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LOSS OF ANDROGEN DEPENDENCE IS ASSOCIATED WITH AN INCREASE IN TUMORIGENIC STEM CELLS AND RESISTANCE TO CELL-DEATH GENES

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Summary—Complete remissions of the androgen-dependent Shionogi mouse mammary carcinoma are observed after androgen withdrawal but invariably the disease recurs and is refractory to further hormonal manipulations. To determine the proportions of androgen-dependent (AD) and -independent (AI) tumorigenic stem cells in parent and recurrent tumors an *in vivo* limiting dilution assay was developed. There was a marked enrichment of stem cells in the recurrent tumors (1/200 tumor cells) relative to the parent tumors (1/4000 tumor cells) when assayed in male hosts. By assaying tumor takes in female mice, the proportion of AI stem cells was found to be 1/370,000 tumor cells in the parent vs 1/800 tumor cells in the recurrent carcinoma; a 500-fold increase in AI stem cells resulting from androgen-withdrawal. Unexpectedly, no enrichment of AI stem cells was evident in regressing parent tumors; rather, the proportion of such cells was very small (1/2,200,000 tumor cells). This finding implies that the AI cells which survive androgen withdrawal may result from the ability of small number of initially AD stem cells to adapt to an altered hormonal environment. This adaptive process was further defined in terms of the disappearance of androgen receptors from the nucleus and the expression of androgen-repressed genes including the proto-oncogenes, c-*fos* and c-*myc*, and the cell death gene, TRPM-2; all of which are constitutively active in recurrent AI tumor cells. Overall, our results indicate: (1) the tumor mass consists mainly of differentiated cells; (2) stem cells initially are AD but at most the killing effect of androgen-withdrawal will be limited to 2–3 logarithms before compensatory adaptive mechanisms supervene; and (3) progression of stem cells to an AI state, in which they are resistant to the killing effects of cell death genes, might be prevented by the inhibition of androgen-repressed adaptive mechanisms which come into play when androgens are withdrawn.

INTRODUCTION

The Shionogi mouse mammary carcinoma has been used by many investigators [1–4] to study the mechanisms through which androgens control cell proliferation and cell death in hormone responsive tumors. With respect to the latter cell-death processes, the regression of androgen-dependent (AD) Shionogi tumors following androgen withdrawal therapy can be described in terms of differential expression of androgen-induced and androgen-repressed genes [5]. However, while complete remissions of these tumors are generally observed after androgen withdrawal, the tumors invariably recur in a form that is refractory to further hormonal manipulations [6]. Thus the Shionogi carcinoma also provides a model system for following the emergence of tumorigenic stem cells during the progression from the androgen-dependent (AD) to the androgen-independent (AI) growth [7, 8].

In the present study, we summarize our findings regarding the activation of cell death related genes during regression of AD Shionogi tumors and the subsequent enrichment in stem cells in recurrent tumors that occurs as a consequence to tumor progression.

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RESULTS

Response of the Shionogi carcinoma to androgen withdrawal

After injecting 5×10^6 cells of the Shionogi carcinoma into male DDS mice, the transplantable parent tumor becomes palpable after about 10 days and is 2–3 g in weight after approx. 15 days. Upon castration of the host, the tumor regresses almost completely within 7–10 days. After a subclinical phase lasting between 20–30 days, when the tumor is either very small or not palpable, recurrent growth is detected and gradually culminates in rapid pro-

