Effects of Androgen Withdrawal on the Stem Cell Composition of the Shionogi Carcinoma

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ABSTRACT

The parent Shionogi mouse mammary carcinoma is androgen-dependent but cells that survive hormone withdrawal progress and give rise to an androgen-independent tumor. To determine whether renewed growth might be attributed to the persistence or partial recovery of an androgenic stimulus, we compared the amount of dihydrotestosterone and nuclear androgen receptor in parent and recurrent tumors. The whole tissue concentration of dihydrotestosterone in the parent tumor before castration was 1.40 ± 0.46 (SE) as compared with 0.22 ± 0.10 pmol/mg of DNA in the recurrent tumor. The initial concentration of nuclear androgen receptor in the parent was 0.65 ± 0.12 pmol/mg of DNA; this was reduced to zero within 24 h after castration. Also in keeping with the androgen independence, no receptor was detected in the nuclear fraction of the recurrent carcinoma. In an attempt to relate malignant potential to nonhormonal factors associated with progression, we compared the proportions of androgen-dependent and -independent tumorigenic (stem) cells in parent and recurrent tumors using an in vivo limiting dilution assay. The difference observed, i.e., one stem cell per 4000 tumor cells in the parent versus one stem cell per 200 tumor cells in the recurrent carcinoma, was consistent with a marked enrichment of stem cells in the latter. The proportion of androgen-independent stem cells was also determined by assaying tumor takes in female hosts. The difference, i.e., one stem cell per 370,000 tumor cells in the parent versus one stem cell per 800 tumor cells in the recurrent carcinoma, demonstrated a striking 500-fold increase in androgen-independent stem cells resulting from androgen withdrawal. Unexpectedly, no enrichment of androgen-independent stem cells was evident in regressing parent tumors; rather, the proportion of such cells was very small, i.e., one androgen-independent stem cell per 2,200,000 regressing parent cells. This finding implies that the androgen-independent state of cells which survive androgen withdrawal may result from the ability of a small number of initially androgen-dependent stem cells to adapt to an altered hormone environment.

INTRODUCTION

The parent Shionogi mouse mammary carcinoma grows rapidly in the presence of androgens and, even if locally advanced, will regress when androgens are withdrawn (1–4); thus, the wild-type tumor is both androgen sensitive and androgen dependent. Serial transplantation of the carcinoma over many generations has yielded several variants which resemble the parent in their requirement for androgens and others which do not. Sublines may be grouped according to whether they are androgen sensitive but androgen independent (3, 5–8); both androgen insensitive and androgen independent, i.e., autonomous (3, 9, 10); or sensitive to estrogens and glucocorticoids in addition to androgens (11–16).

The phenotypic gap which separates the parent and autonomous tumors is linked to progression, a process characterized by a stepwise cumulative loss of hormonal regulation (17–20). Paralleling observations in other model systems (17, 18), the progression of the parent Shionogi carcinoma is accelerated by the withdrawal of steroid; despite active autophagic reduction of the primary tumor to the point of complete remission, such treatment is not curative (4, 6–8, 21–23). On the contrary, the cycle of growth, therapeutic regression, and recurrence in an androgen-poor environment fosters the eventual outgrowth of an androgen-insensitive, androgen-independent carcinoma (4, 23).

In studying the long-term effects of androgen withdrawal on malignancy, we have examined two concepts which might explain the recurrent growth of the Shionogi carcinoma: the first focuses on the inadequate suppression of androgenic mechanisms within the cell; the second, on the amplification of endocrine-related progression in the absence of androgens. Since the amount of nuclear androgen receptor in the recurrent tumor is consistent with the post-castration level of androgen in the host animal (4), the possibility of an extended hormonal response seems unlikely. We now report additional findings which strengthen this conclusion and clearly demonstrate that androgen withdrawal culminates in a more aggressive tumor owing to a massive increase in the proportion of androgen-independent tumorigenic (stem) cells.

MATERIALS AND METHODS

Shionogi Mammary Carcinoma. The Toronto subline (9) of the transplantable SC-115 androgen-dependent mouse mammary carcinoma (4, 24) was used in the following experiments. Castration of male tumor-bearing DD/S strain mice was performed through an abdominal incision. Ether anesthesia was used for surgery and also for sacrificing animals. In hormone replacement experiments, castrated hosts were given injections s.c. with a single dose of 100 µg of testosterone in 1 ml of distilled H2O containing 5% ethanol, 10% polyoxyethylenesorbitan monopalmitate and 0.9% NaCl solution and sacrificed 1 h later.

