The Effect of Estrone-Progesterone Treatment on Cell Proliferation Kinetics of Hormone-dependent GR Mouse Mammary Tumors

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SUMMARY

Hormone-dependent mammary tumors were induced in virgin GR mice by treatment with estrone and progesterone. Discontinuation of hormonal treatment was followed by regression of the tumor. This response to hormone treatment was also observed in the first transplant generation in inbred syngeneic hosts, but after several transplantations the tumor growth became hormone independent.

The hormone dependence of the primary tumors and tumors after a single transplantation was demonstrated by growth curves. Furthermore, the cell proliferation kinetics has been investigated in a growing as well as in a regressing hormone-dependent tumor after a single transplantation from the same primary tumor. The experimental data consist of growth curves, percentage-labeled mitoses curves, and labeling indices. Since these data do not contain information concerning the localization of the cell loss in the cell cycle, they were analyzed by a computer method based on three mathematical models differing in respect to the mode of cell loss. All three models gave approximately the same estimates of the cell kinetic parameters in the growing as well as the regressing tumor.

The results for the growing, hormone-dependent tumor showed a growth fraction of 62%, a cell production rate of 3.4% hr⁻¹, and a cell loss rate of 2.3% hr⁻¹.

Regression of the tumor after hormonal deprivation was accompanied by a decrease in growth fraction to 18% and a decrease in the cell production rate to 0.9% hr⁻¹, while the cell loss rate was unchanged at 2.8% hr⁻¹. Furthermore, the discontinuation of hormonal treatment introduced an increase in the mean transit time of the cell cycle, particularly in the mean transit time of the G1 phase.

The results might indicate that estrone and progesterone treatment stimulated growth of hormone-dependent GR mouse mammary tumors mainly by an increase of growth fraction and cell production rate.

INTRODUCTION

Clinical experience has shown that human mammary cancers are HD or hormone responsive in more than one third of the cases (17). HD mammary tumors of rats and mice are considered to be suitable models for the investigation of the influence of hormones on mammary cancer. The growth of many rat and mouse mammary tumors is known to be influenced by several hormones, e.g., estrogens, progesterone, and pituitary hormones (13). However, the mechanism of the growth-stimulating effect of hormones remains unclear. The purpose of the present work has therefore been to investigate the effect of estrone-progesterone treatment on the cell proliferation kinetics of HD GR mouse mammary tumors.

Treatment with progesterone and estrone can induce mammary adenocarcinomas in GR mice that carry the mammary tumor virus (3, 14, 19). Discontinuation of hormonal treatment is followed by a significant regression of the tumor and, in turn, if hormones are administered again, the tumor resumes its growth. This hormone dependence also can be demonstrated after transplantation of the tumor, but successive transplantations usually lead to hormone independence (3, 14).

Growth curves have been plotted to demonstrate the effect of progesterone and estrone on primary tumor growth and growth of tumors in the 1st transplant generation. Furthermore, the hormonal effect on HD tumor growth has been evaluated on the basis of the following cell kinetic quantities: cell cycle parameters, growth fraction, cell production rate, and cell loss rate. These quantities were estimated on a batch of tumors in the 1st transplant generation, all derived from the same primary tumor. The experimental data were obtained from the tumors during growth in hormone-treated animals and during regression after hormone deprivation. Tumors from the 1st transplant generation were used for the cell kinetic investigations since considerable biological variation was to be expected in primary tumors.

The experimental data consist of a growth curve (TD and TH), PLM curves, and a LI. From these data, cell cycle parameters, growth fraction, cell production rate, and cell loss rate were estimated by a computer method based on 3

1 The abbreviations used are: HD, hormone-dependent; TD, doubling time of growing tumor; TH, half-time of regressing tumor; PLM, percentage of labeled mitoses; LI, labeling index.
Cell Kinetics of HD Mammary Tumors

Chart 1. Growth curves of primary hormone-induced mammary tumors in female GR mice. + and −, hormonal treatment. Each curve, 1 tumor. At Day 0, female mice were spayed and treated with estrone and progesterone. At Day 112, hormonal treatment was discontinued. Repeated hormonal treatment was given to Animal 626, 627, and 628 at Day 175, 175, and 153, respectively.

mathematical models that differed with respect to mode of cell loss. This procedure was used since the experimental data contained no information about the position of the cell loss in the cell cycle. Two of the models were described earlier (6–8, 11), while the 3rd model was developed for the present study.

