Self-Renewal Capacity of Leukemic Blast Progenitor Cells

R. N. Buick,² L. J-A. Chang, H. A. Messner, J. E. Curtis, and E. A. McCulloch³

Abstract

A review is presented of experimental information pertaining to the characteristics of a procedure designed to quantitate the capacity for self-renewal in clonogenic cells of human acute myeloblastic leukemia. In a series of 44 previously untreated patients, a significant correlation (ρ < 0.01) was seen between low capacity for self-renewal and successful remission induction. Three cytotoxic drugs (Adriamycin, 1-ß-d-arabinofuranosylcytosine, and N-[4-(19-acridinylamino)-3-methoxyphenyl]-methanesulfonamide) were tested for preferential effect against self-renewal events. Surviving clonogenic cells to these agents had, respectively, unchanged, lower, and higher capacity for self-renewal. The implications of such drug properties are discussed.

Introduction

Over the last 15 years, methods have been developed that allow analysis of the human hemopoietic cell renewal system by assessment of culture clonogenicity of stem and progenitor cells. Such techniques all depend on the use of viscid or semisolid media (which allow the progeny of a single cell to remain localized as a colony) and the presence in the culture medium of specific stimulators. These developments have been detailed in a number of recent reviews (12, 15). More recently, a number of groups have applied similar technology to the study of leukemic blast cell populations in AML.⁴ The published reports of the groups have indicated a striking dependence on PHA for leukemic growth in culture. Leukemic cells have been stimulated either by preincubation with PHA (8) by culturing in suspension over agar with PHA in both phases (11) or by continuous feeding with conditioned medium from PHA-treated cells (PHA-LCM) (17). The colony assay around which this study is conducted is also based on the characteristics of a procedure designed to quantitate the human hemopoietic cell renewal system to the progenitor's property of self-renewal (as opposed to the PE1, which is a measure of proliferation from a single-cell suspension after a primary growth phase in order to assess secondary colony formation). The procedures developed to study self-renewal are based on replating colonies after a primary growth phase in order to assess secondary colony formation (3). Measurement of this parameter showed considerable promise of prognostic significance in our initial series of 21 patients (3).

The purpose of this paper is to update the information relating to the prognostic significance of self-renewal capacity and to present the results of experiments designed to determine if the parameter can be manipulated in a manner applicable to therapy.

Materials and Methods

The basic colony assay was performed as previously described (4, 13). Briefly, cells from the peripheral blood of AML patients are depleted of T-cells (16) by erythrocyte rosette depletion and then cultured in methylcellulose (0.8% w/v) supplemented by the addition of PHA-LCM (5 to 10%, v/v). Small colonies (20 to 200 cells) of blast-like cells can be enumerated after 5 to 10 days of incubation in a humidified atmosphere of 5% CO₂ in air.

Self renewal during colony formation can be considered to have taken place when one can demonstrate cell(s) within colonies (10, 13) and the cells were blasts by light and electron microscopy (14, 16) and failed to express any lymphoid markers (13).

The results of these initial studies are consistent with the view that a minority population of leukemic blasts has sufficient proliferative capacity to be clonogenic and in fact that these cells represent the population (or part of it) maintaining the tumor in situ. As such, these cells should be the critical population in terms of response to therapy and also should display the biological characteristic of self-renewal (a necessary function for population maintenance, and therefore the defining property of a stem cell). Accordingly, we performed 2 studies to establish conditions for measurement of drug sensitivity of the clonogenic cells (2) and to measure the self-renewal capacity.

Drug sensitivity measured in culture to Adriamycin, daunorubicin, and 1-ß-d-arabinofuranosylcytosine was found to vary considerably from patient to patient. We are in the process of attempting a clinical correlation of these data in approximately 50 patients. Initial analysis in a series of 20 patients found only weak correlation between culture Adriamycin sensitivity and outcome of remission induction with an Adriamycin-containing regimen (1).

Experiments designed to manipulate the self-renewal capacity are performed in the following manner; primary plating is carried out in the presence of (or under the influence of) an agent or cellular manipula-

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⁴ The abbreviations used are: AML, acute myeloblastic leukemia; PHA, phytohemagglutinin; LCM, medium conditioned by leukocytes in the presence of PHA; PE2, secondary plating efficiency; PE1, initial plating efficiency; m-AMSA, N-[4-(19-acridinylamino)-3-methoxyphenyl]-methanesulfonamide.

