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Cell Kinetic Alterations in Murine Mammary Tumors Following Fasting and Refeeding*

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Abstract—*In the present study, nutritional deprivation was shown to markedly alter the growth and cell kinetics of two widely different experimental tumors, the T-1699 transplantable mammary tumor (TMT) and the C3H/HeJ spontaneous mammary tumor (SMT).* In vitro *techniques were used to determine the thymidine labeling index ([^{3}H]TdR LI), the tumor growth fraction, by the primer available DNA polymerase assay (PDP), and the DNA synthesis time (T_S) following fasting and refeeding. In both systems, fasting resulted in suppression of cell proliferation and significant tumor regression. Refeeding re-established the prefasting growth rates and cell kinetic profiles. The kinetic response to fasting in the TMT involved a generalized lengthening of the cell cycle (T_C), while the timing and magnitude of the response to refeeding appear to be dose dependent. In contrast, the kinetic data from the SMT during fasting was consistent with the presence of a cell cycle block in G_1. Removal of this block by refeeding was prompt and was characterized by tumor cell synchronization and recruitment of non-cycling cells.*

INTRODUCTION

SEVERAL previous studies have established that nutritional manipulations, in the form of caloric "underfeeding" [1–3], starvation [4], hyperalimentation [5] and the feeding or underfeeding of specific dietary components [6–11], can markedly affect the growth of malignant cells. Hypocaloric conditions reduced the incidence of spontaneous neoplasms [1] and retarded the establishment and growth of transplantable tumors [2].

Nutritional studies *in vitro* have established a marked disparity between normal cells and their virally transformed counterparts. When deprived of serum or essential amino acids, normal cells became blocked at a point in mid-G_1 termed the "restriction point" [12]. These cells remained blocked until adequate nutritional conditions were re-established. In contrast, transformed cells were delayed randomly with respect to the cell cycle [13, 14], indicating a loss of restriction point function. Similar studies, *in vivo*, with the colonic epithelium have also indicated the presence of a fasting induced G_1-block [15, 16].

From a clinical standpoint, the nutritional status of cancer patients is often dramatically compromised by the anorexia and oligophagia attendant with their disease and/or as a result of oncotherapy [17]. If these various nutritional states are reflected by specific cytokinetic responses in the tumor, it is conceivable that these changes could be exploited to improve the response to therapy. Previous studies have indicated the efficacy of kinetically based chemotherapy schedules [18].

In the present studies, the effect of fasting and refeeding on tumor cell proliferation was determined for two widely differing experimental solid tumors, the T-1699 transplantable tumor (TMT) and the C3H/HeJ spontaneous mammary tumor (SMT).

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MATERIALS AND METHODS

Animals and tumors

Spontaneous mammary tumors (SMT) in female C3H/HeJ retired breeders (Jackson) were studied between 0.5 and 1.0 cm^3. T-1699 transplantable mammary tumors (TMT) were passaged in 6–8 week old male DBA/2J mice (Jackson) by subcutaneous implantation of 10^6 viable cells. Tumors were studied at 14 days, when the tumor volumes were approximately 1.0 cm^3.

Tumor growth in both systems was monitored by daily duplicate caliper measurements in three dimensions. Tumor volumes were estimated from the equation for a prolate spheroid, growth curves constructed and tumor volume doubling times (TD) determined for these curves.

Nutritional studies

Mice were housed singly in metabolism cages with wire bottoms or in plastic cages with sawdust bedding. Fasting was begun between 12 noon and 2 p.m., with water given *ad libitum*. Following fasting for 48 or 72 hr animals were refed *ad libitum* with autoclaved lab chow (Purina). Body weights and tumor volumes were measured daily. In some studies, animals were sacrificed during fasting via cervical dislocation, the tumors removed, weighed wet, dried overnight in a 60°C oven, reweighed and the percentage tumor water determined.

In vitro *labeling*

The techniques employed for the *in vitro* single and double labeling have been described in detail elsewhere [19–21].

