Dormancy and Spontaneous Recurrence of Human Breast Cancer in Vitro

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ABSTRACT

Monolayer cultures of the human breast cancer cell line MDA-361 require insulin for growth and for maintenance of viability, as is evidenced by rapid and complete degeneration of the cells after the removal of insulin from the medium. Detachment from the plastic surface occurs within 24 to 48 hr, and the rare (<0.1%) cell that remains attached doubles every 3 to 4 weeks. Multicellular tumor spheroids, derived from this same tumor cell line, enter a dormant phase which lasts approximately 6 weeks, when insulin is removed from the medium. During this dormant period the multicellular tumor spheroids appear healthy and gradually become less dependent on and more responsive to insulin. This dormant period culminates in spontaneous regrowth in the absence of insulin after the sixth week, and this growth continues at least through 3 months. In this respect these multicellular tumor spheroids parallel the behavior of residual tumors in vivo; the residual tumor remains viable but nongrowing for a prolonged period only to resume growth following escape from the growth-limiting mechanism.

INTRODUCTION

An all too common observation in cancer therapy is that treatment produces a prolonged disease-free interval only to be followed by 1 or more recurrences (3). In many instances the interval between treatment and recurrence far exceeds the time one would expect for a single tumor cell to give rise to a detectable mass, and for this reason the tumor cells responsible for the eventual recurrence are assumed to be in a dormant state. In the absence of knowledge of the mechanisms involved, both dormancy and recurrence must be operationally defined as periods of net tumor mass stability and net tumor mass increase, respectively.

Since the competence of a patient’s immune system often correlates positively with the time before recurrence (6, 13, 15, 17), it has been argued that tumor dormancy reflects the ability of the immune system to control small residual tumor burdens (10) and that recurrence reflects either failure of the growth-controlling factors (4) or escape from control by variant, nonimmunogenic tumor cells (1). In some tumors such as breast (19) and prostate (6), however, immune competence does not correlate with the time before recurrence, and it has been speculated that in these tumors the contribution of immunological factors is more than counterbalanced by the sensitivity of the tumors to hormones (6).

Exactly how hormone sensitivity might overshadow the contribution of the immune system is unknown, but a brief review of the effectiveness of endocrine therapy in breast cancer is instructive. Endocrine therapy alone is seldom curative, but in combination with other forms of therapy (e.g., surgery) it can produce dramatic long-term remissions in certain cases (3). The role of endocrine therapy in improving surgical results appears, however, to be temporary since “recurrence is not prevented, merely delayed” (3).

A clue as to how endocrine therapy can temporarily postpone recurrence can be found in the clinical observation that breast cancers, which are initially responsive to endocrine therapy, are seldom responsive to it at the time of recurrence (7). This would suggest that the bulk of the tumor cells are hormone-dependent at the time of the initial treatment but that few are at the time of recurrence. Endocrine therapy could selectively kill the hormone-dependent cells within the tumor and allow any hormone-independent cells to repopulate the tumor, but the prolonged duration of at least some of the remissions is inconsistent with the continued growth of these hormone-independent cells throughout the period of the remission. Alternatively, hormone-dependent cells may survive the therapy in a viable but nongrowing state, adapt to the absence of hormones either genetically or physiologically, and then resume growth.

This hypothetical explanation of tumor dormancy and recurrence has been considered by a number of investigators, but to the best of our knowledge little data have been put forth to support it. We raise this point again for consideration since we have been able to demonstrate the operation of the hypothesis in vitro. MTS$^3$ derived from the MDA-361 human mammary adenocarcinoma cell line fail to grow in the absence of insulin but remain viable and resume growth after a 45-day dormant period.

MATERIALS AND METHODS

Cells. The MDA-361 cell line used in these experiments was derived from a brain metastasis of a human mammary adenocarcinoma (5) and was supplied to us by Dr. Reida Cailleau of the M. D. Anderson Hospital and Tumor Institute. Stock monolayer cultures were maintained in EBME supplemented with 10% FCS, 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 10 μg of porcine insulin.

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3 The abbreviations used are: MTS, multicellular tumor spheroid; EBME, Eagle’s basal medium; FCS, fetal calf serum; $[^{3}H]dUrd$, $[^{3}H]dododeoxyuridine.$
per ml (Elanco, Elkhart, Ind.). For experimental studies the FCS was first dialyzed (distilled water FCS, 10:1; 3 changes each lasting 8 hr) and absorbed on dextran-coated charcoal to reduce endogenous peptide and steroid hormones to as low a level as possible (2). Unless otherwise stated insulin(+) medium contains exogenous insulin (10 μg/ml) plus whatever remains after dialysis, whereas insulin(−) medium contains only that insulin that dialysis failed to remove. All of the data reported below for insulin(−) medium were indistinguishable from those obtained with undialyzed FCS or with a serum substitute (12). This suggests that the endogenous insulin contained in undialyzed FCS (11) was still too low to affect our experiments. Monolayer cultures were maintained and harvested as described elsewhere (20-22).