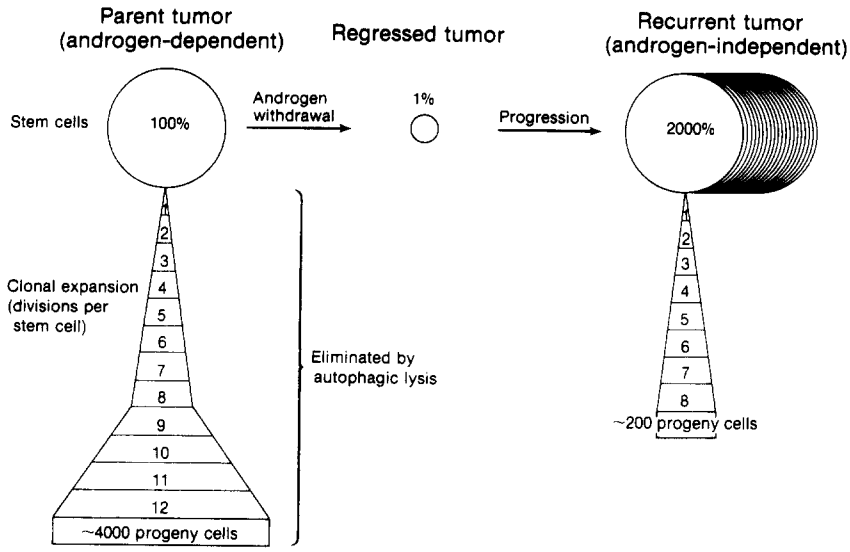

Fig. 1. Effects of androgen withdrawal on stem cell composition [10].

liferation. Unlike the parent cells, the recurrent tumor does not respond to any further androgen-withdrawal therapy. Overall, the Shionogi carcinoma probably mimics the clinical course of prostatic carcinoma better than any other animal model system presently available. Cycles of growth, regression and recurrence can be faithfully reproduced affording ideal conditions for studying the loss of androgen regulation.

Tumorigenic stem cells: theoretical considerations

In an attempt to relate failure of hormone treatment to other factors associated with tumor progression and hormone-independence, we adopted the working hypothesis that is shown in Fig. 1. A hormone-dependent tumor consists of a functional hierarchy of stem cells and differentiated cells which arrive through clonal expansion of the stem cell compartment. Androgen withdrawal triggers a process of programmed cell death which results in autophagic lysis and elminates the differentiated progeny cells. By definition, some stem cells are AI and are immune to autophagic killing. Progression and further growth of surviving stem cells results in the recurrent autonomous tumor with an enriched stem cell population.

Measurement of tumorigenic stem cells

A limiting dilution assay [9] was developed in our laboratory to measure the stem cell content of solid tumors [8, 10]. The analysis is done by injecting a decreasing number of dispersed tumor cells into several groups of male or female animals and noting the incidence of tumor takes in each group. The data obtained is analyzed by computer methods [11] and the proportion of stem cells in the tumor is estimated (Fig. 2). Roughly speaking, at the point where the proportion of tumor takes falls to 37%, there is an average one clonogenic cell per innoculum. Since the number of tumor cells in the latter is known, an estimate is obtained of the proportion of stem cells in the overall population of tumor cells. A shift in the curve to the right denotes an increased proportion of stem cells while a shift to the left denotes a decreased proportion.

Within the framework of this model system and the application of the limiting dilution assay, we addressed three questions. First, whether the recurrent tumor contained more stem cells than the parent tumor. Second, whether the proportion of stem cells in the parent tumor increased after castration owing to the selective survival of presumed AI stem cells.

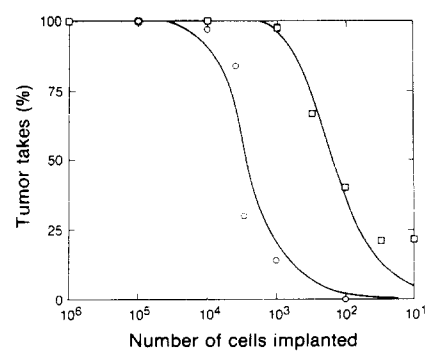

Fig. 2. Limiting dilution assay: computer plot for parent (○) and recurrent (□) tumors.

Third, whether there was a difference in the effect of androgen withdrawal on parent as compared with recurrent tumor stem cells.

