Effect of Local or Systemic Treatment Prior to Primary Tumor Removal on the Production and Response to a Serum Growth-stimulating Factor in Mice[1]

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ABSTRACT

This report indicates that not only does the preoperative administration of cyclophosphamide or radiation prevent the kinetic changes observed in a distant tumor focus following tumor removal but that the preoperative administration of the antiestrogen tamoxifen and the luteinizing hormone-releasing hormone analogue Zoladex are equally effective in that regard. It also provides evidence indicating that serum obtained from mice treated with those therapies when transferred to a recipient bearing a tumor of a similar type to that in the donor fails to stimulate DNA synthesis in the tumor of the recipient. In contrast, an increase in labeling index occurs following transfer of serum obtained following tumor removal from untreated mice. Moreover, when tumor-bearing mice were treated by each of the four modalities prior to receiving serum obtained from untreated donors following removal of a tumor, no kinetic changes were observed in the tumor of the serum recipient.

INTRODUCTION

Investigations by us have indicated that, following removal (1-3) or radiation (2, 3) of a primary tumor (but not after amputation of a non-tumor-bearing leg), there occurs an increase in the LI[3] of a distant tumor (focus/metastases) which results in an acceleration of tumor growth. We subsequently conducted studies to determine whether there was an optimal interval between operation and the administration of adjuvant chemotherapy which was important for preventing those kinetic changes (4). The most effective control of residual tumor growth and augmentation of survival occurred when the largest tolerable dose of chemotherapy was administered prior to primary tumor removal. Preoperative radiation also eliminated the increase in the proportion of tumor cells undergoing DNA synthesis following tumor removal (3).

Those findings relative to the kinetic changes in metastases and the effect of preoperative therapy in the animal model used provide a biological rationale for considering clinical trials to test the hypothesis that the use of adjuvant therapy prior to tumor removal, by eliminating the ensuing kinetic response, will result in an improvement of patient outcome. In a companion paper to this report, we noted that the kinetic changes following tumor removal were not unique to the C3H tumor model which we had initially used but were also present when a variety of other tumors were used (5). Moreover, we provided evidence to indicate that the stimulation of cell growth resulting from tumor removal or radiation is due to the presence of a circulating growth-stimulating factor. Those findings provide additional evidence to justify carrying out a clinical trial.

The current studies were undertaken to determine (*a*) whether

a number of preoperative therapies differing in their mode of action could prevent the kinetic changes occurring in residual tumor following removal of the primary, (*b*) whether serum obtained from animals so treated continues to contain a growth-stimulating factor, and (*c*) whether tumors in treated recipients of serum containing the growth factor remain responsive to the factor. This report presents the findings from those studies.

MATERIALS AND METHODS

Experimental Animals and Tumor Used. Young adult female C3HeB/FeJ mice from The Jackson Laboratory, Bar Harbor, ME, were used in these experiments. A spontaneous mammary adenocarcinoma which arose in a C3H/He mouse and has been carried by transfer in C3HeB mice was used.

Tumor Cell Preparation. Tumor cell suspensions for animal inoculation were prepared by mincing tumor fragments with scissors on an 80 mesh nylon screen and washing the cells through the screen with Medium 199 (Difco Laboratories, Inc., Detroit, MI). Preliminary experiments were done to determine the size of inoculum required to produce a 3- to 5-mm tumor in the left leg and a 5- to 7-mm tumor in the right in 14 days. In all animals containing two tumor foci, the larger is designated as the "primary" and the smaller one as the "metastatic" or residual focus. Serum recipients were animals containing a single tumor focus which was similar in size and duration of growth to the smaller of the tumors in animals with two foci. Tumors were removed by amputation of the leg under light ether anesthesia.

Cell suspensions for LI determination were prepared by mincing the entire tumor in Dulbecco's Ca^+- and Mg^+-free PBS (Gibco, Grand Island, NY). Cells were washed and resuspended in the PBS.

