Influence of Factors Derived from EMT₆ Tumors and from Bone Marrow of Tumor-bearing Mice on Tumor and Bone Marrow Stem Cell Kinetics

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ABSTRACT

Untreated EMT₆ tumors in BALB/c mice were used to assess the regulatory mechanisms of tumor growth in these animals. This tumor can be quantitated for clonogenic cells by in vitro techniques, and the hydroxyurea suicide method makes it possible to determine the kinetic status of the clonogenic cells. The untreated EMT₆ tumor does not seem to have internal humoral regulatory mechanisms explaining tumor growth kinetics. However, the exponentially growing EMT₆ experimental tumor releases a factor capable of stimulating quiescent splenic colony-forming units into cycle. This is also true of bone marrow taken from tumor-bearing mice.

This study was made possible using an in vivo-in vitro technique which separates the effector cells from the responder cells by a Millipore filter floating on the culture medium.

The relationship between tumor growth and normal hematopoietic tissue of the tumor-bearing animal is discussed.

INTRODUCTION

It is well documented that the growth rate of solid tumors decreases with time after inoculation of cells (14, 29). This is attributed to a decreased growth fraction and increased cell loss, but the mechanisms of these phenomena are not well known.

In normal tissues, regulation of cell proliferation seems to be due, at least in part, to humoral factors capable of inhibiting (12, 15) or stimulating (11, 22) cell division. Inhibitors have been found in some tumors, but these studies were not related to tumor growth kinetics (2, 21). We therefore decided to investigate the possibility that there are humoral factors involved in regulating tumor kinetics.

In addition, we undertook a study of the effects of the EMT₆ tumor on bone marrow stem cell kinetics. This is of some clinical importance, inasmuch as such information may help to explain the hematological disorders of cancer patients bearing nonhematological tumors and should aid in monitoring the chemotherapy regimens of those patients whose bone marrow may be erroneously considered as normal.

A certain number of investigations have described changes in the hematopoietic systems resulting from the growth of implanted experimental tumors. During the growth of certain tumors, the number of bone marrow CFU-S² has been shown to decrease (4, 6, 19) and in other cases to increase (17, 20, 24, 25). The proportion of CFU-S in DNA synthesis was found to increase in NCTC fibrosarcoma-bearing mice (5).

Some authors have shown that CFU-S increase (6, 17, 19) in tumor-bearing mice.

The unipotent granulocyte-macrophage stem cell level was shown to decrease in the bone marrow and to increase in the spleen of Ehrlich ascites tumor-bearing mice. (16).

In previous publications, we reported the existence of a stimulating factor secreted by bone marrow (11, 13) and by EMT₆ tumor (13) after treatment by drugs or irradiation. Others (22) have subsequently demonstrated that regenerating bone marrow releases a factor capable of stimulating CFU-S into cycle.

In this paper, we report the evidence for an in vitro elaboration of diffusible factors originating from the tumors which stimulate CFU-S into cell cycle.

MATERIALS AND METHODS

Animals

The animals used were 1350 BALB/c OLA females (Bicester, England) ages 10 to 12 weeks for the CFU-S and bone marrow studies and 310 BALB/c OLA females ages 14 to 18 weeks for the tumor grafts.

Tumors

EMT₆ cells of the BALB/c-derived mammary carcinoma were passaged alternately in vitro and in solid form in vivo, as has been described previously (27). The solid tumors were obtained by a single s.c. injection of 4 × 10⁵ cells from the cell cultures. Three days after cell inoculation, the dimensions of the palpable tumors were measured and their volume, assuming a hemiellipsoid, was calculated according to the formula of Dethlefsen (9).

We chose 3 stages of tumor growth for our experiments: beginning of the exponential phase equivalent to 20 ± 10 (S.E.) cu mm, (4 days after cell inoculation); the exponential phase equivalent to 100 ± 20-cu mm tumor (7 days after cell inoculation); beginning of the reduced growth phase equivalent to 220 ± 40 cu mm (10 days after cell inoculation).