Primer-dependent DNA polymerase assay (PDP)

Tissue imprint preparations were made from the freshly cut surface of either tumor on acid cleaned slides. These cell preparations were then assayed via the primer available DNA polymerase assay (PDP) which is described in detail elsewhere [22–24]. Briefly, in this system, nuclei are provided with all the necessary ingredients for DNA synthesis with the exception of α polymerase and DNA capable of acting as primer template. When both of these latter elements are present, DNA synthesis occurs and can be quantitated by autoradiography. The results are expressed as the fraction of labeled nuclei, the PDP index (PDP-I). The PDP-I has been shown to accurately estimate the tumor growth fraction in these experimental tumors [23, 24].

Autoradiography

Liquid photographic emulsion (Kodak NTB-2) was applied to slides by the dipping method, with gold activation autoradiography employed to shorten the exposure period [19].

Calculations

Other tumor cell kinetic parameters, including the cell cycle time (T_C), DNA synthesis time (T_S), the rates of cell production (K_P) and cell loss (K_L), were calculated as described previously [25–27].

RESULTS

Tumor growth

The effects of fasting and refeeding on the growth of the T-1699 TMT and C3H/HeJ SMT are given in Fig. 1. Untreated control T-1699 TMT's had a T_D of approximately 72 hr while fasting animals showed tumor regression (5–20%) and thus negative T_D values. By definition this would represent the time required for the tumor volume to regress by 50%. Refeeding at 48 hr resulted in a sharp increase in tumor growth and attainment of age-matched control size by day 6. Refeeding at 72 hr resulted in a slower recovery. The T_D during refeeding was similar to the prefasting value (72 hr), while in the age-matched controls the T_D had increased to 168 hr.

The TMT mice exhibited a 5 and 10% loss in body weight after 48 and 72 hr of fasting respectively. Tumor water content was similar before ($70.6 \pm 4\%$) and after ($77 \pm 2\%$) fasting. Animal survival consistently exceeded 90% after a 48 hr fast but was reduced to approximately 75% after 72 hr.

The T_D for the untreated SMT was approximately 13 days (Fig. 1). During the 72 hr fast the reduction in tumor volume ranged from 20 to 60% with a mean of $40 \pm 7\%$. Refeeding resulted in a prompt resumption of SMT growth, however, tumor volumes remained below age-matched control values for the remainder of the observation period.

During the fast a 20% body weight loss in the SMT mice was routinely observed. Tumor water content following fasting ($79 \pm 0.5\%$) was similar to the control value ($80 \pm 1.0\%$). In general, C3H/HeJ mice tolerated the fasting stress well with usually less than 10% mortality. In some groups

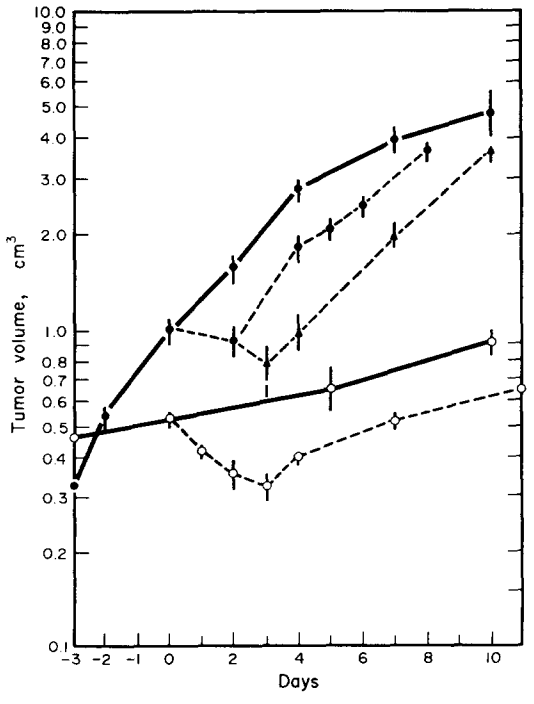

Fig. 1. The effect of fasting and refeeding on tumor growth in T-1699 TMT's and C3H/HeJ SMT's: T-1699 untreated (●——●), 48 (●--●) and 72 hr (▲--▲) fasting; C3H/HeJ control (○——○) and 72 hr fast (○--○). Mean of 5–10 mice per point ± 1 S.E.

with older mice, however, mortality occasionally exceeded 50%. Thus, for the subsequent kinetic studies, fasting periods were reduced to 60 hr with little or no resultant mortality.