LI. The LI was determined by autoradiography using single cell suspension (3 to 5 × 10^7 cells/ml) incubated with [^{3}H]thymidine (14 to 17 Ci/mmol) (Du Pont NEN Products, Boston, MA). The method has been repeatedly described (1, 2, 5, 6). The percentage of tumor cells undergoing DNA synthesis (LI) was determined by counting 1000 cells identified by their morphological characteristics as tumor cells. Labeling indices were determined on groups of animals sacrificed 1, 3, 5, and 7 days after tumor removal. In studies involving serum transfer, the LI was determined 1 day after serum administration.

Serum. Mice were bled from the retroorbital plexus, and the serum was separated at once, pooled, and stored at -90°C until used. Preliminary investigations showed that storage at this temperature did not alter the effectiveness of the serum. Sera were collected either with the tumors in place or at intervals after primary tumor removal. Serum from nontumorous mice was also collected. One ml of serum was administered to recipient mice i.p.

Irradiation of Primary Tumors. Irradiation of tumors was carried out by anesthetizing mice with pentobarbital sodium. They received 50 Gy from a Therapi 4 linear accelerator in a period of 12 min. That dose of radiation was selected because preliminary evaluation indicated that it was the maximum tolerated dose which retarded tumor growth. Control animals were sham radiated, that is, they were anesthetized with pentobarbital for the same period of time as were the radiated animals.

Drug Administration. CY was diluted so that 240 mg/k were contained in 0.01 ml/g of body weight. It was administered i.p. as a single dose 5 days prior to tumor removal or to tumor-bearing recipients 5 days before their inoculation with serum. Zoladex (ICI 118,630) is a LHRH analogue that induces pituitary down-regulation resulting in a medical castration which is reversible on cessation of therapy. It is administered as a sustained release depot containing 0.05 mg of the drug. The depots were implanted s.c. on the abdomen of the mice 14
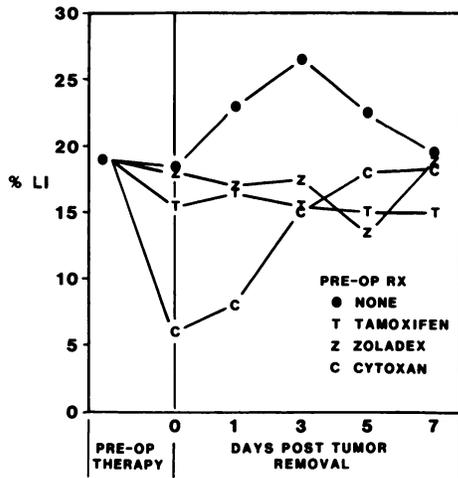
Fig. 1. Effect of treatment of mice with cyclophosphamide (Cytoxan), tamoxifen, or Zoladex prior to removal of a C3H mouse tumor on the LI of a residual tumor focus over time (1 to 7 days). Each *point* represents the average value of at least six mice. Animals receiving no preoperative therapy prior to tumor removal are designated as "none."

days prior to tumor cell inoculation and were present during the entire experiment. TAM (Nolvadex) is a nonsteroidal compound with estrogenic activity in the mouse. As the free base in powder form, it was mixed with laboratory chow at a concentration of 1 mg/g of ground chow and fed to mice from the day of tumor cell inoculation and throughout the entire experiment. Both the Zoladex and the tamoxifen were supplied by ICI Pharmaceuticals, Wilmington, DE.

RESULTS

Effect on LI of Residual Tumor. The increase in LI observed in residual tumor following excision of a primary tumor was completely prevented if animals were treated prior to operation (Fig. 1). The administration of a single dose of CY 5 days before tumor removal resulted in a marked decrease in the LI so that at operation, it was 6 ± 0.6% instead of 18 ± 0.4%. Following tumor removal, the LI in the residual tumor focus gradually returned to the control level. Thus, the 44% increase in the LI of residual tumor in nontreated animals was prevented. Feeding TAM or implanting Zoladex prior to tumor removal was also effective in completely preventing the kinetic change in metastases observed after operation. The effect of pretreatment in preventing an increase in LI 24 h following primary tumor removal is displayed for individual animals (Fig. 2).