Proportion of tumorigenic stem cells in parent and recurrent tumors

As summarized in Table 1, when the stem cell compositions of parent and recurrent tumors were measured in the presence of androgens (i.e. male hosts), the proportion of stem cells in the recurrent tumor, at 1 per 200 cells, was 20-fold greater than the proportion of stem cells in the parent tumor at 1 per 4000. In the absence of androgens (i.e. female hosts), the proportion of stem cells in the parent tumor drops to 1 in 370,000. This difference is equivalent to a 100-fold or 2 logarithm cell kill. By comparison in the recurrent tumor, the proportion of stem cells in the presence of androgens is 1 in 200 and this drops to 1 in 800 in the absence of androgens. This decrease represents only a four-fold reduction in the number of stem cells. Thus stem cells in the parent tumor are much more sensitive to the effects of androgen withdrawal than those in the recurrent tumor.

If one assumes that the stem cells that are measured in the absence of androgens are AI, then there is clearly a very large increase in the proportion of AI stem cells in the recurrent tumor. The difference between 1 in 370,000 and 1 in 800 is almost 500-fold.

Proportion of tumorigenic stem cells in regressing tumors

The stem cell composition of the 7-day regressed parent tumor was next measured. The number of stem cells in the tumor was compared before castration and 7 days after castration when the tumor had regressed to about 20% of its pre-castration weight. In this experiment the 7-day regressed tumor was assayed both in the presence (male hosts) and absence (female hosts) of androgens. As indicated in Table 1, the proportion of stem cells in the regressed tumor did not increase; rather it fell from 1 in 4000 in the non-regressed tumor to 1 in 70,000 in the 7-day regressed tumor. In this experiment tumor cells were rescued by androgen replacement after 7 days; but in the absence of androgen replacement, the proportion of stem cells fell to the extremely small number of 1 in 2,200,000. These data clearly demonstrated that stem cells are AD and the overall killing effect of androgen withdrawal is about 3 logarithms. Since virtually all of the stem cells are AD, the question arises whether there are any AI stem cells in the parent tumor at the initiation of therapy. As there is no enrichment of AI stem cells during tumor regression, it is likely that they arise as a consequence of an adaptive or epigenetic change in surviving AD stem cells rather than a clonal selection mechanism.

Table 1. Summary of stem cell estimates in parent, regressing, a recurrent Shionogi carcinomas

	Number of tumor cells per stem cell	
Tumor	Male host	Female host
Parent	4000	370,000
Regressing	70,000	2,200,000
Recurrent	800	200

Effects of androgen withdrawal on tumor kinetics

We next determined the effect of androgen withdrawal on the doubling time (T_D) of parent and recurrent tumors [10]. The time for the parent tumor to reach 0.5 g in weight was measured after the injection of decreasing numbers of cells into groups of male and female animals. The slopes of the lines ($T_D/\log_e 2$) obtained from this data are directly related to the doubling time (T_D). Since the slopes of both lines are parallel (data not shown), the doubling time of the tumor in both male and female animals is the same and approx. 12 days. Thus androgen withdrawal does not affect the doubling time.

When the same measurements were repeated on the recurrent tumor, the slopes of the 2 lines obtained were also parallel; indicating that the doubling time of the tumor is again the same in both male and female animals. An estimated T_D of 6 days indicates that the recurrent tumor grows at a slightly faster rate than the parent tumor. These results imply that there are no female specific growth factors that influence the doubling time of either the parent or recurrent tumors.

Activation of cell death genes

In AD cells, androgen receptors act as repressors of genes involved in programmed cell death [12]. TRPM-2 (testosterone repressed prostatic message) was originally isolated from the involuting rat prostate [13], but subsequently, this gene has been found to be expressed abundantly in other epithelial cells undergoing programmed cell death; such as the uterus, mammary gland and kidney [14, 15]. Recently, it was reported that the TRPM-2 gene has considerable homology to a gene coding for a serum factor whose function is to inhibit comp-

lement-induced cytolysis of epithelial cells in the male reproductive tract [16]. Thus it is uncertain whether the protein product of TRPM-2 directly participates in autophagic lysis or whether it plays a protective role in surviving cells.

The activation of cell death genes during regression of the AD Shionogi mouse mammary carcinoma and the killing effects of these genes on tumorigenic stem cells were determined. Within 12 h following castration, Northern analysis of parent tumor mRNA revealed that there was a transient peak of expression of the proto-oncogene c-*fos* [5]. By comparison, c-*myc* exhibited a very low level of expresssion that gradually increased over the 6-day period examined. The concentration of the mRNA encoding TRPM-2 rose from very low to very abundant only when tumor regression was most evident (Fig. 3). At this point the proportion of stem cells had decreased from 1/4000 to approx. 1/70,000.