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tion. Surviving colonies are pooled and then replated for measurement of PE2. A comparison can then be made between survivors of a given manipulation and control, untreated cells. These measurements therefore rely on the ability to measure self-renewal (PE2) independently of primary colony growth (PE1). This concept is complicated by the fact that primary growth is necessary for expression of self-renewal capacity. However, we have demonstrated that PE2 and PE1 are not correlated (3).

Results

**Characteristics of PE2.** Secondary colony growth has satisfied certain conditions which allow us to equate PE2 with the process of self-renewal. For example, the colonies obtained by replating dispersed primary colonies are indistinguishable from primary colonies in size, colony morphology (14), cellular markers (peroxidase, lack of lymphoid surface markers), and optimum PHA-LCM concentration (3). The extent of renewal capacity is limited. We have succeeded in transferring single colonies 3 times but never a fourth (3). Of course, we cannot estimate the extent to which physical damage limits the ability to be clonogenic on repeated transfers. The time at which transfer is made is extremely important, with 6 to 7 days in primary culture being optimal. The clonogenicity in the PE2 assay is linear with respect to cell number in the operative range (3). PE2 is heterogeneous with respect to cell size consistent with the theory that blast cells form a lineage undergoing progressive loss of self-renewal capacity (6).

Of considerable interest is the status of PE2 in relation to disease state. In the limited experience thus far available, we can show that PE2 is a stable characteristic. For instance, when comparing PE2 from a patient’s leukemic blasts at presentation and after regrowth from cytoreduction, the values are similar. PE2, therefore, seems to be a heritable characteristic of the clone rather than reflecting its physiology.

**Prognostic Indication of PE2.** On a theoretical basis, self-renewal capacity might be expected to be related to the inherent aggressiveness of leukemic clones. We have performed measurements of PE2 on 44 evaluable, previously untreated patients. The extent to which PE2 correlates with remission induction (with an Adriamycin-1-β-D-arabinofuranosylytosine protocol) (7) is shown in Chart 1. In this nonselected group, 47.7% remission induction was achieved, and there was a highly significant correlation between low PE2 and successful remission induction (0.001 < p < 0.01; n = 44, rank sum test). Other variables such as age, presenting blasts, and platelet count have been implicated as prognostically important (9, 18). None of these factors, however, is correlated with PE2, indicating that the effect of PE2 on remission induction is not acting through another identifiable variable.

We are in the process of analyzing data on drug sensitivity of the clonogenic cells. Preliminary data indicate that drug sensitivity is correlated with remission induction, although with much less statistical significance than is PE2. Clearly, it is to be hoped that the addition of the laboratory-derived parameters to a multivariate analysis of prognostic factors in AML will lead to increased confidence in prediction of remission induction with existing drug regimens and also to point out those patients in whom novel therapy may be attempted.

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Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug concentration (μg/ml)</th>
<th>PE1</th>
<th>PE2</th>
</tr>
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<tbody>
<tr>
<td>Adriamycin (10 min preexposure)</td>
<td>0</td>
<td>193 ± 8*</td>
<td>37.5 ± 2.5</td>
</tr>
<tr>
<td>0.25</td>
<td>160 ± 12</td>
<td>49.6 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>99.5 ± 4.5</td>
<td>38.0 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>64 ± 11.5</td>
<td>46.5 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>16.5 ± 4.5</td>
<td>32.0 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>m-AMSA (continuous contact)</td>
<td>0</td>
<td>1186 ± 252</td>
<td>24.0 ± 2.4</td>
</tr>
<tr>
<td>10^−5</td>
<td>1085 ± 65</td>
<td>47.0 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>10^−4</td>
<td>798 ± 77</td>
<td>53.7 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>10^−3</td>
<td>398 ± 169</td>
<td>30.5 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>10^−2</td>
<td>326 ± 66</td>
<td>22.7 ± 9.4</td>
<td></td>
</tr>
<tr>
<td>10^−1</td>
<td>213 ± 26</td>
<td>23.7 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-β-D-Arabinofuranosylcytosine (continuous contact)</td>
<td>0</td>
<td>202 ± 18</td>
<td>38.0 ± 4</td>
</tr>
<tr>
<td>10^−3</td>
<td>188 ± 40</td>
<td>42.5 ± 8</td>
<td></td>
</tr>
<tr>
<td>10^−2</td>
<td>78 ± 9</td>
<td>6.5 ± 2</td>
<td></td>
</tr>
<tr>
<td>10^−1</td>
<td>34 ± 6</td>
<td>14.25 ± 1</td>
<td></td>
</tr>
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* Mean ± S.E. of quadruplicate plates.

Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Comparative inhibitory effect on PE2 or PE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>PE2 = PE1 (6)*</td>
</tr>
<tr>
<td>m-AMSA</td>
<td>PE2 &lt; PE1 (4)</td>
</tr>
<tr>
<td>1-β-D-arabinofuranosylcytosine</td>
<td>PE2 &gt; PE1 (3) or PE2 = PE1 (2)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of patients in study.

**Discussion**

The experience with a large series of AML patients confirms and in fact strengthens our initial claims (3) as to the prognostic indication of the self-renewal capacity of the leukemic progenitors. Our data show that low PE2 is significantly associated with remission induction (0.01 > p > 0.001) using a rank sum test. The strength of this correlation leads us to propose that this culture parameter may prove to be a very important additional prognostic indicator to those already described (9, 18).

Drug sensitivity measured in culture also proves to have prognostic indication although with much less significance than PE2. However, they are independent variables and therefore will be additive in a test of multivariate factors in AML.

As a corollary to those clinical observations, it is clear that the self-renewal capacity of the clonogenic cells is an important target for intervention. Since patients with low self-renewal do best clinically, can we lower the self-renewal capacity in those patients with aggressive disease? We have attempted to investigate this possibility in 2 areas; fortuitous change in self-renewal caused by cytotoxic drugs used for cytoreduction or designed intervention based on interaction of noncytotoxic agents with the process.

An analysis of 3 cytotoxic drugs in terms of their effect on primary colony growth and self-renewal capacity point to a number of areas for future development. The apparent selectivity of 1-β-D-arabinofuranosylcytosine in inhibiting self-renewal suggests a key biological role for this agent in successful treatment of AML. This would seem to be reflected in the almost obligatory inclusion of this agent in remission induction protocols. Adriamycin, on the other hand, would seem to have its major effect in a cytoreductive mode. An important area of future work will be to determine whether the patient-to-patient heterogeneity with respect to the effect of 1-β-D-arabinofuranosylcytosine on self-renewal will be related to the outcome of attempted remission induction. From the point of view of the future selection of agents useful in the treatment of AML, it would be advantageous to choose compounds with a dual role; cytoreduction and inhibition of self-renewal. m-AMSA consistently allows increased self-renewal in survivors of treatment, whereas on a molar basis it seems as efficient as Adriamycin in terms of cytoreduction. On a theoretical basis, these characteristics would be consistent with a drug which was efficient in remission induction but which would not be associated with lengthy remissions.

The possibility also exists for manipulation of self-renewal by more physiological agents. As a preliminary to these studies, attempts to manipulate PE2 by simple alteration of culture conditions have been strikingly unsuccessful. For example, primary colonies growing with low efficiency in medium deficient in PHA-LCM have a PE2 identical to that for colonies grown in optimum conditions. Clearly, the capacity for renewal is expressed even in situations where primary colony growth is limited by deficiency of growth factors. However, some studies have indicated that self-renewal can be manipulated by agents which are thought to act at levels other than cytotoxicity.

Chang and McCulloch (5) have, however, shown that low concentrations of tumor promoters, such as tetradecanoylphorbol acetate, cause increased self-renewal during colony growth, and Taetle et al. (18) have shown a specific inhibitory action of human fibroblast interferon on self-renewal.

In terms of designing intervention at the biological level, we need to know a great deal more about the control of the self-renewal process. From our initial attempts to alter PE2 by simple alterations to the culture medium, we know that the renewal process is not easily manipulated. Since, in conventional thinking, the alternative to self-renewal in the cell is differentiation, it is possible that agents known to induce hematopoietic differentiation may be the same agents that we are seeking as self-renewal inhibitors. In this regard, it is of interest that 2 cases of manipulation of PE2 by tetradecanoylphorbol acetate (5) and interferon (6) have been brought about by agents thought to act at the level of control of differentiation.

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**References**

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