The in Vivo Experiments

Three mice were killed per experimental group. The cells were flushed from the femurs and the tibias of the donor mice with a syringe using NCTC 199 medium containing 0.02 M 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer, pH 7.3 (Eurobio, Paris, France). The cells were suspended in 1 ml of medium per leg and counted.

The percentage of CFU-S in S phase was determined by the thymidine suicide technique of Becker and McCulloch (1) as follows. Two vials were prepared. One contained 200 µCi of [³H]dThd (specific activity, 15 Ci/mmol; CEA, Saclay, France) in 1 ml of NCTC 199 medium. The second vial contained 1 ml of the culture medium without [³H]dThd. After the addition of...
an equal aliquot of bone marrow (2.5 × 10⁶ cells) to each vial, the final volume was adjusted to 2 ml with NCTC 199. The 2 vials were incubated for 20 min at 37°. Eight ml of cold NCTC 199 were then added to each vial to dilute the concentration of cells to approximately 2.5 × 10⁵ cells/ml and to stop further incorporation of [³H]dThd. Of the final solutions with [³H]dThd 0.2 ml/mouse was injected i.v. into 8 mice (Group A), and of the solution without [³H]dThd 0.2 ml/mouse was injected into another group of 8 mice (Group B). The recipient mice were exposed 24 hr before injection to 740 rads total body irradiation from a ¹³⁷Cs source; 8 to 10 days after the injection, the recipients were killed, and their spleens were fixed in Bouin’s solution. The nodules on the spleens were counted to give an estimate of the number of CFU-S. The percentage of CFU-S susceptible to [³H]dThd suicide was calculated by:

\[
\text{% in } S = \frac{\text{Mean no. of spleen nodules in Group B} - \text{Mean no. of spleen nodules in Group A}}{\text{Mean no. of spleen nodules in Group B}}
\]

The in Vivo-in Vitro Technique

The release of soluble factors was assayed by a method described previously (11). The system was comprised of 2 cellular compartments separated by a Millipore filter (0.45 µm) (Millipore S.A., Malakoff, France); the filter prevents cell transit but allows free diffusion of molecules (Chart 1). The fact that cells are not capable of passing through or around the filter in this system has been verified by the observation that, when a [³H]dThd-labeled cell population was placed in the upper compartment, no labeled cells could be found in the lower compartment.

Tissue fragments to be tested were placed on the Millipore filter. The filter was then floated on the surface of the culture medium. At the bottom of the dish were placed the responder cells which were not treated and were in a known proliferative stage. After 24 hr of incubation, the responder cell population was tested to determine the effect of whatever factor was suspected to have passed into the lower compartment from the upper one.

With this technique, the 2 populations can be of the same nature (bone marrow-bone marrow, tumor-tumor) or of a different nature (tumor-bone marrow, bone marrow-tumor).

Responder Cells

Bone Marrow. The cells were flushed from the femurs and the tibias of the donor mice with a syringe using α medium (Eurobio) with antibiotics (penicillin, 200 units/ml; streptomycin, 0.2 mg/ml). The cells were suspended in 4 ml of medium plus 15% FCS (Eurobio, Paris, France) at a concentration of 2.5 × 10⁵ nucleated cells/ml, and placed in 25-cm² tissue culture dishes (Corning SA, Paris, France) (Chart 2).

EMT₆ Tumor. The tumors were removed from the host mice when their volume was 100 cu mm. Three tumors were used per experimental group. The tumors were trypsinized, and the number of cells was enumerated. An appropriate dilution was then made to give a suspension at 4 × 10⁵ cells/ml. Two × 10⁶ cells were placed in the tissue culture dishes with 5 ml of Waymouth’s medium (Eurobio) containing 15% FCS (Chart 2).