Treatment of the Serum Donor. The treatment of serum donors with CY, TAM, or Zoladex before removal of a primary tumor prevented an increase in the LI of tumors in recipients such as occurred when the serum was derived from operated animals that were untreated (Fig. 3). The mean LI (18 to 20%) in recipients receiving serum from treated animals was equivalent to that observed when serum from normal mice or those bearing an unremoved tumor was transferred (18%). Transfer of serum obtained from non-tumor-bearing mice that received tamoxifen failed to affect the LI in recipient tumor (not shown), indicating that the inhibition was not related to tamoxifen or its metabolites present in the serum transferred. Radiation of the primary tumor in the serum donor prior to its removal also abrogated the increase in LI of the recipient tumor following serum transfer (Fig. 3).

Pretreatment of Serum Recipient. The transfer of serum following removal of a tumor from untreated animals to tumor-bearing recipients treated with CY, TAM, or Zoladex failed to increase the LI in tumors as was observed when such serum was transferred to nontreated recipients (Fig. 4). When the recipient tumor had been treated with radiation prior to the transfer of such serum, no effect on tumor LI was observed.

DISCUSSION

The present findings clearly indicate that the administration of cyclophosphamide, tamoxifen, or Zoladex prior to the re-

Fig. 3. Effect of serum obtained from donors treated with cyclophosphamide (Cytoxan, CY), tamoxifen (TAM), Zoladex (Z), or irradiation (XRT) prior to tumor removal on the LI of recipient tumor. Animals receiving no preoperative therapy are designated as "none."
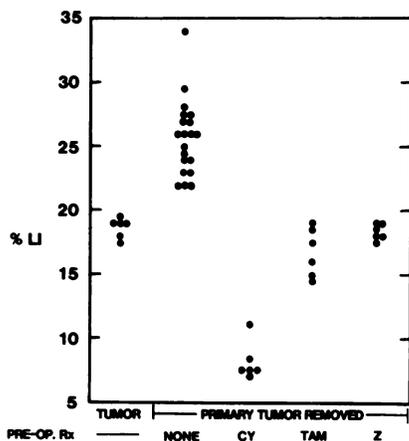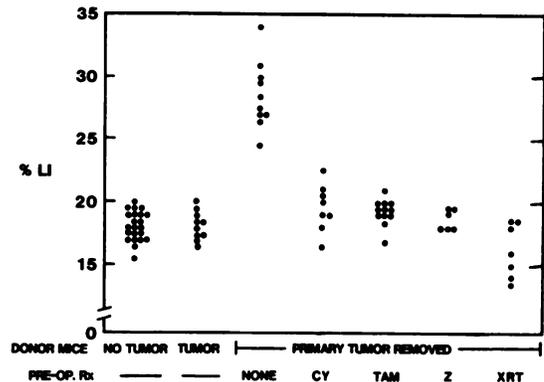

Fig. 2. Effect of treatment of mice with cyclophosphamide (CY, Cytoxan), tamoxifen (TAM), or Zoladex (Z) prior to removal of a C3H mouse tumor on the LI of a residual tumor focus of individual mice after 24 h. Each *dot* in all subsequent figures represents the LI of a residual tumor in a single mouse.
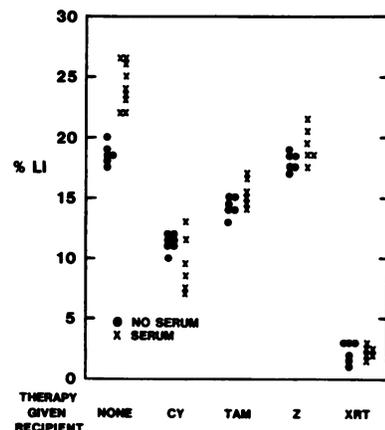

Fig. 4. Effect of serum obtained from mice following tumor removal on the LI of tumors in recipients treated with CY, TAM, Zoladex (Z), or XRT prior to serum transfer.

