Influence of the Interval between Primary Tumor Removal and Chemotherapy on Kinetics and Growth of Metastases

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ABSTRACT

In many animal models, primary tumor removal produces increased proliferation of cells in metastatic foci. The present investigations using a murine mammary tumor were carried out to determine how a variation in the time interval between primary tumor removal and administration of a single dose of cyclophosphamide (CY) affected labeling indices of residual tumor cells, their growth, and animal survival. The CY (240 mg/kg) had a more favorable effect when given on the day of tumor removal than 3 days after, a time when the labeling index (LI) of metastases was at a peak. It was least effective if given at 7 days following primary tumor excision, when the LI had returned to the preoperative level. The greatest effect occurred when the CY was given prior to operation. It completely prevented the increase in LI resulting from tumor removal, more effectively suppressed the growth of residual tumor, and prolonged survival to a greater extent than was noted under any other circumstance. The interval between tumor removal and administration of a relatively small amount of CY (60 mg/kg) was critical. When given on the day of tumor removal, an increase in LI of the residual focus occurred which was greater than that occurring as a result of tumor removal. When given 3 days after tumor removal, the smaller dose was almost as effective in suppressing LI as was the larger. From a kinetic standpoint, there was no advantage in reducing the tumor burden prior to the use of chemotherapy. The tumor response in this model suggests that, for the most effective control of metastases, the largest tolerable dose of chemotherapy would best be used at the time of or before primary tumor removal. The results provide a biological rationale for the use of perioperative adjuvant chemotherapy.

INTRODUCTION

Investigations over the years have indicated in many animal models that the presence of a primary tumor inhibited (1, 2, 6, 7, 10–12, 20, 22) and its removal accelerated (9–12, 15, 17, 21) metastatic tumor growth. Those studies exclusively used gross tumor growth as the index of effect. More recently, information has accumulated regarding the kinetic characteristics of both primary and metastatic tumors, but few investigations have determined the effect of removal or manipulation of a primary tumor on the kinetics of tumor cells in metastases. Simpson-Herren et al. (18, 19) described the consequences of excision of a Lewis lung tumor on the kinetics of lung metastases, and Schiffer et al. (16) briefly noted the alteration in kinetics of one tumor after complete or partial removal of a second of the same type. Our own studies (8) described in detail the kinetic changes occurring in a residual tumor focus following removal of a transplantable C3H mammary tumor. The increased cellular proliferation occurring in metastases, noted in all of the investigations, has received little attention, despite the potential importance of the observation. This kinetic perturbation, because of its potentially harmful effect through acceleration of metastatic growth, requires consideration as to how it may be either abrogated or exploited for therapeutic benefit. To our knowledge, no studies have been specifically directed toward that end. Such an effort could produce information which might provide a more precise biological rationale for the use of perioperative adjuvant chemotherapy.

The present report provides information concerning the effect of the time interval between primary tumor removal and CY3 therapy on the LI of metastatic or residual tumor cells. It correlates those findings with the tumor growth and survival of animals.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old female C3H/HeJ 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 maintained by transplant in C3HeB/FeJ 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 10⁶) were inoculated s.c. in the left hind leg proximal to the popliteal node to induce tumors arbitrarily designated as "primary," and 3 x 10⁶ tumor cells were injected into the right hind leg at the same location to induce tumors designated as being "metastatic;" or "residual tumor focus." Primary tumors were removed by amputation of the left tumor-bearing leg.

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.

CY. CY was dissolved in distilled water, and 60, 120, or 240 mg/kg were injected i.p. at times indicated.

In Vitro LI. Animals were sacrificed by cervical dislocation, and tumors were removed immediately. A single-cell suspension was prepared by mincing the tumor in 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 10⁶ cells/ml) was incubated for 1 hr at 37°C in fresh medium with 2.5 µCi of [3H]thymidine per ml (14 to 17 Ci per mmol). 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 the cells were washed, they were digested in 0.25% Bacto-trypsin (Difco Laboratories, Inc., Detroit, Mich.) plus DNase (0.1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) and incubated for 10 min at

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3 The abbreviations used are: CY, cyclophosphamide; LI, labeling index.

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They were centrifuged at 500 x g for 3 to 5 min and resuspended in fresh medium, and 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.

**Autoradiography and Counting Procedure.** Autoradiography was carried out as described in detail previously (3). To intensify the latent image, slides were incubated in KAuCu4 for 20 min at 15° and rinsed in distilled water just prior to development. The slides were then developed in Kodak Amidol developer for 15 min at 15°, fixed, washed, air dried, and stained with hematoxylin. Gold activation reduces the exposure time to 1 day. With this method, background labeling was approximately 4 grains/cell, while the grains over labeled cells were usually too dense to count. To determine LI, 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. LI is the percentage of labeled cells in the sample.