Preparation of Tissue Fragments Placed on the Filter

Bone Marrow. All 4 bones from each of 4 mice were used per experimental group. Six to 8 bone marrow plugs were placed on each Millipore filter. The Petri dishes containing the responder cells, medium, and filter with marrow plugs were then placed carefully in an incubator (37°; 5% CO₂ in air) for 20 to 24 hr.

EMT₆ Tumor. Three mice were used per experimental group. A thin central slice (0.5 mm) of each tumor was placed on the Millipore filter, one tumor slice per filter. The incubation was performed for 24 hr as described for the bone marrow.

Chart 1. Schematic presentation of the incubation system.

Chart 2. In vivo-in vitro technique for assessing tumor and CFU-S kinetics under the influence of stimulating factors. Procedure for measuring the presence of stimulating factor(s) from either tumors (left) or bone marrow plugs (right) capable of modifying the kinetic state of responder cells. The responder cells may themselves be tumoral (A-cells) or dispersed bone marrow (B-cells). After incubation, the percentage of A-cells in DNA synthesis is evaluated by hydroxyurea (HU) suicide. The percentage of cycling CFU-S in the B-cells aliquot is measured by [³H]dThd suicide. x-irrad., x-irradiation.
Assessment of Effects on Responder Cells

Response of CFU-S. For each experimental group, a pool of cells from 3 incubated Petri dishes was made by aspirating and discarding the incubation medium and then resuspending the responder cells adhering to the bottom of the Petri dishes in 1 ml of fresh NCTC 199 medium.

The percentage of CFU-S in S phase was determined by the thymidine suicide technique of Becker described previously. To each vial, 5 x 10^6 cells were added for the incubation, and 10^6 cells were injected i.v. per recipient mouse.

Response of EMT6 Tumor Cells. The filters and culture medium were removed from the Petri dishes, and the cells adhering to the bottom of the dishes were washed with 2 ml of fresh Waymouth’s medium without serum. Two Petri dishes were used per group.

To obtain the percentage of viable clonogenic cells and the proportion of these in DNA synthesis, we utilized the hydroxyurea suicide technique described by Rockwell et al. (26). After harvesting, the responder cells were counted and diluted. Two groups of tissue culture dishes were prepared. One (control) contained 4.5 ml Waymouth’s medium with 15% FCS plus 0.5 ml cell suspension to 800 to 1000 cells/ml. The other (suicide) contained 4.0 ml Waymouth’s medium with 15% FCS, 0.5 ml cell suspension, and 0.5 ml hydroxyurea solution (10 mM) (generously donated by Upjohn, Paris, France). The two groups were incubated for 1.5 hr. By the end of the incubation period, the clonogenic cells had adhered to the dishes. The supernatant was removed carefully, and 2 ml of Waymouth’s medium was added to both groups of dishes. This was then removed after a few min, and the procedure was repeated once again in order to remove residual hydroxyurea. Finally, 5 ml of Waymouth’s medium with 15% FCS was added to the dishes for an incubation period of 10 to 12 days. The clones were fixed, stained with crystal violet, and counted. Results were calculated as in [3H]dThd experiments.

RESULTS

In Vivo Experiments

Table 1 shows the proliferation pattern of bone marrow CFU-S in the mice in the course of the growth of EMT6 tumor. The bone marrow CFU-S were quiescent during the first days after inoculation. The percentage of CFU-S in S phase was 8.5 ± 3.9; this value was not significantly different from the control value (5 ± 2.7). The bone marrow CFU-S were in active DNA synthesis when the tumor volume reached 100 cu mm (36 ± 12.6% CFU-S in S phase) and remained in proliferation as the tumor increased to a volume of 220 cu mm (31 ± 3.9% in S phase).

Table 1

<table>
<thead>
<tr>
<th>Bone marrow CFU-S S phase</th>
<th>% of bone marrow CFU-S in S phase</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal bone marrow</td>
<td>5 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Bone marrow from EMT6 tumor-bearing mice</td>
<td>20-cm tumor volume</td>
<td>8.5 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>100-cm tumor volume</td>
<td>56 ± 12.6</td>
</tr>
<tr>
<td></td>
<td>220-cm tumor volume</td>
<td>31 ± 3.9</td>
</tr>
</tbody>
</table>

NS, not significant.

