Effect of Surgical Removal on the Growth and Kinetics of Residual Tumor

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ABSTRACT

Findings from this study using a transplantable C3H mammary tumor failed to indicate interaction relative to growth parameters between two foci present in the same host. Whether they were growing alone or in the presence of a second focus, tumor growth rates were similar until the combined mass of multiple tumors approached that which was incompatible with survival. Only then was a difference in growth observed. Cytokinetic parameters, i.e., labeling index, primer-dependent DNA polymerase index or growth fraction, DNA synthesis time, tumor doubling time, and cell cycle time, were also similar whether tumors grew alone or in the presence of a second focus. Following removal of a tumor, changes were observed within 24 hr in the kinetics of the residual focus. There was an increase in labeling index (duration = 10 days) and primer-dependent DNA polymerase index with a decrease in the tumor doubling time. Minimal change was noted in DNA synthesis time and cell cycle time. The kinetic changes observed were reflected in a measurable increase in tumor size = a week following tumor removal. Absence of an alteration in DNA synthesis time and cell cycle time indicates that the increase in tumor growth was probably due to a conversion of noncycling cells in G0 phase into proliferation. Relationship of the findings to the use of adjuvant chemotherapy is considered.

INTRODUCTION

Numerous investigators have reported that the presence of a primary tumor inhibits growth of metastases (1, 2, 4, 5, 8-10, 12, 15, 19) and that its removal accelerates their growth (7-9, 12, 15, 19). The degree of inhibition varied relative to the type of tumor and its volume (6). Findings from those studies were obtained by observation of gross tumor growth. None supplied information relative to cellular change. With the advent of methodology for ascertaining cytokinetic parameters, considerable information has accumulated regarding the growth characteristics of both primary and metastatic tumors. Few investigations, however, have been carried out to determine the effect of removal or manipulation of a primary tumor on the kinetics of metastases. The reports by Simpson-Herren et al. (16, 17) describing the consequences of excision of primary s.c. Lewis lung tumors on kinetics of lung metastases provide, to our knowledge, the only information in that regard. They observed that noncurative excision resulted in an increase in the LI and growth rate with minimal changes in the Tc and Ts of the metastases. There was a consistent decrease in median life span as a result of stimulation of growth of lung nodules. Since much of the rationale for the use of postsurgical adjuvant chemotherapy has arisen from those contributions, there is clearly a need for further investigation utilizing a variety of models to determine the universality of the findings. As a consequence, the present studies using a C3H mammary tumor have been carried out to determine the cell kinetics of multiple tumor foci and to ascertain whether kinetic changes occur in a residual tumor following removal of a distant focus.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old female C3HeB/FeJ mice from The Jackson Laboratory, Bar Harbor, Maine, were used. All animals were housed in separate cages and fed standard laboratory chow and water ad libitum.

Tumors. The spontaneous mammary adenocarcinoma originating in a female C3H/HeJ mouse was passed by biweekly s.c. transplant in C3HBe/F-eJ mice. Tumor cell suspensions were prepared by mincing tumor fragments with scissors on an 80-mesh nylon screen and washing the cells through the screen with Medium 199. The cells were counted using trypan blue exclusion as a test of viability. Viable tumor cells (2 x 10^6) were inoculated s.c. in the left hind leg proximal to the popliteal node to induce tumors arbitrarily designated as "large," and 3 x 10^6 tumor cells were injected into the right hind leg at the same location to induce tumors designated as "small." In some animals, large tumors were produced in both legs by inoculation of 2 x 10^6 cells.

Tumor Growth. Tumor growth was followed by caliper measurement of 2 perpendicular diameters. Tumor volumes were calculated, and curves of mean volume versus time were plotted on semilog paper. Td was derived from the slope of the curve.

Experimental Design. Eighty-six animals were given injections of 2 x 10^6 and 3 x 10^6 tumor cells in the left and right hind leg, respectively, at the same time from the same suspension and divided into 6 groups consisting of 12 to 15 mice each. The large tumors were removed from 3 of the groups at 14, 21, or 28 days after inoculation of tumor cells. The remaining 3 groups served as controls for the tumor-amputated animals. The left tumor-bearing leg was removed by amputation under ether anesthesia. There was no operative mortality following removal of 14- and 21-day tumors. Eighty % of animals survived removal of 28-day tumors.