Preparation of Single Cell Suspensions. The excised tumors were minced with scissors, and the fragments were washed in 20 ml of warm (37°C) Dulbecco's modified Eagle's tissue culture medium. The clumps were suspended in 0.5% trypsin (1:250) and 0.025% EDTA in Ca++- and Mg++-free saline and incubated for 10 min at 37°C with constant stirring. Large particles of tissue were allowed to settle, and the supernatant was decanted through a 150-µm Nitex filter (Tetko, Inc., Elmford, NY). Centrifugation of the filtrate at 250 × g for 3 min yielded a pellet which was resuspended in 5 ml of tissue culture medium; the particles of tissue collected after the first period of digestion were reincubated in trypsin-EDTA solution, and the cycle of incubation, filtration, and centrifugation was repeated. The 2- to 5-ml cellular suspensions were combined, passed through a 48-µm Nitex filter, and centrifuged at 350 × g for 5 min. The final pellet was resuspended in 10 ml of tissue culture medium from which an aliquot was removed for the counting of cells in a hemocytometer. The percentage of viable cells, determined by trypan blue exclusion, routinely was greater than 90%. The cells were then either diluted with tissue culture medium in preparation for injection or stored in 20% dimethyl sulfoxide for analysis by flow cytometry.

Limiting Dilution Assay for Stem Cells. The preparations of single cells were serially diluted with tissue culture medium (20°C) yielding a decreasing number of tumor cells ranging from 10 to 10^5 in a final volume of 0.5 ml. Groups of 10 to 60 male and female mice were given injections s.c. into the interscapular region with the cell suspensions. The incidence of tumors in each group of animals was recorded over a...
period of 6 to 7 mo; a tumor take was judged positive when the tumor had attained a size of approximately 0.5 g; however, the animals were not sacrificed until the estimated weight of the tumor reached 2 to 3 g. Results were expressed as the proportion of tumor takes, and 50% confidence intervals were calculated assuming a binomial distribution.

Statistics. The number of viable tumorigenic (stem) cells in each implanted tumor was assumed to follow a binomial distribution with parameters n and p, where n is the total number of cells implanted and p is the proportion of viable stem cells in the donor tumor (25). It was further assumed that the implantation of a single viable stem cell would subsequently lead to the development of a tumor. The proportion of takes in an experiment is therefore given by 1-(1-p)n. Relationships of this form were fitted to the data using the method of maximum likelihood. The parameter p was estimated for each series of experiments. The estimated value of p was then used to plot the predicted proportion of tumor takes as a function of the number of cells implanted. The statistical significance of differences in the proportion of stem cells between two series of experiments was assessed using the asymptotic distribution of the difference in log-likelihoods which yields a χ² statistic with 1 d.f.

Preparation of Tissue Fractions. All procedures were performed at 4°C. Reagents for buffers and other solutions were obtained from Sigma Chemical Co. (St. Louis, MO). Tumor tissue was homogenized in a Dounce apparatus as described previously (4), and aliquots were taken for estimation of the whole tissue concentration of dihydrotestosterone (26), protein (27), and DNA (28). Nuclei were then purified from the remaining homogenate using established methods (4, 10); aliquots of this fraction were taken for measurement of the concentrations of dihydrotestosterone (26), nuclear androgen receptor (29), and DNA.

