Sensitivity of Human Granulopoiesis in Vitro to Adriamycin before and after Exposure in Vivo

Hans-Peter Lohrmann and Wolfgang Schreml

Division of Hematology, Department of Internal Medicine, University of Ulm, Federal Republic of Germany

ABSTRACT

The present studies were performed to determine whether the sensitivity of a normal human granulopoiesis to Adriamycin changes after repeated exposure to the drug in vivo. A single-layer agar culture for the in vitro growth of granulocytic colonies from committed stem cells was used as the assay system. The in vitro sensitivity of the human granulopoiesis to different doses of Adriamycin was determined from the reduction in colony incidence (a) following exposure of marrow cells to Adriamycin for 30 min before initiation of cultures and (b) in the presence of Adriamycin during the total culture period. Using both test systems, the sensitivity of granulopoiesis in vitro remained unchanged after patients had received six courses of chemotherapy incorporating Adriamycin. Sensitivity to Adriamycin appeared to increase by Day 4 after chemotherapy, probably as a result of the increased proliferative activity of granulopoietic precursor cells at this time. The data thus fail to provide evidence that a granulopoietic subpopulation with increased resistance to the cytotoxic effects of Adriamycin emerges after repeated in vivo exposure to the drug.

INTRODUCTION

Acquired resistance of tumors to chemotherapeutic agents represents a major problem in the treatment of cancer patients. Such failure of tumors further to respond to a chemotherapy protocol originally effective for tumor growth control results from a change in the sensitivity of tumor stem cells to the drug(s) used (15), for which different mechanisms may be responsible (5, 9). It is of interest whether normal mammalian proliferating cellular systems respond in a similar way to repeated in vivo exposure to cytotoxic drugs. The hematopoietic cell renewal systems are rather easily accessible for repeated examination and are therefore particularly suited to study the question of whether a change in the sensitivity to cytotoxic drugs occurs during and/or after a limited period of intermittent cytotoxic chemotherapy.

In patients with a history of previous cytotoxic drug administration, increased hematopoietic toxicity after standard doses of chemotherapy is a common clinical experience. From this, one might conclude that the cells of the hematopoietic systems fail to develop increased resistance to cytotoxic drugs. However, we have demonstrated a long-lasting defect of granulopoiesis persisting after completion of chemotherapy (12). Therefore, increased resistance at the cellular level may well exist after chemotherapy but might be outweighed by a hypoplastic hematopoietic system. Therefore, the question of altered sensitivity of hematopoietic cells after chemotherapy can be approached only by studying well-defined hematopoietic cell populations in vitro.

This paper describes the results of studies performed to investigate possible changes in the in vitro sensitivity of granulopoietic cells to Adriamycin after in vivo exposure to this agent. A protocol of adjuvant chemotherapy for primary breast cancer appeared to be ideally suited for these studies. Here, a patient population with normal hematopoiesis is exposed to chemotherapeutic agents for a limited period, after which no further chemotherapy is administered. This clinical setting therefore allows the study of possible changes of hematopoiesis persisting after cessation of chemotherapy.

MATERIALS AND METHODS

Patients. Thirteen female patients were studied during and after adjuvant chemotherapy for primary breast cancer after radical mastectomy. Adjuvant chemotherapy consisted of Adriamycin (50 mg/sq m i.v.) plus cyclophosphamide (500 mg/sq m i.v.) given at 4-week intervals for a total of 6 courses. Details of patient selection have been reported (12).

Hematological Studies. Bone marrow was obtained before the first and before the sixth (last) chemotherapeutic courses, and 55 days after the last drug administration. In 9 patients, bone marrow was also studied 4 days after administration of the first course; and in 7 patients, it was studied 4 days after administration of the sixth course. Following local anesthesia, 4 to 5 ml of marrow were aspirated from the posterior iliac crest into syringes containing 1 ml of isotonic disodium EDTA solution. The further preparation of the marrow cells has been described (11, 12).

CFU-C Culture. For the in vitro culture of hematopoietic stem cells committed to granulopoiesis-monocytopoiesis (CFU-C), a single-layer agar culture system was utilized, with human placenta-conditioned medium as a source of colony-stimulating activity (6, 11). Details of the culture method have been described (11, 12). After 10 days of culture, colonies (defined as aggregates of more than 50 cells) were scored using a stereomicroscope.