Tumor cell kinetics

Baseline kinetic parameters for the fast growing, poorly differentiated T-1699 TMT and the slow growing, well differentiated SMT are given in Table 1. In the T-1699 TMT, 48 hr of fasting resulted in a 13% reduction in the PDP index (significant at $P \leqq 0.05$) with a concomitant reduction in the [^{3}H]TdR LI of approximately 45%. The T_S was increased by a factor of 2 while the T_C was increased by a factor of 3. The K_P was reduced by 74% with a 45% reduction in K_L. The potential doubling time (T_P) was increased from 25 to 96.1 hr. The TMT cytokinetics after 72 hr of fasting were of similar magnitude to those observed after 48 hr.

Although fasting in the C3H/HeJ SMT resulted in no significant change in the PDP index, an 80% reduction in the [^{3}H]TdR LI was observed. The T_S increased slightly to 12.0 hr, while the T_C lengthened by a factor of 5. The K_P was reduced by 80% while the K_L was increased by a factor of 2. The T_P was increased from 123 to 601 hr after fasting.

The cytokinetic response to fasting and refeeding in the T-1699 TMT is presented in Fig. 2. Refeeding after 48 hr of fasting resulted in a recovery to age-matched control PDP-I's by 24 hr. Refeeding after 72 hr of fasting, however, resulted in a slower recovery of the PDP-I with an increase above age-matched controls observed between days 3 and 7. The [^{3}H]TdR LI following refeeding (48 hr fast) exhibited a sharp increase to control levels within 6 hr, a subsequent reduction to the fasting levels at 24 hr and

Table 1. Cell kinetic parameters for the control and fasting T-1699 TMT and C3H/HeJ SMT

	T-1699 TMT			C3H/HeJ SMT	
	Control	48 hr fast	72 hr fast	Control	60 hr fast
PDP-I (%)	70.6 ± 3.0	61.4 ± 4.1	49.9 ± 4.0	16.6 ± 0.4*	18.5 ± 3.5
[^{3}H]TdR LI (%)	25.4 ± 1.2	13.4 ± 2.1	15.2 ± 1.9	6.9 ± 0.6*	1.5 ± 0.7
T_S (hr)	6.9 ± 0.2	15.2 ± 0.7	12.4 ± 0.1	10.9 ± 0.2*	12.0 ± 0.5
T_C (hr)	17.6	58.9	35.9	20.5	111.3
K_P	0.0277	0.0072	0.0096	0.0055	0.0011
K_L	0.0180	0.0098	0.0128	0.0033	0.0079
T_D (hr)	72	−264	−216	312	−96
T_P† (hr)	25.0	96.1	71.9	123.8	601.8
ϕ‡	0.652	1.364	1.332	0.603	7.260
λ§	0.922‖	0.847‖	0.882‖	0.784¶	0.752¶

*From reference [28].
†T_P = potential tumor doubling time [26].
‡ϕ = cell loss factor [26].
§λ = correction factor for nonlinear distribution of S through the cycle [26].
‖G_2 = 3.9 hr [24].
¶G_2 = 2 hr [27].