Monolayer Experiments. All attempts to plate MDA-361 cells in insulin(−) medium failed, i.e., less than 0.1% of the cells attached and those rare cells that did attach doubled every 3 to 4 weeks. We, therefore, harvested cells from stock cultures, plated approximately 105 in 100-mm plastic Petri dishes along with 10 ml of insulin(+) medium, and allowed them to attach for 24 hr. Plates were harvested and counted (20-22), and then were refed with either insulin(+) or insulin(−) medium. The cultures were harvested and counted at 3-day intervals for 2 weeks, with new medium being added every third day.

MTS Studies. The MTS’s were produced according to methods described elsewhere (20-22). Briefly MDA-361 cells were harvested from monolayer cultures and suspended in insulin(+) or insulin(−) medium. Approximately 104 cells in 10 ml of liquid medium were plated in 100-mm plastic Petri dishes that had been base-coated with 2 to 3 mm of 0.75% noble agar (Difco Laboratories, Kalamazoo, Mich.) in insulin(−) EBME. Cultures that lacked insulin failed to produce MTS’s within a 60-day observation period. Plating of the cells in insulin(+) medium, however, allowed for the development of MTS’s that measured 250 to 300 μm within 2 weeks. These MTS’s were harvested by means of a sterile pasteur pipet and were transferred to insulin(+) or insulin(−) medium (12 MTS’s/5 ml in 60-mm agar-based plates) for a 24-hr pretreatment prior to analysis. Pretreatment was used to allow the MTS’s to recover from the variable and unknown effects of crowding in the plates used to mass produce them and to allow the residual insulin and its induced effects to decay, as described below.

Growth experiments involved 12 to 24 MTS’s/group, with each MTS being cultured in an individual 16-mm agar-based well, along with 1 ml of the appropriate medium. MTS’s were sized 3 times weekly, and the medium was changed twice weekly.

The techniques for determining [3H]IdUrd incorporation by MTS’s have been described elsewhere (20). In brief, MTS’s were placed in 1 ml of appropriate medium along with 1 μCi of [3H]IdUrd for varying periods, washed 3 times with cold 5% trichloroacetic acid, and then counted. For the “decay of insulin activity” experiments, the MTS’s were pretreated for 24 hr in insulin(−) medium as described in the text. For analysis of the concentration of insulin required to support maximum [3H]IdUrd incorporation, all MTS’s were pretreated for 24 hr in insulin(−) medium.

RESULTS

Insulin Requirement in Monolayer. Twenty-four hr after MDA-361 cells were plated in insulin(+) medium, they were refed with insulin(+) or insulin(−) medium, and then the doubling time of the cultures was determined. In the presence of insulin, the cells doubled every 97 ± 3.8 (S.E.) hr, but in insulin(−) medium the cultures rapidly degenerated; by the end of the first week fewer than 0.1% of the original cells remained attached. These rare cells required longer than 3 weeks to divide and could not be rescued by readdition of insulin(+) medium after 1 week in insulin(−) medium. We conclude, therefore, that monolayer cultures of MDA-361 require an exogenous source of insulin not only for growth but also for maintenance of viability. To the best of our knowledge, this is the most extreme case of an insulin requirement yet reported for breast cancer cells (9, 14, 16, 18).

Insulin Dependence of MTS’s. It was not possible to produce MTS’s in insulin(−) medium, so we had to produce them in insulin(+) medium and then transfer them to insulin(−) medium. Before comparisons were made of the MTS’s growing in the 2 types of medium, it was necessary to establish the amount of time it required for the residual insulin and its induced effects to decay. To determine this we compared the [3H]IdUrd incorporation by 300-μm MTS’s which had been pretreated and analyzed by insulin(+) medium (Chart 1, +/+), with those pretreated with insulin(+) medium but analyzed in insulin(−) medium (Chart 1, +/−). In Chart 1 the +/+ group shows a linear increase in incorporation as a function of time in insulin(+) medium supplemented with [3H]IdUrd (1 μCi/ml). The +/− group, which was similarly pretreated but which was analyzed in insulin(−) medium, showed incorporation levels comparable to that of the +/+ group through the 12th hr of analysis, after which time no further increases in incorporation were observed (Chart 1). We interpret this result as indicating that the combined effects of residual insulin and insulin-induced effects subside within 12 hr after transfer to insulin(−) medium.