In Vitro Test of Adriamycin Sensitivity. The above-described agar culture system for the in vitro growth of granulopoietic stem cells was used as an indicator system for the sensitivity of granulopoietic cells to Adriamycin. Colony formation in this system represents a clonal growth...
of granulopoietic cells in vitro (for references, see Ref. 13). Two different methods were applied to study the sensitivity of granulopoietic cells to Adriamycin in vitro. First, Adriamycin was added directly to the culture plates and left there for the total culture period. After preliminary experiments had shown negligible or no colony growth at Adriamycin concentrations of $2 \times 10^{-4}$ g/liter, and usually no inhibition of colony growth at Adriamycin concentrations of $6.6 \times 10^{-7}$ g/liter, the following concentrations (g/liter) of the drug were chosen for a dose-response analysis: $2 \times 10^{-4}$; $13.2 \times 10^{-4}$; $6.6 \times 10^{-4}$; $2 \times 10^{-4}$; and $6.6 \times 10^{-7}$. A commercially available preparation of Adriamycin (Adriblastin; Montedison Farmaceutica, Freiburg, Germany; also used for the treatment of the patients) was dissolved to a concentration of $2 \times 10^{-2}$ g/liter and stored frozen at $-20\degree$ until use. With regard to the photosensitivity of Adriamycin, care was taken to expose the drug solutions as little as possible to light. Immediately before cultures were set up, Adriamycin was thawed and added to the cultures to yield the above-mentioned final concentrations. Colony growth (the mean colony number of triplicate cultures) was expressed as a percentage of the colony incidence in cultures grown in the absence of Adriamycin.

Decay of Adriamycin may cause changes in the drug concentration during the 10-day incubation period, although evidence has been obtained that Adriamycin is stable under similar in vitro conditions for at least 8 hr (J. A. Belli, personal communication). However, if such decay occurs, it will probably be nonvariable under identical culture conditions, thus allowing comparison of the colony-inhibiting action of the drug in this system. This first method then determines the sensitivity of the human granulopoiesis in vitro as a population with different levels of differentiation; it does not permit assessment of the sensitivity of a particular granulopoietic population.

Therefore, in order to characterize the sensitivity of a defined subpopulation of granulopoietic cells, the CFU-C, a second method was applied. Here, bone marrow cells were first exposed for 30 min at $37\degree$ to different concentrations of Adriamycin and then washed twice before cultures were set up. Since by definition only CFU-C give rise to granulocytic colonies in the culture system used, any colony growth inhibition observed after such preincubation of marrow cells with Adriamycin may be ascribed to the cytotoxic effect of Adriamycin on CFU-C. Preliminary studies led us to use the following concentrations (g/liter) of Adriamycin for the short-term incubation: $2 \times 10^{-3}$; $13.2 \times 10^{-4}$; $6.6 \times 10^{-4}$; $2 \times 10^{-4}$; $2 \times 10^{-4}$. Again, the mean colony number of triplicate cultures was expressed as a percentage of the colony incidence in cultures incubated and processed identically, but in the absence of Adriamycin.

Statistical analysis was performed using a computer program for a desk computer (Hewlett-Packard Model 9830 A) utilizing the 2-tailed Mann-Whitney U test.

RESULTS

Dose-response curves of a normal, unperturbed human granulopoiesis (i.e., before initiation of chemotherapy) are demonstrated in Chart 1. The short-term incubation method required approximately 100-fold higher drug concentrations to cause a colony growth inhibition comparable to that seen in cultures which contained Adriamycin during the total culture period. The results of the serial studies are listed in Table 1. The data show an identical drug sensitivity of granulopoiesis before the first and before the sixth chemotherapeutic courses, regardless of the test method. Furthermore, Adriamycin sensitivity determined 55 days after the last drug administration was again not different from the pretreatment values. On Day 4 of the first and sixth courses, the sensitivity of granulopoietic cells to Adriamycin appeared somewhat increased at the higher drug concentrations.

DISCUSSION

Using different cell lines, several groups (2–4, 8, 10) have described an extreme in vitro sensitivity of mammalian cells to Adriamycin. The cytotoxic effect of the drug was shown to be greatest on cells in S phase of the cell cycle (3, 10) or at the late S-G2 boundary region (8) of the cell cycle, although cells in the other cell cycle phases were also sensitive to the drug. Razek et al. (14) described the effect of in vivo administration of Adriamycin on normal murine hematopoietic stem cells; studies on the in vitro sensitivity of hematopoietic cells to Adriamycin in humans have not been reported.