**Experimental Design.** In these experiments, groups of animals bearing 14-day-old tumors were subjected to primary tumor removal or CY administration. Those having their tumors removed on this day received either CY at the same time, CY 3 days or 7 days later, or no chemotherapy. When CY was the initial treatment, tumor removal was performed 5 or 7 days later.

**RESULTS**

**Effect of Tumor Removal.** Mice bearing both a large primary and a small secondary tumor focus were subjected to removal of the larger tumor. The LI of the residual focus was found to increase (Chart 1). By 1 day following tumor removal, there was a mean increase of 32% in LI; by the third day, it was 55% greater than that found at time of tumor removal. By 7 days, the average LI had returned to the control level. At each time, there was considerable variation in the LI of tumors. Amputation of a normal leg in mice with a single tumor of a size equivalent to the residual focus in animals with 2 tumors resulted in no increase in tumor LI. Removal of the primary tumor was followed by accelerating growth (p = 0.02) of the distant focus (Chart 2) in keeping with the observed changes in LI.

**Influence of the Interval between Primary Tumor Removal and CY.** CY was administered on the day of tumor removal. The effect on the LI of the residual focus was related to the amount of drug used (Chart 3). When 60 mg/kg were used, the LI of tumors after 24 hr was greater than that observed in concomitant controls which received no chemotherapy following tumor removal. At the third and fourth days, however, LIs were considerably lower than those in untreated tumor-removed controls but were little different from those observed prior to treatment. At 7 days, the LI in both untreated and treated mice were at the level observed at the time of tumor removal. When CY (120 or 240 mg/kg) was administered, no increase in LI was observed at any time subsequent to tumor removal; in fact, a marked depression was noted which was sustained for at least 7 days when the larger dose was used. When tumor growth curves were examined, they reflected the kinetic changes (Chart 4). The growth of tumors in animals receiving the small dose of CY (60 mg/kg) at the time of amputation was uninhibited. When 240 mg/kg were used
used, the greatest tumor growth retardation was observed.

If CY was given 3 days following tumor removal at the peak of the increase in LI, the depression of LI noted on the first 3 days following chemotherapy was similar when CY (60, 120, or 240 mg/kg) was used (Chart 5). When the 60- or 120-mg/kg dose was used, the peak depression occurred 3 days after drug administration, with a return to control levels by the seventh day after therapy. A greater depression occurred at the fourth day following 240 mg/kg, and by 7 days the LI had not yet returned to control levels. The effect on tumor growth when CY was given 3 days after amputation was, as when therapy was given at the time of amputation, dose related.

Administration of CY (240 mg/kg) 7 days following tumor removal resulted in a reduction in LI during the next 7 days (Chart 6). Removal of a primary tumor 5 days following CY administration, at the nadir of the LI resulting from the chemotherapy, failed to produce an increase in LI greater than that which occurs during recovery from chemotherapy (Chart 6). By 12 days (7 days following tumor removal), the level of the LI had almost returned to that in non-CY-treated tumors. The nadir of the LI was similar when CY (240 mg/kg) was given at the time of, 3 or 7 days following, or 5 days prior to tumor removal.

When compared with animals having primary tumors removed and no chemotherapy (Group A), those receiving CY (240 mg/kg) either at the time of tumor removal (Group B) or 3 (Group C) or 7 (Group D) days later all demonstrated retardation of the growth of a residual tumor focus (Chart 7). When CY was given 5 or 7 days prior to tumor removal (Group E), a similar effect was noted. Because of the concordance in growth, the findings were combined. In each circumstance, a different growth pattern emerged. For the first 10 to 12 days, the most effective suppression of growth occurred in the animals (Group E) receiving CY followed by tumor removal 5 or 7 days later. Progressively delaying the onset of chemotherapy following amputation suppressed the growth of successively larger tumors. In all groups receiving CY, the length of the initial retardation was variable, as was the subsequent growth rate. By ≈ 3 weeks, the size of tumor in the various treated groups was similar.

Survival of animals was evaluated relative to the interval between primary tumor removal and CY administration (Chart 8). Mortality during the first 4 weeks following the initiation of treatment was considerably lower when the CY was administered prior to or at the time of primary tumor removal. Survival during that time decreased with a prolongation of the interval between tumor removal and CY administration, but even when given 7 days later, there was a notable benefit. Subsequently,
the mortality rate was more rapid in those who received CY at the time of tumor removal than in those receiving the drug prior to operation; median survival was 28 days in the former and 34 days in the latter.