Assay of Nuclear Androgen Receptor. Purified nuclei were resuspended in 0.5 ml of TES buffer* containing 50 mM NaCl and allowed to stand for 30 min at 4°C. The swollen nuclei were then sonicated with five 10-s pulses of 50,000 cycles of a Bronwill Biosonic III sonicator. An equal volume of TES buffer containing 1.15 M NaCl was added, and the suspension was sonicated again with 5 10-s pulses. After an incubation period of 30 min, the sample was centrifuged at 17,000 × g for 20 min to obtain a nuclear extract free of membranous debris. The cycle of sonication, extraction, and centrifugation was repeated 1 to 2 more times. The supernatants were pooled and divided into a number of equal fractions; these were incubated with 12 concentrations of [1,2-3H]dihydrotestosterone (0.2 to 20 nmol/liter). The remainder were incubated with 2 concentrations of [1,2-3H]dihydrotestosterone (1.2 and 6.0 nmol/liter) and a 100-fold excess of nonradioactive dihydrotestosterone to verify nonspecific binding. An aliquot (10 μl) was taken from each sample to confirm the concentration of [1,2-3H] dihydrotestosterone. Triplicate aliquots of 100 μl were analyzed for receptor using pyridoxal-5'-phosphate and protamine sulfate precipitation (29). Data were analyzed using the Ligand computer program (30) to obtain estimates of the dissociation constant and maximum number of nuclear binding sites.

Measurement of 5α-Reductase Activity. A standard assay which provides optimum conditions for detecting 5α-reductase activity was developed in earlier studies (26) and used again in the present experiments without modifications. The rate of metabolism of testosterone was determined from the relationship (concentration of steroid substrate multiplied by the percentage of conversion) divided by (concentration of protein) to yield a result in terms of pmol/30 min/mg of protein. To obtain the V₅₀ and Kₘ₅ parameters of the Michaelis-Menten equation, the data were analyzed by the Lineweaver-Burk reciprocal plot (1/v versus 1/[S]) (31).

Flow Cytometry. Preparations of single cells were stained for DNA content with propidium iodide (32) and then analyzed on a Coulter Epics V laser flow cytometer. A DNA index was calculated from the relationship, intensity of fluorescence of aneupeic peak divided by intensity of fluorescence of diploid peak (33).

Radioactive Materials. [1,2,3H]Dihydrotestosterone (50 to 60 Ci/ mmol) and [1,2,3H]Testosterone (50 to 60 Ci/mmol) were purchased from Amersham Canada, Ltd. (Oakville, ON, Canada). Purity was checked by thin-layer chromatography, and the steroid was considered acceptable only if it was 95 to 100% pure. In preparation for incubation, radioactive steroid in benzene:ethanol (9:1, v/v) was dried under N₂, dissolved in 100% ethanol, and diluted with TES buffer to a final concentration of 50 pmol/ml. Radioactivity was counted in a Beckman LS-7500 liquid scintillation system; the counting efficiency was about 48%.

RESULTS

Growth Characteristics of Parent and Recurrent Tumors. The sequential pattern of growth, regression, and recurrence of the Shionogi carcinoma is shown in Fig. 1. Following the injection of 5 × 10⁶ parent tumor cells into adult male mice, the tumor becomes palpable on approximately the tenth day and subsequently enlarges rapidly and continuously unless checked by treatment. Despite castration, the tumor continues to grow for 1 to 2 days before involution begins; in the interval between 3 and 10 days after castration, the tumor regresses rapidly, losing as much as 50% of its mass within 72 h. By 15 to 17 days after castration, the bulk of the tumor has disappeared, and in 20% of the animals the remission is complete. About 40 to 50 days after the transplant, recurrent growth begins, gradually accelerates, and finally culminates in a stage of rapid proliferation.

For experimental purposes, both the parent and recurrent tumors were usually harvested when 2 to 3 g in weight. If the recurrent tumor is subsequently transplanted into intact male mice, castration fails to induce regression and, thus, the tumor is androgen independent (data not shown).

Concentration of Dihydrotestosterone in Parent and Recurrent Tumors. To confirm that the recurrent tumor develops the capacity to grow in an androgen-poor environment, the concentration of dihydrotestosterone was compared in the parent and recurrent tumors; the results are presented in Table 1. Before castration of the host animal, the parent tumor is characterized by a whole tissue concentration of dihydrotestosterone of 1.40 pmol/mg of DNA, virtually identical to the mean concentration in the nucleus, 1.06 pmol/mg of DNA (t test, P > 0.05). These results indicate that 75% or more of the intracellular dihydrotestosterone is concentrated in the nucleus. In the first 2 to 8 h

