range of in vivo levels achievable with
pharmacological doses in humans (28). Although the profound
and selective suppression of leukemic cells was noted with
these concentrations, we were tempted to test a higher con-
centration (1 mM) to see the maximum suppression of leukemic
cells. No additional suppression of leukemic cells was noted.
Instead, normal myeloid colonies started to be suppressed at
this concentration which is, however, beyond the level achiev-
able in humans, and therefore no clinical relevance can be
given.

With this culture system (20) and another similar system (7,
23), it has been shown that there is a good correlation between
in vitro cytotoxicity of chemotherapeutic drugs on malignant
cells freshly obtained from the patients and the clinical re-
sponse of the same patients to the same drugs. It is therefore
conceivable that L-ascorbic acid might be selectively suppres-
sive to the leukemic cells in vivo as in vitro. It is feasible to
study this clinically because the lowest concentration shown to
suppress leukemic growth in vitro (0.1 mM) can be attained in
vivo easily and safely with a pharmacological dose of L-ascorbic
acid (28). If such a clinical study is contemplated, a preliminary
in vitro test should be done in all patients to exclude those in
whom L-ascorbic acid enhances the growth of leukemic cells.

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Human Leukemic Cell Suppression by L-Ascorbic Acid

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