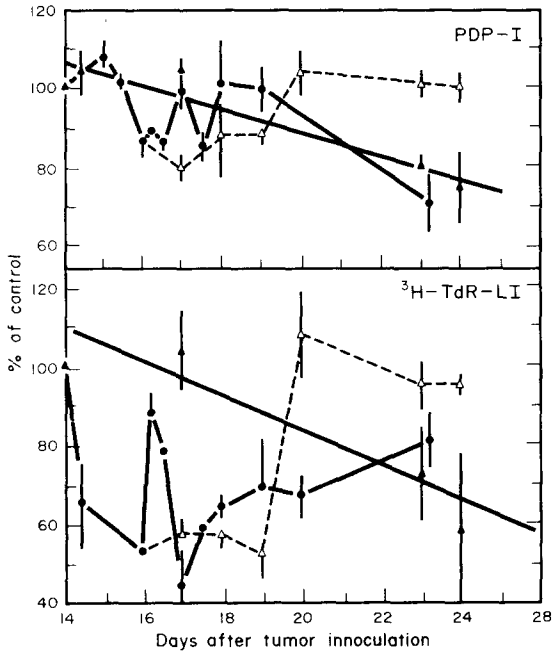

Fig. 2. Effect of a 48 hr (●——●) *or* 72 hr (△- △) *fast plus refeeding on the PDP-I and* [^{3}H]*TdR LI of the T-1699 TMT vs nonfasted controls* (▲——▲). *Mean of* 5 *mice per point* ± 1 S.E.

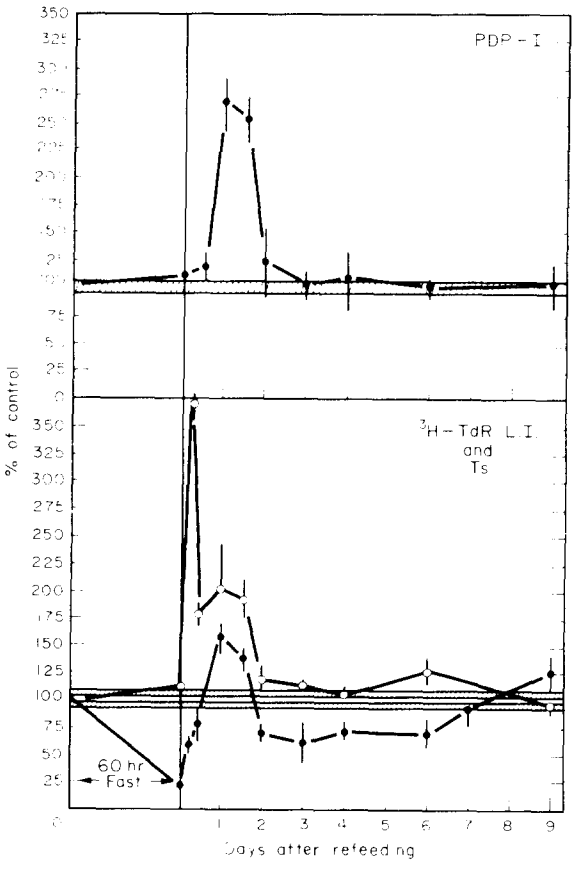

Fig. 3. Effect of a 60 hr *fast plus refeeding on the PDP-I* (●——●), [^{3}H]*Tdr LI* (●——●) *and* T_S (○——○) *of the C3H/HeJ SMT. Mean of* 3–5 *mice per point* ± 1 *S.E. Horizontal slashed line delineates control PDP-I and* [^{3}H]*TdR LI values* ± S.E. *Horizontal open line delineates control* T_S *values* ± 1 S.E.

then a recovery to normal levels during the next 72 hr. After 72 hr of fasting, increases in [^{3}H]TdR LI were not observed until 72 hr after refeeding. As with the PDP-I, increases above age-matched controls were observed between days 3 and 7 after refeeding. The T_S for the T-1699 TMT (not shown) was reduced to near control values within 6 hr of refeeding and remained unchanged thereafter.

The cytokinetic response to refeeding in the C3H/HeJ SMT is presented in Fig. 3. A 2.7-fold increase in the PDP-I was observed at 24 hr of refeeding, with a regaining of control values by 72 hr. The [^{3}H]TdR LI increased within 6 hr of refeeding reaching a maximum by 24 hr. The [^{3}H]TdR LI subsequently decreased to subnormal levels between days 2 and 6 and regained control values by day 7.