MATERIALS AND METHODS

Growth Curves and TD(TH). Growth curves were established for primary mammary tumors as well as for their 1st transplant generation. Primary tumors were induced in spayed virgin mice of the GR/FIB strain by treatment with progesterone and estrone. Estrone was administered in the drinking water in a concentration of 0.5 μg/ml, and 5 to 10 mg of progesterone were injected s.c. as pellets each week (14). Macroscopic tumors were detectable after about 8 to 12 weeks of treatment (3). Tumor tissue for transplantation was minced with scissors, and 0.1 ml was inoculated into each animal. Castrated male mice treated with progesterone and estrone served as hosts. Two animals not treated with hormones were inoculated simultaneously; if tumors were not palpable on these animals after 2 months of observation, the tumor was considered HD. Growth of the tumor was followed by measurements of the tumor size according to the method of Rockwell et al. (12). Twice a week the length, width, and height (l, w, and h) were measured with a slide caliper. No correction was made for skin thickness. The volume (V) of the tumor was calculated from the formula:

\[ V = 0.5236 \times l \times w \times h \]

Tumors less than 50 cu mm were not scored. On the basis of the tumor volume, growth curves were drawn (Charts 1 and 2).

The 1st transplant generation of tumors for further cell kinetic investigations was established from a single primary tumor. This tumor was treated with the enzymes collagenase, hyaluronidase, and Pronase (20) to obtain a cell suspension (about \(4 \times 10^6\) cells were obtained per tumor) that was inoculated s.c. into castrated male mice treated with progesterone and estrone. Previous experience (P. Briand, unpublished observation) in our laboratory has shown that the tumor cell dose that takes in 50% of the inoculated animals for these tumors is around \(0.5 \times 10^6\) cells. To assure a maximal number of takes, we inoculated \(10^7\) cells/mouse. Therefore, tumor cells from 1 primary tumor could be inoculated to a maximum of 40 mice. Mean tumor volume was measured with an interval of 1 to 2 days as an average of 6 tumors. The growth curve is seen in Chart 3. TD was calculated from the slope of the tangent of the curve on Day 23 after transplantation. On Day 30 hormonal treatment was discontinued by removing estrone from the drinking water and stopping the weekly inoculation of progesterone pellets. This means that estrone administration was stopped immediately, whereas the uptake of progesterone from the pellets decreased gradually. The TH was defined as the time for a 50% reduction of the
volume of an exponentially regressing tumor. $T_H$ was calculated from the slope of the tangent of the growth curve in Chart 3 on Day 34 after transplantation. The $T_D(T_H)$ values are recorded in Table 1.

**PLM Curves and LI.** The PLM experiments and LI measurements were carried out on the same animals used for the growth curve experiment in Chart 3. The PLM curve was measured on Day 23 (growing tumor) and on Day 34 (regressing tumor) after transplantation. As was mentioned earlier, a total of 40 tumor-bearing animals were used for the PLM experiments on the growing tumor and the regressing tumor. $^3$H-Labeled thymidine (Radiochemical Centre, Amersham, England; specific activity, 2 Ci/m mole), in an amount of 50 $\mu$Ci/mouse, was injected i.p. into animals bearing a tumor of 1000 to 2000 cu mm. After the injection, the animals were sacrificed at time intervals. Due to the limited number of animals in the experiment, only 1 animal was sacrificed for each point on the PLM curve, thus achieving the most frequent sampling possible. The tumors were removed and fixed in 5% formaldehyde, and cut sections were dipped in K-2 or K-4 Ilford nuclear emulsion and kept for 2 weeks of exposure. After development and fixation, the slides were stained with hematoxylin and eosin. Sixty to 120 metaphases were counted in each tumor. The experimental PLM values are recorded in Chart 4.

The fraction of labeled cells (LI) 1 hr after injection of $^3$H-labeled thymidine was measured on Days 23 and 34 after transplantation. To determine the LI, labeled cells were counted in a total of 2000 tumor cells. The LI values are recorded in Table 1.

**Computer Method.** PLM curves, LI, and $T_D(T_H)$ were analyzed by a computer method based on 3 mathematical models (Chart 5).

Model A was developed for an earlier purpose (8), and a more complicated form has been described several times (6,
Chart 4. PLM curves of a HD GR mammary tumor in the 1st transplant generation. O, experimental PLM data; —, theoretical PLM curves representing the best fit to the experimental data. Growing and regressing tumors were investigated on Day 23 and 34 after transplantation, respectively. Hormone treatment was discontinued on Day 30.

Chart 5. Three mathematical models of cell proliferation kinetics used in the computer analysis of experimental data from HD GR mammary tumors in the 1st transplant generation. $G_1$, $S$, $G_2$, and $M$, compartments of proliferative cells; $Q_1$, compartment of resting cells.

parameters mentioned for $G_1$, $S$, $G_2$, and $P$ are valid for the nonrandom processes.