If the MTS’s were both pretreated and analyzed in insulin(−) medium, no incorporation above background was observed during the 20-hr period of analysis (data not shown). However, pretreatment in insulin(−) medium followed by analysis in insulin(+) medium (Chart 1, −/+ +) indicated that it required 4 hr before the MTS’s would respond to the insulin, after which time the rate of increase in incorporation paralleled that of the +/+ group. The parallel rates of incorporation that followed the 4-hr lag indicate that the cessation of DNA synthesis induced by the pretreatment in insulin(−) medium was not the result of blocking cells at a specific stage of the cell cycle. These patterns of DNA synthesis alteration have been confirmed in our own laboratory with [3H]thymidine (F. Bennett, unpublished data).

Since 12 hr in insulin(−) medium allowed the residual effects of insulin to decay, we elected to use a 24-hr pretreatment in insulin(−) medium prior to using any of the MTS’s for insulin(−) medium studies. The lack of DNA synthesis after 12 hr in insulin(−) medium suggested that

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Chart 1. Incorporation of \([^{3}H]\)ldUrd by MDA-361 MTS's (300 µm) as a function of time. (+/+, pretreated for 24 hr in insulin(+) medium and then analyzed in insulin(+) medium containing 1 µCi of \([^{3}H]\)ldUrd per ml; (+/-), pretreated as above but analyzed in insulin(−) medium; (−/+), pretreated as above but in insulin(−) medium and then analyzed in insulin(+) medium. Pretreatment and analysis (−/−) did not yield detectable incorporation. Insulin(+) medium contains 10 µg of exogenous insulin per ml, whereas insulin(−) medium contains only that amount remaining after dialysis.

Chart 2. Growth of MDA-361 MTS's as a function of time in EBME containing 0 or 10 µg of exogenous insulin per ml.

Chart 3. Growth rate (O) and 24-hr, \([^{3}H]\)ldUrd incorporation (•) by MDA-361 MTS's as a function of the insulin concentration in the medium. Bars, S.E.

the MTS’s would fail to grow in this medium, as was observed. Chart 2 is a plot of MTS size versus time for MTS’s that were maintained in insulin(+) or insulin(−) medium. In the presence of insulin, the MTS’s grew at a rate of 12 ± 1.3 µm/day. As shown elsewhere (22) and as follows, the growth rate under these conditions is stable and ranges from 11 to 15 µm/day. In insulin(−) medium, however, the MTS’s failed to grow and actually shrank during the 14 days of observation (Chart 2). The maximum shrinkage amounts to a loss of 12 to 40% of the MTS volume; however, we were unable to detect significant numbers of tumor cells in the medium surrounding the MTS’s. During this 2-week period in insulin(−) medium, the MTS’s appeared normal and merely reduced in size.

Before proceeding with a further analysis of the insulin dependence of these MTS’s, we considered it necessary to determine whether this effect operated within the physiological range. A concentration of 10 µg/ml is far beyond the physiological range (16) but was chosen to compensate for the loss of biological activity in the twice weekly changes. Chart 3 shows that concentrations of insulin as low as 1 µg/ml can support maximum growth, even with twice weekly changes, and that concentrations as low as 10^{-2} µg/ml can support maximum 24-hr \([^{3}H]\)ldUrd incorporation. Therefore, growth and DNA synthesis are insulin concentration-dependent and probably operate in the physiological range of 10^{-3} µg/ml once decay of insulin activity in the medium is taken into account.

On the basis of the normal surface morphology of the MTS’s being maintained in insulin(−) medium, we considered it possible that at least some of the cells in the MTS’s were viable and that transfer of these MTS’s to insulin(+) medium might allow the resumption of growth. We, therefore, maintained MTS’s in insulin(−) medium for as long as...
31 days and then transferred them into insulin(+) medium and determined their growth rate over the next 10 to 14 days. Not only were the insulin-deprived MTS's capable of resuming growth, but all of them grew at a faster rate than did MTS's that had never been insulin-deprived. Chart 4 is a plot of the growth rate in insulin(+) medium as a function of the duration of insulin deprivation and demonstrates that the growth rate increases linearly with the duration of insulin deprivation through 31 days. Longer periods of insulin deprivation (up to 3 months) did not result in a further increase in the growth rate upon transfer to insulin(+) medium (data not shown). As shown in Chart 5, however, these longer periods of insulin deprivation did result in a reduced requirement for insulin to maintain any given growth rate.

We have not pursued precise quantitation of this reduced insulin requirement (Chart 5), since it became apparent that the MTS's were able to adapt in such a way that they could grow in insulin(-) medium after long periods of deprivation. Chart 6 is a plot of the size of insulin-deprived MTS's as a function of time up to 3 months. Between the 30th and 45th day in insulin(-) medium, the MTS's resume growth at a rate of 4.1 ± 0.56 µm/day, and they continue to grow in the absence of exogenous insulin through the 90th day. This resumption of growth was not due to an error in formulating the insulin(-) medium, since the same medium used to feed the cultures failed to support the growth of more recently isolated MTS's. Further, this resumption of growth is repeatable, and its mechanism(s) are presently under study in our laboratory.