Unexpectedly, Northern analysis of mRNA from recurrent tumors revealed that c-*fos* and c-*myc* were expressed constitutively and that the concentration of transcripts for TRPM-2 was as high or higher than that measured in the parent tumor of 6-day castrates (data not shown). Furthermore, when the recurrent tumor was transplanted into non-castrated males, the expression of this gene was apparently switched off. After castration, there was a transient rise in the level of TRPM-2 transcripts; peaking at approx. 12 h and then declining to pre-castration levels. Thus despite the absence of measurable androgen receptors, the TRPM-2 gene was apparently sensitive to fluctuations in hormonal levels; suggesting that some processes in the recurrent tumor remain AD.

DISCUSSION

Our results with the Shionogi carcinoma indicate progression of the tumor cannot be ascribed to the incomplete suppression of androgenic mechanisms within the cell. Rather, progression is associated with a major change in the proportion of stem cells in the overall tumor cell population (Fig. 1). For each stem cell in the AD tumor there are approx. 4000 progeny cells which arise through clonal expansion of a parent stem cell (Fig. 2). The daughter cells may undergo up to 12 divisions and during this period of limited self-renewal acquire the capacity for programmed cell death. Thus, androgen withdrawal results in the elimination of the majority, and possibly all, of the progeny cells [6]. Coincidentally, the proportion of stem cells in the regressed tumor declines to 1–6% of the number of the parent tumor. Progression of surviving tumor cells is associated with the development of an AI recurrent tumor in which the proportion of stem cells is increased at least 20-fold over the fraction measured in the parent tumor. For each stem cell in the recurrent tumor there are approx. 200 progeny cells; this observation indicates that the differentiation at AI stem cells does take place but stops after eight divisions.

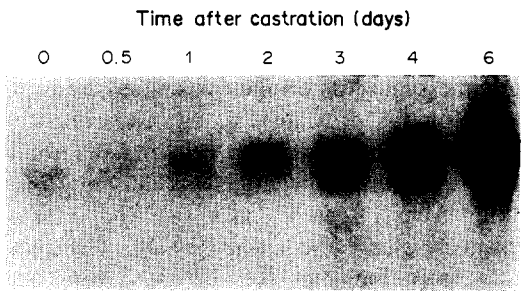

Fig. 3. Effects of castration on the expression of TRPM-2 in parent tumors.

Surprisingly, no enrichment of AI stem cells was evident in regressing parent tumors; rather, the proportion of such cells was very small, i.e. one AI stem cell per 2,200,000 regressing parent cells (Table 1). This finding implies that the AI cells which survive androgen withdrawal may result from the ability of small number of initially AD stem cells to adapt to an altered hormonal environment. This adaptive or epigenetic process was further defined in terms of the disappearance of androgen receptors from the nucleus and the expression of androgen-repressed genes including the proto-oncogenes, c-*fos* and c-*myc*, and the putative cell-death gene, TRPM-2 (Fig. 3), all of which are constitutively active in recurrent AI tumor cells. Other androgen-repressed genes may code for autocrine or paracrine growth factors that substitute for androgens in stimulating the division and differentiation of surviving parent stem cells.

The foregoing observations indicate that there are several factors that should be taken into consideration when androgen-withdrawal is used to check tumor growth. First, the bulk of the tumor mass initially consists of differentiated cells which are programmed to undergo autophagic lysis when androgens are withdrawn. Second, stem cells initially are also AD but at most the killing effect of androgen-with-

drawal will be limited to 2–3 logarithms before compensatory adaptive mechanisms supervene and provide the support mechanisms for renewed growth. Lastly, progression of stem cells to an AI state, in which they can circumvent the killing effects of cell death genes, might be prevented by the inhibition of androgen-repressed adaptive/epigenetic mechanisms which come into play when androgens are withdrawn.

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