The growth fraction is the percentage of cells in $P$, cell production rate is the percentage of cells produced per hr, and cell loss rate is the percentage of cells lost per hr.

The basic assumption behind the models is exponential growth or exponential regression. This means that the models can be applied to experimental data obtained during a time interval in which no transient variations can occur in the cell kinetics. In this time interval the tangent to the growth curve, recorded in a semilog plot, is a good approximation to the curve.

The probability density functions for the transit times in the various compartments are simulated with normal distributions, and in Model C this simulation yields the nonrandom processes. With this choice of probability density function, it was possible to develop analytical expressions for the PLM curve, the growth fraction, the cell production rate, and the cell loss rate.

The 1st step in the computer analysis was to fit a theoretical PLM curve to the experimental data in order to estimate the cell cycle parameters. The best fit between the theoretical curves and the experimental data was obtained
by the maximum likelihood method. This optimizing procedure gives a rather well-defined best fit, because parameter estimates obtained from different initial conditions show differences that are negligible. Based on $T_D(T_H)$, LI, and values of the aforementioned parameters, the final step was to estimate growth fraction, cell production rate, and cell loss rate.

For purely mathematical reasons, Models A and B give identical results with respect to all cell kinetic values presented in this study, whereas the results estimated with Model C are slightly different. All estimated parameter values are recorded in Table 1.

**RESULTS**

Growth curves of 5 primary tumors are seen in Chart 1. During hormonal treatment the tumors had almost the same growth rate. When hormonal treatment was discontinued, all tumors regressed. By visual inspection of Chart 1, the growth curves for the regressing tumors seem to be exponential, but $T_H$ varies considerably from tumor to tumor. Resumption of hormone treatment to 3 of the tumor-bearing animals resulted in a rapid regrowth of the tumor.

Three types of growth were seen in tumors investigated in the 1st generation of transplantation as is shown in Chart 2. One type of tumor (HD) regressed completely when hormonal treatment was discontinued, and regrowth occurred when the hormone treatment was started again (Type I). Another type regressed significantly, but regrowth occurred without repeated hormonal treatment (Type II). In a 3rd type of tumor, hormonal treatment had no effect on the growth rate (Type III).

Chart 3 shows the growth curve of the HD tumor on which PLM and LI studies were carried out. The hormone dependence of this tumor is clearly demonstrated by the fact that discontinuation of hormone treatment is followed by a significant regression of the tumor within 4 days. Thus the tumor growth corresponds to the 1st part of Type I and Type II in Chart 2.

The experimental PLM data from the growing and regressing HD tumor measured, respectively, on Days 23 and 34 after transplantation are plotted in Chart 4 (circles). In the same figure, theoretical PLM curves representing the best fit to the experimental data are recorded (solid lines). By visual inspection of the curves it is obvious that $T_C$ has increased after removal of the hormones.

$T_D(T_H)$ and LI measured on Days 23 and 34 after transplantation are given in Table 1, which also shows the cell kinetic parameters estimated by the computer analysis. The most predominant change following regression of the tumor was the decrease of growth fraction from 62% to 18% and of cell production rate from 3.4% to 0.9%/hr. The cell loss rate scarcely changed. As mentioned, $T_C$ has increased in the regressing compared to the growing tumor, mainly because of an increase of the $T_D$.

**DISCUSSION**

Three stages can be considered in the development of hormone-induced mammary tumors in GR mice. In the 1st stage, the hormones act on the mammary tissue and a tumor develops. In the 2nd stage, the established tumor grows although only in the presence of hormones. The 3rd stage is achieved when the HD tumor becomes independent, i.e., it grows without hormones. This stage may occur in the primary tumor and is usually seen after several transplantations (3, 14, 18).

In a HD mammary tumor of GR mice we have found that discontinuation of hormonal treatment was followed by a decrease of the tumor with a considerable decrease in growth fraction and cell production rate, whereas the cell loss rate was more or less unchanged. Furthermore, an increase in the mean transit time of the cell cycle, particularly in the mean transit time of the G1 phase, was observed. These results might indicate that the mechanism of hormone action on growth of HD mammary tumors is a regulation of the size of the proliferative pool and of the cell production rate, and affects the mean transit time of

<table>
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<td>Cell loss rate (%) / hr</td>
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Table 1

$T_D(T_H)$, LI, cell cycle parameters, growth fraction, cell production rate, and cell loss rate in a HD growing and regressing mammary tumor of GR mice
the cell cycle to a smaller extent it does not involve changes of the cell loss rate.