In Vitro Labeling Methods. Animals were sacrificed by cervical dislocation, and tumors were removed immediately. A single-cell suspension was prepared by mincing the tumor in

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2 To whom requests for reprints should be addressed, at Department of Surgery, University of Pittsburgh School of Medicine, 914 Scaife Hall, 3550 Terrace Street, Pittsburgh, Pa. 15261.
3 The abbreviations used are: LI, labeling index; Tc, cell cycle time; Ts, DNA synthesis time; Td, tumor doubling time; dThd, thymidine; PDP, primer-dependent DNA polymerase; PDPI, primer-available DNA polymerase index.
McCoy's medium with 20% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) at room temperature and filtered through a nylon screen. The single-cell suspension (3 to 5 x 10^7 cells/ml) was incubated for 1 hr at 37° in fresh medium with 2.5 μCi [3H]dThd per ml (14 to 17 Ci/mmol) in single-label studies and for an additional 30 min with 0.25 μCi [14C]dThd per ml (54 mCi/mmol) for double labeling. Labeling was terminated by inserting tubes containing cells into ice. The cells were washed with cold Ca2+-Mg2+-free Eagle's minimal essential medium (Grand Island Biological Co.). After washing, the cells were digested in 0.25% Bacto-trypsin (Difco Laboratories, Inc.) plus 0.1 mg DNase per ml (Sigma Chemical Co., St. Louis, Mo.) and incubated for 10 min at 37°. They were centrifuged at 500 x g for 3 to 5 min and resuspended in fresh medium. Viability tests were made by trypan blue exclusion. Cell viability was in excess of 90% (100% viability was not infrequent). The cells were centrifuged at 500 x g for 3 to 5 min, resuspended in cold 0.1 M citric acid for 2 min, and centrifuged again at 500 x g for 3 to 5 min. They were resuspended in Carnoy's fixative for 5 min at 4°, and "drop" preparations were made.

**PDP Assay.** The growth fraction was estimated in vitro by means of PDP assay (13, 14). Tumor imprints were made from the freshly cut surface of tumors on acid-cleaned slides. The slides were air-dried, dipped in 0.25% agar solution, and dried again. The slides were incubated for 45 min at 37° in 0.5 ml of an incubating mixture containing dATP, dGTP, and dCTP; 5 mM MgCl2; Ficoll (Sigma Chemical Co.); 5 μCi [3H]TTP (17 to 54 Ci/mmol). The incubation mixture was buffered with Tris-HCl, pH 7.4 at 25°. Following incubation, slides were fixed in methanol, rinsed thoroughly in tap water and triple-distilled water, and air-dried. The nuclei are provided with all the necessary components for DNA synthesis with the exception of DNA polymerase and DNA capable of acting as primer template. When all the necessary elements are present, DNA synthesis occurs. Incorporation of [3H]TTP was detected on the slides by autoradiography. The fraction of PDP-positive cells/total cells was determined. In this paper, the PDP index is referred to as the growth fraction.

**Autoradiography.** Autoradiography was carried out as previously described in detail (3) using gold activation to intensify the latent image in the photographic emulsion, thus shortening the exposure time. With this technique, the exposure time for single-labeled samples is reduced to one day and for PDP samples to 5 to 6 days. Double-emulsion autoradiography was used to distinguish cells labeled with only [3H]dThd and those labeled with [14C]dThd.

**Counting Procedure.** To determine LI, PDPI, and Ts, 700 to 1500 cells were counted for each tumor sample. Slides were counted by either 2 or 3 observers, and the results, which were in good agreement, were pooled to obtain a mean value. The equations used in calculation of the kinetic parameters are those previously described by us (3).

**RESULTS**

**Effect of the Presence of a Separate Tumor Focus on the Growth and Kinetics of an Associated Tumor.** Growth rates were similar until the final stages of tumor growth, whether the tumors were growing alone or in the presence of a second focus (Chart 1). Only then were tumors in mice with a single focus observed to grow at a greater rate than did their counterparts with 2 tumor foci. This occurred whether tumors arose from large or small tumor cell inocula. The cytokinetic parameters were similar whether the tumor grew alone or in the presence of an additional focus (Chart 2). There was an equivalent decrease in LI during the period of observation, i.e., from the second to fourth weeks of tumor growth. A similar decrease in PDPI (growth fraction) occurred in both circumstances. A similar increase in Ts and Tc occurred in the single growing tumor and the one accompanied by a second tumor focus. The kinetics during the 2 to 4 weeks of tumor growth when a small growing tumor was used for comparison were similar to those for a larger growing tumor and were not affected by the presence of a second focus.