Influence of CY in the Presence or Absence of a Primary Tumor. When CY was administered to a group of animals on the day of their tumor removal and to a second group whose tumors were left intact, no significant difference in the LI of metastatic tumors in the 2 groups was observed (Chart 9). Both the decrease and subsequent increase of LI were similar in the groups. These findings occurred whether CY (120 mg/kg) or CY (240 mg/kg) was used. When the administration of CY was delayed for 3 days, although the LI in the group with the tumor removed was higher at the time of CY administration, the subsequent depression and recovery of LI were similar in the 2 groups. Growth curves were in keeping with findings relative to LI. No significant difference was observed in the growth of metastases between those having a primary tumor removed and those having a primary tumor left intact.

DISCUSSION

The current findings provide additional support to those noted by us previously (8) indicating that a transient increase in the LI of a residual tumor focus occurs during the first 3 to 4 days following primary tumor removal but not following amputation of a normal leg. The observed kinetic changes are associated with more rapid growth of metastases. These studies demonstrated that tumor removal was not associated with shortening of cell cycle or DNA synthesis times. Thus, the increase in LI was due to noncycling cells becoming proliferative and therefore 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.

The present investigations tested this hypothesis. They indicate the importance, in this model, of the interval between tumor removal and the initiation of adjuvant chemotherapy. The shorter the time after operation that therapy is begun, the more complete is the abrogation of the kinetic changes in distant tumor foci. Consequently, the more effective becomes the suppression of residual tumor burden and the more prolonged is the survival. A single large dose of CY was more effective when given the day of operation than when given after an interval of 3 days, a time when the LI of the metastasis was at a peak. This benefit may be attributed to the effect of CY on tumor cells in the G0-G1 compartment which are about to go into S phase and which are stimulated to do so by tumor removal. The therapy was least effective if the interval was 7 days when the LI had already returned to the preoperative level and an additional increment of tumor growth had occurred. Administration of CY and delay of operation until the LI was at the nadir completely prevented an increase in LI following tumor removal and was associated with a more effective suppression of residual tumor burden and prolongation of survival than occurred under any other circumstance.

The findings provide a biological rationale for the administration of perioperative adjuvant chemotherapy which is different from that which provided the basis for such therapy in the late 1950's and early 1960's (4, 5, 13, 14). At that time, it was used for the purpose of destroying those tumor cells which were disseminated at the time of operation and which were considered to be responsible for the development of metastatic tumor. The modest effect of the therapy in those early studies may well have been due, at least in part, to the destruction of cells in existent metastatic foci which had been stimulated by the removal of the tumor rather than by the elimination of circulating tumor cells. Of interest is the observation that when CY (240 mg/kg) was used at different times in relation to tumor removal, i.e., before, at the time of, or at various times after, the nadir of the LI was essentially the same, despite the variation in the proportion of labeled cells existing at the time of drug administration. That finding indicates that the effectiveness of the drug in suppressing cell division was related to the proportion of cells undergoing DNA synthesis at the time of its administration.

The findings also indicate that, when a relatively small dose of CY (60 mg/kg) was used, the interval between its administration and tumor removal was critical. Its effectiveness was related to the proliferative activity of the metastases. When given to an animal whose metastatic focus contained more cells in S phase because of primary tumor removal, i.e., at 3 days, it was almost as effective in suppressing LI as was the larger dose of the same drug. Recovery of cell proliferation was, however, more rapid following the lower dose. When the smaller amount was admin
lished at the time of primary tumor removal, an increase in LI resulted which was greater than that occurring as a consequence of the tumor removal. That increase is difficult to explain, since the administration of the same dose of drug to animals with a single intact tumor was earlier found to result in a depression of the LI (3). The tumor response in this model suggests that, for chemotherapy to be most effective for controlling micrometastases, it should be given using the largest tolerable dose at the time of or before primary tumor removal.

It is generally assumed that chemotherapy is apt to be more effective when there is a lower total tumor burden in a host. The present finding that the reduction in LI of metastases following CY administration was similar whether the primary tumor had or had not been removed does not support that thesis. It suggests that from a kinetic standpoint there is neither an advantage nor a disadvantage when surgical efforts are used to reduce tumor burden ("debulking") prior to the use of chemotherapy.

It is not to be inferred from these studies that a delay in administration of chemotherapy beyond the time when the kinetic perturbation in a distant tumor focus after primary tumor removal had disappeared will negate all benefit from such therapy. In these studies, prolongation of survival was observed no matter when the therapy was instituted. The advantage of earlier therapy is that it eliminates the transient acceleration of growth resulting from removal of the primary tumor. By delaying the onset of therapy, it will be directed toward a larger tumor burden having a different sensitivity to the drug. Although there is no information relative to whether, and if so when, primary tumor removal in the human affects the kinetics of residual tumor microfoci, it would seem worthwhile to use adjuvant therapy at the time of and/or prior to tumor removal in order to test the thesis that by eliminating a kinetic response to tumor removal there will result an improvement in patient outcome.

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REFERENCES

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