moval of a primary tumor prevents the increase in the LI of a distant tumor focus observed in untreated animals. Such a kinetic perturbation has previously been shown by us to result in enhanced tumor growth. In a companion paper we provide information indicating that the changes in LI are related to a serum-stimulating factor which promotes DNA synthesis in the cells of a distant metastatic tumor focus. Since in the model system used both the primary tumor and distant metastases were in the same animal, they should be similarly affected by the systemic therapies administered preoperatively. Consequently, studies were conducted to determine whether the production of growth factor is prevented by the therapies used and/or whether the treatments inhibit response to the stimulating factor. From the findings presented it seems that both functions are impaired by the treatment. Since serum obtained following tumor removal from animals which had received CY, TAM, Zoladex, or XRT failed to alter the LI of tumors in untreated recipients bearing an isologous tumor, it would seem that the production of a serum factor is inhibited. The failure of tumors in treated recipients to respond to the serum obtained from untreated donors following tumor removal indicates that there is an altered response of recipient tumors to the growth-stimulating factor as a result of the treatment.

Consideration has been given to how the various therapies used might impair both the production and the response to the stimulating factor. We had concluded from previously obtained data that tumors producing the factor are those which are capable of responding to it. The current findings tend to support that conclusion. They suggest that, since both cyclophosphamide and irradiation affect cycling tumor cells and tamoxifen arrests the cells in the G_0-G_1 phase, it is possible that the stimulating factor is elaborated during the cell cycle and that the more cells in cycle the greater is the amount of factor elaborated. Since the same kinetic changes occur in the cells of tumors in recipients following their treatment with the various modalities and they fail to respond to the serum-stimulating factor, it is likely that cycling cells, particularly those in the G_0-G_1 phase, are those which are responsive to the stimulating factor.

The LHRH agonist used in this study (ICI 118630; Zoladex) has been shown by numerous investigators to inhibit the growth of animal tumors and human mammary and prostatic cancers. Its mechanism of action is via a complex hormonal pathway, one of the end results of which is suppression of ovarian activity and consequently of estrogen. The tumor responses provided by this compound at high dose levels are equal to those observed following oophorectomy or tamoxifen treatment. Our investigations with the drug were initially directed toward comparing its effect with that of tamoxifen on estrogen receptor and cell kinetics in a mouse mammary tumor model. Zoladex inhibits estrogen production, and tamoxifen acts as an agonist resulting in increased estrogen output by ovaries. The current findings indicate that both agents, despite their disparate mechanisms of action, when administered prior to tumor removal equally prevent the increase in LI observed in metastatic foci. The serum transfer experiments indicate that both inhibit the production of and the response to a growth-stimulating factor.

Preoperative systemic therapy, sometimes referred to as "neoadjuvant" therapy, has been used in the treatment of pa-

tients with certain solid tumors (6–8). Its use has often resulted in a decrease in tumor size permitting less extensive surgery. No randomized trials, however, have been conducted to determine whether such treatment more effectively controls micrometastatic disease than does the same therapy administered after operation. Until recently there has been no clear biological rationale to justify the conducting of clinical trials to answer that question. With the accumulation of evidence from experimental systems to indicate that noncurative reduction of a tumor cell burden results in an increase in the proliferation of residual tumor cells (1–3) and with the present findings indicating that chemotherapy, tamoxifen, or radiation therapy given prior to operation prevents the increase in such cell proliferation, there is provided a basis for conducting trials to determine the worth of such therapy. Justification is also supplied by the Goldie-Coldman hypothesis which proposes that, as a tumor cell population increases, there is an ever-expanding number of drug-resistant phenotypic variants arising due to spontaneous somatic mutations (9). It is hypothesized by them that the initiation of therapy as early as possible will be of benefit by minimizing the risk of resistant cells occurring.

The two concepts, *i.e.*, that by Goldie and that relating tumor removal to kinetic changes in metastases, are not mutually exclusive. Appropriate preoperative therapy should prevent cell proliferation following tumor removal and, consequently, prevent an increase in the number of resistant cells in the metastatic population.

There is no assurance that similar kinetic changes observed in animal models occur following removal of tumors in the human and that the temporal pattern of the kinetic changes (should they occur) is similar; nor is there certainty that the Goldie-Coldman hypothesis has relevance to patients who have tumors which by the time of diagnosis have been present for months to years. None of these hypotheses justify the evaluation of preoperative therapy in the clinical setting. Such a trial is currently being carried out by the National Surgical Adjuvant Breast and Bowel Project.

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