**Effect of Tumor Removal on Growth and Kinetics of Cells in a Residual Tumor Focus.** Mice, each bearing a large and a small tumor focus, were randomly assigned to one of 3 groups.
Growth of Residual Tumors after Surgery

In one of the groups, the large tumors were removed at 14 days of growth, in another group at 21 days, and in the third at 28 days of growth. Such removal resulted in acceleration of the growth of the residual smaller tumor focus in all groups but more prominently when 14- and 21-day tumors were amputated (Chart 3). Following removal of a 14- or 21-day-old tumor, a decrease in Td was evident for about a week when compared to the Td of single tumors of the same size (isometric) (Table 1). When tumor removal was delayed for 28 days, no decrease in Td was observed.

The cell kinetics of residual tumor foci were studied following removal of large tumors after 14, 21, or 28 days of growth (Chart 4). When compared with control values, i.e., those values on the day of operation, the LI was found to have increased. While there was an increase noted 1 day after removal of tumors in all groups, the increase was greatest when tumors were removed after 14 or 21 days of growth (125% of controls in the former and 144% in the latter). The LI remained greater than control values for ≈10 days after tumor removal in those 2 groups. Removal of the large tumor at a later stage of growth (28 days) also induced an increase in LI (115% of control) but to a lesser extent than when 14- or 21-

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**Table 1**

<table>
<thead>
<tr>
<th>Age of tumor removed (days)</th>
<th>Time after removal (days)</th>
<th>Residual tumor (hr)</th>
<th>Single isometric tumor (control) (hr)</th>
<th>Residual tumor (hr)</th>
<th>Single isometric tumor (control) (hr)</th>
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<tr>
<td>14</td>
<td>0</td>
<td>38 ± 9.6*</td>
<td>34 ± 3.1</td>
<td>11.5 ± 1.2</td>
<td>11.8 ± 0.4</td>
</tr>
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<td>1</td>
<td>30</td>
<td>31 ± 1.6</td>
<td>31 ± 1.4</td>
<td>10.8 ± 0.8</td>
<td>10.2 ± 0.4</td>
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<tr>
<td>3</td>
<td>54</td>
<td>62 ± 10.5</td>
<td>62 ± 10.5</td>
<td>13.0 ± 0.8</td>
<td>12.2 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>99 ± 6.4</td>
<td>99 ± 6.4</td>
<td>11.1 ± 0.4</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>131</td>
<td>156 ± 10.5</td>
<td>156 ± 10.5</td>
<td>10.5 ± 0.8</td>
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<tr>
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<td>207</td>
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<td>217 ± 10.5</td>
<td>12.4 ± 0.4</td>
<td>12.0 ± 0.4</td>
</tr>
<tr>
<td>21</td>
<td>217</td>
<td>214 ± 12.0</td>
<td>214 ± 12.0</td>
<td>12.0 ± 0.4</td>
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<tr>
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<td>12.2 ± 0.5</td>
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<td>89 ± 5.8</td>
<td>10.9 ± 0.6</td>
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<tr>
<td>3</td>
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<td>102 ± 1.6</td>
<td>12.6 ± 0.8</td>
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<tr>
<td>7</td>
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<td>145 ± 11.5</td>
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<tr>
<td>10</td>
<td>158</td>
<td>303 ± 12.4</td>
<td>303 ± 12.4</td>
<td>10.7 ± 0.3</td>
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</tr>
<tr>
<td>14</td>
<td>253</td>
<td>370 ± 15.1</td>
<td>370 ± 15.1</td>
<td>14.3 ± 0.6</td>
<td>13.9 ± 0.8</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>129 ± 15.1</td>
<td>129 ± 15.1</td>
<td>13.9 ± 0.8</td>
<td>13.1 ± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>168</td>
<td>150 ± 12.8</td>
<td>150 ± 12.8</td>
<td>12.8 ± 0.6</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>136</td>
<td>144 ± 12.3</td>
<td>144 ± 12.3</td>
<td>12.3 ± 1.0</td>
<td>12.1 ± 0.6</td>
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<tr>
<td>7</td>
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<td>154 ± 12.4</td>
<td>154 ± 12.4</td>
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<tr>
<td>28</td>
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<td>129 ± 15.1</td>
<td>129 ± 15.1</td>
<td>13.9 ± 0.8</td>
<td>13.1 ± 0.6</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 animals.
day tumors were removed. The PDPI of residual tumors also increased to a maximum of 125, 121, and 108% of controls following removal of tumors at 14, 21, or 28 days, respectively. The Ts of residual tumors remained relatively constant following removal of large tumors (7.9 ± 0.12 (S.E.) hr). Following removal of a tumor after 14 days of growth, the Tc of the residual tumor focus remained relatively unchanged throughout the duration of the experiment (11.6 ± 0.3 hr) (Table 1). Similar findings were noted when tumors 21 or 28 days of age were removed.