Other studies on the effect of steroid hormones on cellular kinetics have been carried out mainly in mouse uterine tissue. It has been found that estrone stimulates the growth of uterine mucosa by shortening the cell cycle, particularly the G1 and S phases, and by increasing the growth fraction (9). Other authors have reported varying results, as pointed out by Das (5). Also, mouse mammary tissue seems to react to steroid hormones by a shortening of the cell cycle, particularly the S phase (1, 2).

The present cell kinetic investigation is based on 38 tumors; however, in the conclusion of the results it must be taken into consideration that all tumors are derived from the same primary tumor. The limited number of tumors that could be transplanted from 1 single tumor allowed only 2 PLM experiments to be carried out. Growing and regressing tumors were chosen for the cell kinetic experiment and, consequently, tumors that were regrowing because of repeated hormone treatment were not investigated.

The cell kinetic parameters discussed above (Table I) are estimated by a computer analysis of the experimental data, i.e., PLM curves, TD(th), and LI. As these data do not contain enough information to localize the position of the cell loss in the cell cycle, 3 models differing in respect to the mode of cell loss have been used in the computer analysis.

It is obvious that the cell kinetics as assumed in Model B can be ruled out as a model for a regressing cell population since the Q1 population cannot decrease with an influx in the compartment and no efflux. Consequently, if the growing HD tumor has a cell loss mechanism as assumed in Model B, there must be a shift in the mode of cell loss after removal of hormones. However, as the cell loss rate does not change significantly from a growing to a regressing tumor, it is most unlikely that the mode of cell loss should change, and Model C can, therefore, be excluded for the growing tumor as well.

As mentioned earlier, Models A and B give identical results with respect to all estimated kinetic parameters. It is furthermore shown that the cell cycle parameters estimated with Model C are almost identical to those of Models A and B. Table I also demonstrates that the growth fraction is slightly higher when estimated with Model C than with Models A and B, and, with respect to cell production rate and cell loss rate, it is opposite.

A basic assumption behind the models is exponential growth or exponential regression, which means that the models are not valid in case of sudden alterations introduced in the cell kinetics, e.g., by removal of the hormones. However, the models provide an adequate tool to demonstrate the general behavior of the cell kinetic parameters in relation to hormone treatment, since the models are applied to data obtained in periods when exponential growth or regression is an acceptable approximation. Thus, the analyzed data from the regressing tumor are obtained 4 days after discontinuation of hormonal treatment, at which time possible transient variations should have vanished.

There is some question as to how accurate the parameter estimates in Table I are. It is not possible on the basis of the mathematical models used in this work to give S.D.'s on the estimated parameter values and to perform a statistical test between the estimates of 2 experiments. However, 2 things can be stated.

(a) By visual inspection of Chart 4 it is conceivable that an actual increase of the mean transit time of the cell cycle has occurred from the growing to the regressing state of the tumor. (b) An increase of Tc from 16 to 22 hr would not alone give a significant difference in the estimates of growth fraction, cell production rate, and cell loss rate.

In this work, the critical experimental data for the estimation of growth fraction, cell production rate, and cell loss rate are TD(th) and LI. Since TD is estimated during growth and TH is estimated during regression, and since the LI under the same circumstances drops from 27% to 7.5%, it is reasonable to believe that the differences in growth fraction and cell production rate demonstrated in Table I are real; whereas it is most probable that the estimates for the cell loss rate do not show changes due to the hormone treatment.

Curtis et al. (4) have developed a mathematical model for nonexponential tumor growth, and this model has also been used in the analysis of data from a regressing tumor. Since regression is introduced by radiation, the model is developed with a subpopulation of cells that can undergo several divisions before dying. As early as 1966, Steel et al. (15) proposed the alternative modes of cell loss described in this paper, and a subsequent study (16) demonstrated, on the basis of mathematical models, how a shift in the mode of cell loss would influence the cell kinetics. Fried (10) has developed a model with a random transition of cells from P to Q1, a transition of cells from M to Q1, and a random cell loss from Q1. M. Takahashi (personal communication) has introduced a similar model but with random cell loss from both P and Q1. However, the experience with application of the mathematical Models A, B, and C in this work indicates that the mode of cell loss is not critical for the estimation of cell kinetic parameters in the present tumor system.

An attempt has been made to clarify the cell kinetic mechanism of the growth-stimulating effect of hormones on HD mammary tumors. The results might indicate that hormone treatment of GR mouse mammary tumors in vivo induces growth by increasing the growth fraction and cell production rate, while there are only insignificant changes in the mean transit time of the cell cycle and in the cell loss rate.

REFERENCES

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