In Vivo-in Vitro Experiments

Controls

Previous studies have shown that the CFU-S of normal bone marrow are quiescent in both in vivo and incubated controls. Moreover, as is shown in Table 2, plugs of normal bone marrow do not trigger into cycle the CFU-S of responder cells, the DNA synthesis fraction of which remained at 5.3 ± 2.2%.

EMT6 responder cells were derived from 100-cu mm tumors. After 24 hr of in vitro incubation, there were 28 ± 3.1% clonogenic responder cells in DNA synthesis (average percentage for the combined 9 experiments).

Relation of Tumor to Tumor

Tumors of 20, 100, and 220 cu mm versus Normal Bone Marrow. As can be seen in Table 2 the proportion of CFU-S of normal responder bone marrow in DNA synthesis are 37.4 ± 7.9 and 33.6 ± 3.8% after incubation with fragments from tumors of 20 and 100 cu mm, respectively, and 28 ± 2.8% after incubation with fragments from 220-cu mm tumors.

Bone Marrow from Tumor-bearing Mice versus Normal Bone Marrow. We found (Table 2) a stimulation of CFU-S (31.4 ± 7.5%) in DNA synthesis after incubation with plugs originating from mice bearing 100-cu mm tumors. The marrow from mice with 20-cu mm tumors showed a slight effect which was not statistically significant [17.2 ± 10.3% (not significant)]. The bone marrow from the mice with the 220-cu mm tumors was not able to stimulate the responder cell population over the values obtained for the quiescent marrow of the normal controls, as indicated by the value of 4.7 ± 4.7% being in DNA synthesis.

Bone Marrow versus Tumor. Table 2 shows that there was no significant difference, in the percentage of EMT6 responder cells in DNA synthesis between EMT6 responder cells alone (28 ± 3.1%) or the same cells incubated with either normal bone marrow plugs (25 ± 3.9%) or bone marrow from mice bearing 20-, 100-, and 200-cu mm tumors (32.7 ± 4.7, 28.5 ± 1.6, and 22.3 ± 4.2%).

DISCUSSION

In a previous study (13), we reported data indicating that EMT6 tumors can respond to regulatory mechanisms after 1-β-D-arabinofuranosylcytosine treatment. Kinetic studies of the phenomenon showed that the first event was the release of a stimulating factor by the tumors. This factor was then found in

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the serum, with the response to stimulation observed 12 hr later in the responding tumor population.

The aim of the present work was 2-fold. We wished to determine whether untreated tumors, at various sizes and growth rates, release a factor capable of affecting tumor growth. This is of interest because it is known that the growth rate of tumors as well as the growth fraction decrease with tumor size and age (14, 29), but the mechanism of tumor growth regulation is not well understood as yet. Moreover, some studies have shown the existence of soluble factors stimulating the proliferation of the hemopoietic cells (13). Older tumors do not secrete inhibitory factors for tumor cells, and the identical behavior of bone marrow from tumor-bearing mice, since no tumor cells have been found in these bone marrows (3). However, this does not explain why older tumors do not release such factors.

Based on the results reported here, we were not able to demonstrate the release of a factor by a nontreated tumor, whatever its size, capable of modifying the number of DNA-synthesizing tumor cells in the responder population.

It seems, therefore, that, at least in the EMT6 tumor, there are no internal humoral regulatory substances when the tumor is not perturbed by treatment.

The second aim of this work was to determine whether tumors release factors capable of stimulating bone marrow CFU-S. This was of interest because we have shown that bone marrow CFU-S, quiescent in normal mice, are in cycle in tumor-bearing mice. This has been observed previously by Croizat and Frindel (5) and Lala (19). Also, changes in splenic CFU-S concentration and proliferation state have been noted in Ehrlich ascites tumor-bearing mice (20).