Before treatment, measurement of the in vitro cytotoxic effect of Adriamycin on normal human CFU-C revealed a
Adriamycin Sensitivity of Granulopoiesis in Vitro

Table 1

<table>
<thead>
<tr>
<th>Adriamycin concentration</th>
<th>First course</th>
<th></th>
<th>Sixth course</th>
<th></th>
<th>Day 55 after last course</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before drugs</td>
<td>Day 4</td>
<td>p&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Before drugs</td>
<td>Day 4</td>
</tr>
<tr>
<td></td>
<td>n = 12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>n = 9</td>
<td></td>
<td>n = 14</td>
<td>n = 7</td>
</tr>
<tr>
<td>A. In plates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>6.8 ± 2.8</td>
<td>1.6 ± 1.2</td>
<td>NS&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6.0 ± 2.3</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>13.2 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>24.8 ± 4.0</td>
<td>10.0 ± 4.8</td>
<td>0.05</td>
<td>19.6 ± 4.6</td>
<td>14.9 ± 6.2</td>
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<td>6.6 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>59.6 ± 5.1</td>
<td>41.7 ± 5.0</td>
<td>0.05</td>
<td>54.8 ± 5.8</td>
<td>31.9 ± 10.2</td>
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<tr>
<td>2 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>78.8 ± 5.5</td>
<td>72.7 ± 5.5</td>
<td>NS</td>
<td>82.6 ± 6.8</td>
<td>77.0 ± 5.5</td>
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<tr>
<td>6.6 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>95.3 ± 3.5</td>
<td>94.2 ± 4.0</td>
<td>NS</td>
<td>97.3 ± 5.4</td>
<td>92.1 ± 8.2</td>
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<td>n = 7</td>
<td>n = 3</td>
<td></td>
<td></td>
<td>n = 7</td>
<td>n = 6</td>
</tr>
<tr>
<td>B. During incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1.0 ± 0.7</td>
<td>0</td>
<td>NS</td>
<td>0.8 ± 0.5</td>
<td>2.0 ± 2.0</td>
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<tr>
<td>13.2 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>7.8 ± 4.9</td>
<td>0</td>
<td>NS</td>
<td>11.0 ± 3.5</td>
<td>2.6 ± 2.6</td>
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<tr>
<td>6.6 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>29.3 ± 8.5</td>
<td>4.5 ± 1.5</td>
<td>0.056</td>
<td>39.0 ± 6.3</td>
<td>23.8 ± 7.0</td>
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<tr>
<td>2.0 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>76.4 ± 12.3</td>
<td>65.3 ± 13.2</td>
<td>NS</td>
<td>80.2 ± 18.5</td>
<td>79.8 ± 8.2</td>
</tr>
<tr>
<td>2.0 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>88.2 ± 8.5</td>
<td>95.0 ± 5.0</td>
<td>NS</td>
<td>94.9 ± 5.8</td>
<td>104.6 ± 16.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> All values not significantly different from those determined before the first course.
<sup>b</sup> When compared to the values determined before the sixth course.
<sup>c</sup> When compared to the values determined before the first course.
<sup>d</sup> n, number of patients studied.
<sup>e</sup> Mean ± S.E.
<sup>f</sup> NS, not significant.

100-fold difference between minimally and maximally cytotoxic concentrations of the drug. The Adriamycin concentrations that inhibited colony formation after a 30-min incubation of human marrow cells are in the range reported by others to inhibit proliferation of various mammalian cell lines under similar conditions (3, 4, 8, 10). When Adriamycin was present during the total 10-day culture period, it inhibited colony formation at concentrations that were about 100-fold lower than those required to achieve the same degree of colony inhibition by short-term incubation. Such an increased cytotoxic effect of Adriamycin with extension of cell exposure to the drug has been observed previously (4, 8, 10).

The present studies were performed in patients who repeatedly received combination chemotherapy incorporating Adriamycin at intervals long enough to allow restoration of the cytotoxic drug-induced perturbation of granulopoiesis (12). On Day 4 after chemotherapy, in vitro Adriamycin sensitivity of granulopoiesis was increased. This is most likely the result of the increased proliferative activity of granulopoiesis at this time, with a greater fraction of CFU-C being in the more Adriamycin-sensitive S phase of the cell cycle (12). However, the data in Table 1 demonstrate that, following granulopoietic regeneration from repeated chemotherapy-induced perturbation, the in vitro sensitivity of the human granulopoiesis to Adriamycin remained unchanged. It then seems reasonable to assume that the pluripotent hematopoietic stem cells, at present not accessible to analysis in humans, similarly failed to develop resistance to Adriamycin. One can only speculate on differences in biological properties that permit the emergence of resistant tumor stem cells upon exposure to cytotoxic drugs (15), whereas stem cells of normal cell renewal tissues appear to retain their original sensitivity after in vivo drug exposure. Most likely, the low mutation rate of normal, nonneoplastic cells (1, 7) causes this stability even under the conditions of repeated perturbations.

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REFERENCES

H-P. Lohrmann and W. Schreml

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