After 90 days in insulin(-) medium the MTS's were transferred to a variety of other media for further study; all continued to grow and the rate of growth was dependent on the insulin concentration (Chart 5), but insulin was not required. From a combination of these data in Charts 3 and 5, it can be seen that an insulin concentration could be selected that would not allow growth initially but would eventually promote growth at a faster rate than normal.

**DISCUSSION**

In these experiments we have compared the responses of monolayers and MTS's of the MDA-361 line in 2 types of media: one in which exogenous insulin has been added and one in which the only insulin present would be that remaining after dialysis [insulin(+), and insulin(-), respectively]. We are not suggesting that our insulin(-) medium is totally devoid of insulin, but we view our data in a relative sense. Whatever the exact insulin concentration in the insulin(-) medium, it is unable to support growth at all in monolayer, unable to support growth initially in the MTS system, but eventually is able to support growth of MTS's. Since the most interesting aspect of these studies is the long-term adaptation of the MTS's to the insulin(-) medium, we will avoid further discussion of the monolayer data. However, the cell-cell attachments of the MTS's appear to be less dependent on the addition of exogenous insulin than do the cell-plastic attachments of the monolayers. Whether similar levels of adaptation could be observed in monolayer if the attachment problem were solved remains to be determined. The few monolayer cells that remained attached did not appear to adapt over a 5-month period in the limited analyses we performed.

The data presented have demonstrated that MDA-361 MTS's, which had been produced in insulin(+) medium, shrink upon transfer to insulin(-) medium, become less dependent on and more responsive to this hormone, and eventually resume growth. This pattern is analogous to
tumor dormancy and recurrence of hormone-dependent tumors, at least in terms of overall kinetics. Ignoring for a moment the specific growth factor involved in our experiments, insulin, one can compare the processes in vivo and in vitro (Table 1). In both cases progressive tumor growth occurs in the presence of the factor, shrinkage occurs upon its removal, stability persists for a prolonged period, and then growth resumes (Table 1). Although it would be premature to propose that the mechanisms are the same, the similarity of the kinetics is impressive.

In this particular study insulin is the growth factor involved, but we are not proposing that insulin is a universal or even a critical growth factor in breast cancer or other hormone-dependent cancers. Most of the data on the role of insulin in breast cancer comes from model systems, and in these studies insulin can stimulate (14, 16, 18) or inhibit (9) breast cancer growth, or have no effect (22), depending on the tumor line studied. What we are proposing is that the MTS system can be used to study the development of therapeutic agents can result in MTS's that are drug resistant or sensitive and resistance to chemotherapeutic agents in-whether similar patterns are observed for other growth ant. These latter studies are particularly interesting since they would be manifested at a level that would escape detection in monolayer culture. We are particularly interested in the possibility that chemotherapeutic agents not only select for "mutant" clones, which are resistant to their action, but also are responsible for the induction of these clones.

Returning to these present data, the question arises as to how the MTS's adapted to the insulin(−) medium. Two broad possibilities may be considered, but neither is totally satisfactory: either evolution of genetic variants that do not require insulin for growth; or physiological adaptation induced by the absence of insulin. If the adaptation is genetic, the variant cells either had to exist within the MTS at the time that they were transferred to the insulin(−) medium, or they had to develop during the course of the insulin deprivation. If the mutants were always present in the MTS's and the cultures that gave rise to them, we would expect that they would eventually take over the cultures because they grow faster than do the wild-type cells in the presence of insulin (Chart 5). For preexistent mutants to account for the data, it must be argued that they have no growth advantage in monolayer culture, i.e., the insulin independence is only expressed in the MTS form. Any argument based on the appearance of mutants during the course of insulin deprivation also must point out that the rate of appearance is far higher in MTS's than in monolayer.

Physiological adaptation to insulin deprivation could occur in many ways, including the development of increased numbers of receptors in the absence of insulin (8). These elevated receptor levels must, however, remain elevated for at least 2 weeks after return to insulin to be compatible with the prolonged elevation of the growth rate upon return to insulin. Since the MTS's grow too large to study beyond 2 weeks, we are presently adapting insulin-deprived MTS's to monolayer culture for further study. If the change is genetic, it should persist even following serial passage.

Whatever the mechanisms involved, selection for genetic variants or physiological adaptation, it is clear that escape from hormonal restriction of growth can be simulated in the laboratory.

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