Moreover, some studies have shown the existence of soluble factors stimulating the proliferation of the hemopoietic cells either in the serum of tumor-bearing mice (23, 24) or in tumor extract (7, 8).

The data presented in this paper demonstrate that exponentially growing EMT6 tumors are capable of stimulating CFU-S responder cells into synthesizing DNA. The larger tumors, however, are incapable of stimulating the CFU-S. Bone marrow from tumor-bearing mice has the same effect as the tumors: stimulation when tumors are in exponential growth; no effect when the host tumors reach a reduced growth rate.

In order to explain the absence of stimulation by older tumors and the identical behavior of bone marrow from tumor-bearing mice, one must keep in mind that the tumor is a heterogeneous population consisting not only of tumor cells but also of macrophages and lymphocytes (28). The experiments that we have conducted thus far cannot determine which of these cell populations are responsible for the release of the stimulating factors. Preliminary experiments with nude mice seem to indicate that the tumor free of T-lymphocytes does not release such factors.

It seems unlikely that macrophages are the responsible cells because older tumors are infiltrated with macrophages (28) and yet do not release stimulating factors.

T-lymphocytes may be involved with either directly secreting a factor effective on bone marrow cells or a factor that would induce tumor cells to secrete this factor. This could explain the preliminary nude mouse data and the behavior of the bone marrow from tumor-bearing mice, since no tumor cells have been found in these bone marrows (3). However, this does not explain why older tumors do not release stimulating factors. This phenomenon may be due to the fact that, although macroscopically the older tumors are not necrotic, they show numerous necrotic zones under the microscope. This confirms data on bone marrow that only live cells can secrete these factors (13). Older tumors do not secrete inhibitory factors for EMT6 cycling cells. However, it is possible that the older tumors may release factors inhibiting CFU-S proliferation if these inhibitors are not tissue specific. This possibility will be explored.

In summary, it would appear as though untreated EMT6 tumors at different stages of growth were not capable of modifying the kinetics of other tumor cells. These tumors do, however, stimulate CFU-S into cycle, most probably via a long-range diffusible factor. This phenomenon did not exist when tumors reached a volume of 200 cu mm or when tumors were grown on nude mice. The bone marrow of tumor-bearing mice behaved in the same manner as did the tumor itself. Experiments testing the role of lymphocytes are not as yet sufficiently

### Table 2

<table>
<thead>
<tr>
<th>Tissue fragments</th>
<th>Normal bone marrow</th>
<th>EMT6 tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of experiments</td>
<td>% of CFU-S in S phase</td>
</tr>
<tr>
<td>Control</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ± 2.2</td>
</tr>
<tr>
<td>Normal bone marrow plugs</td>
<td>10</td>
<td>17.2 ± 10.3</td>
</tr>
<tr>
<td>Bone marrow from tumor-bearing mice&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-cu mm tumors</td>
<td>4</td>
<td>31.4 ± 7.5</td>
</tr>
<tr>
<td>100-cu mm tumors</td>
<td>7</td>
<td>4.7 ± 4.7</td>
</tr>
<tr>
<td>220-cu mm tumors</td>
<td>3</td>
<td>2.8 ± 2.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Normal bone marrow or EMT6 tumor cells grown in vitro.

<sup>b</sup> ND, not done; NS, not significant.

<sup>c</sup> Mean ± S.E.

<sup>d</sup> Bone marrow plugs taken from mice bearing tumors of the volumes indicated.

* Equal-sized slices from tumors grown in vivo to the volumes indicated.
convincing and need further confirmation which will be reported subsequently.

The fact that a tumor is capable of provoking CFU-S entry into cycle bears importance for clinical chemotherapy treatments of non-hematological tumors.

REFERENCES

Influence of Factors Derived from EMT6 Tumors and from Bone Marrow of Tumor-bearing Mice on Tumor and Bone Marrow Stem Cell Kinetics

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