The T_S values, as measured by the double label technique, exhibited a 3.7-fold increase over control at 6 hr and a 2-fold increase between 12 and 36 hr. Control values were re-established at 48 hr and remained constant for the remainder of the observation period.

DISCUSSION

In the present studies the kinetic responses of two tumor models to fasting and refeeding were examined. These tumors were selected for their divergent cell kinetic and morphological characteristics. The C3H/HeJ SMT is a slow growing, well differentiated adenocarcinoma, while the T-1699 is a rapidly growing, poorly differentiated adenocarcinoma.

In both tumors nutritional deprivation resulted in a significant reduction in the tumor volumes. Since the tumor water content was unchanged by fasting, these observed regressions probably reflect an actual loss of tumor tissue mass. Following refeeding, both tumors recovered rapidly, regaining their prefasted growth rates within 1–2 days.

In both systems, the volume changes can be attributed to a pronounced depression in cell production coupled with a continued cell loss. In the C3H/HeJ SMT the cell loss rate was increased during fasting, suggesting a starvation enhanced cell senescence. Increased cell death has been demonstrated for nutritionally deprived cells *in vitro* [13]. In contrast the T-1699 TMT showed a reduced cell loss rate during fasting. Since the T-1699 TMT's exhibit a high degree of host cell

infiltration and cellular necrosis at the sizes studied, this reduced cell loss during fasting may reflect a transitory compromise of the immune response [29].

The kinetic basis for the diminished cell production was unique to each tumor system. In the T-1699 TMT, fasting induced a generalized lengthening of the cell cycle as evidenced by the 2–3-fold increase in both T_S and T_C. Upon refeeding, increases in the [^{3}H]TdR LI and PDP-I were observed within 24 hr after a 48 hr fast while a delay in the response was observed after a 72 hr fast. This may suggest a dose–response relationship for the fasting stress in this poorly differentiated tumor.

In contrast, the C3H SMT showed a marked increase in the T_C but only a slight increase in T_S together with an 80% reduction in the [^{3}H]Tdr LI. Although the response in the [^{3}H]TdR LI was initiated promptly after refeeding, increases in the PDP-I were not observed until 24 hr. These observations are consistent with the presence of a cell cycle block near the G_1/S border, which is released promptly upon refeeding resulting in a synchronous movement of tumor cells into S phase. Synchronization is further evidenced by the marked lengthening of T_S immediately after refeeding, reflective of a large cohort of cells entering S without a concomitant movement of cells into G_2. This would tend to artificially elevate the T_S measurement. The sustained increase in T_S observed between 12 and 36 hr could be accounted for by the movement of cells into S exceeding the movement of cells into G_2, as would be expected during a recruitment period. Further evidence for the recruitment of noncycling tumor cells is given by the increase in the PDP during refeeding. The magnitude of this increase (270%) is greater than that which would be expected from the division of the synchronous cohort of cells in mitosis (200%).

This study was designed to evaluate the modification of tumor kinetics through fasting and refeeding. While being a nonspecific stress, these nutritional manipulations were shown to produce distinct cell kinetic responses in both tumor systems. What must be considered now is how does the nutritional status of cancer patients influence the tumor cell kinetics and could these changes have clinical importance for a kinetically based sequential or combination oncotherapy. In the present studies, a pretreatment therapy of fasting plus refeeding would have enhanced the response of the C3H SMT to cycle active agents. What must also be considered is to what extent do the nutritional side effects of oncotherapy influence the resultant tumor response(s)? These questions are currently under investigation.

Of additional interest is the disparity of response between these two adenocarcinomas. The ability of the C3H/HeJ SMT to respond to the nutritional stress as would be expected for normal cell types [12, 15, 16] may reflect this tumor's well-differentiated profile. Conversely, the undifferentiated TMT responds as predicted for virally transformed cells *in vitro* [12]. These observations may reflect the degree of malignancy of these tumors.

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