DISCUSSION

The present investigation indicates that in the model used there was little interaction between multiple tumor foci which affected their growth rate. Only when the combined mass of multiple tumors approached that which was incompatible with survival was a slower rate observed. Cytokinetic studies also failed to indicate any interaction between the 2 tumor foci. In contrast to our findings, DeWys (2) and Simpson-Herren et al. (16) have shown that in mice bearing 2 simultaneous s.c. tumor implants, the total mass of the 2 tumors during their growth was equivalent to that of a single s.c. implant. Our observations are more in keeping with those of Rockwell and Kallman (11) who reported that there was no significant difference in either the growth rate or cell kinetics of single and multiple tumors in mice bearing a sarcoma. Numerous other investigators have also reported on the influence of a primary tumor on the growth rate of metastases or on a second tumor transplant (1, 2, 4–10, 16–18, 20). Despite their efforts, whether a primary tumor influences the growth of metastases or vice versa remains unclear because of the divergence of findings which are, in all probability, related to differences in host, tumor type, size of inoculum used, immunological variation, and other factors.

Despite lack of evidence in this model to indicate interaction relative to growth parameters between 2 separate tumor foci present in the same host, removal of one was found to affect the other. Changes in the kinetics of the residual tumor were observed within 24 hr. An increase in LI and PDPI (growth fraction) together with a shortening of the Td indicated an acceleration in growth of the residual tumor. Since there was minimal change in Ts and Tc, the increase in growth following removal of the "primary" tumor was probably not the result of a more rapid proliferation of the dividing cells but was more probably due to conversion of noncycling tumor cells in G0 phase into proliferation. Since the increase in the growth fraction observed at 1 and 3 days after tumor removal was no longer evident by 7 days, it is possible that these stimulated cells returned to the nonproliferating population after a few cell divisions. The increase in LI with minimum changes in Ts and Tc in lung metastases reported by Simpson-Herren et al. (16) following removal of a s.c. Lewis lung tumor is in accord with our findings. While the kinetic changes observed were not immediately reflected in a measurable increase in tumor size, the effect of removal of a tumor on the size of the residual focus was evident after 1 week. The growth of that focus was then more rapid than if the second tumor had remained in place.

If the rapid but transient change in the population kinetics of the residual focus after removal of a "primary" tumor, as described here and reported by others, is a consistent finding under a variety of settings, it could have significant implication relative to the use of adjuvant chemotherapy. At present, it is considered that certain factors negatively influence the success of chemotherapy given following removal of a primary tumor. Nonproliferating (G0) cells which retain their potential for proliferation, while sensitive to cell cycle nonspecific agents to some extent, are less responsive than those in the proliferating pool to cytostatic manipulation. Other factors which determine the responsiveness of a tumor population to chemotherapy are the variations of Tc and the degree of synchronization of cell cycles. Although the present investigations failed to demonstrate that tumor removal affected Tc or Ts and although they provided no information relative to synchronization, they did indicate that noncycling cells became proliferative and thus could become more vulnerable to cytostatic agents. The rapidity of the onset of the kinetic changes and their relatively short duration provide a rationale for the use of adjuvant chemotherapy as soon as possible following primary tumor removal. Confirmatory evidence from other host-tumor systems is necessary to support or deny this hypothesis.

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