![Fig. 1. Growth, regression, and recurrence of androgen-dependent Shionogi carcinoma cells. Male DD/S mice were given implants of about 2 × 10⁷ tumor cells. After 17 days, when the tumors had reached a mean weight of 6 g, the animals were castrated. Each animal had only one tumor mass, and tumor diameters were measured in mm with callipers. The formula (length × width²)/2 = mass (mg) was used to estimate the mean weight of the tumors (34). Points, mean from 7 to 10 tumors; bars, SE.](https://cancerres.aacrjournals.org/download/2276/fig1.jpg)
after castration, there is little change in the whole tissue concentration of dihydrotestosterone (1.10 pmol/mg) compared with the 77% fall in the nuclear concentration to 0.24 pmol/mg; this asymmetrical change implies that dihydrotestosterone is initially discharged from the nucleus into the cytoplasm before effluxing from the cell. By 16 to 24 h after castration, the whole tissue and nuclear concentrations of dihydrotestosterone, 0.12 pmol/mg of DNA and 0.38 pmol/mg of DNA, respectively, are both significantly below the corresponding levels in the parent tumor before castration (t test, P < 0.05). In the recurrent tumor, the whole tissue and nuclear concentrations of dihydrotestosterone at 0.22 pmol/mg of DNA and 0.13 pmol/mg of DNA are only 12 to 16% of the precastration levels. Thus, the change in the androgen-dependent state of the parent tumor to the androgen-independent state of the recurrent tumor is not associated with any alterations in the baseline castrate levels of dihydrotestosterone in either whole tissue or the nuclear compartment. These results clearly eliminate the possibility that recurrent growth is due to the rebound of tissue dihydrotestosterone from precursor androgens secreted by the adrenal glands.

Concentration of Nuclear Androgen Receptor in Parent and Recurrent Tumors. In previous studies, we have demonstrated the presence of nuclear binding sites for dihydrotestosterone by gel exclusion chromatography (4, 9, 10). The nuclear androgen receptor is firmly bound to chromatin, and attempts to release it by salt dissociation or micrococcal nuclease digestion have not been successful. However, the receptor can be precipitated by protamine sulfate affording a sensitive method for studying its concentration under different physiological conditions. Specific and nonspecific binding can be demonstrated as shown by the results in Fig. 2A, and a Scatchard plot of the specific binding, as shown in Fig. 2B, yields a dissociation constant, $K_d$, of 5 nmol/liter and a maximum binding capacity of 4600 molecules/nucleus.

The results of similar determinations on the parent and recurrent tumors under different conditions of androgen withdrawal and replacement are summarized in Table 2. The concentration of nuclear androgen receptor in the parent tumor before castration is 0.65 pmol/mg of DNA, dropping to 0.14 pmol/mg of DNA 2 to 8 h after castration and to a nondetectable level after 16 to 24 h. The s.c. injection of 100 μg of testosterone restores the concentration of nuclear androgen receptor to the precastration level within 1 h. In contrast, the recurrent tumor is devoid of nuclear androgen receptor, and the replacement of testosterone is without effect. Since the receptor mechanism for mediating the action of androgens is missing, it may be concluded that the growth of recurrent malignancy is androgen independent.

$5α$-Reductase Activity in Parent and Recurrent Tumors. To study the potential of recurrent tumor cells to accumulate dihydrotestosterone, the activity of $5α$-reductase in parent and recurrent tumors was compared. As can be seen from the results presented in Table 3, one-half of the parent tumors contains a small amount of enzyme activity; in contrast, no activity is detectable in the recurrent tumors. These findings are in accord with the conclusion that recurrent growth takes place in what is essentially an androgen-free state.

Stem Cell Composition of Parent and Recurrent Tumors. Androgen withdrawal might be expected to alter the relative proportion of tumorigenic (stem) cells and non-stem cell descendants. It has been assumed that stem cells are characterized by an ability to survive in the androgen-free state and are therefore

![Fig. 2. Estimation of dissociation constant and maximum binding capacity of nuclear androgen receptor. Parent tumor (2 to 3 g) was harvested during growth phase. An extract of purified nuclei was prepared as described in "Materials and Methods" and labeled with 12 concentrations of [1, 2-3H]dihydrotestosterone. After incubation with pyridoxal-5′-phosphate, the receptor was precipitated with protamine sulfate and counted for radioactivity. A: O, total binding; ---, nonspecific binding as given by a Ligand computer program (30). B: Scatchard plot of specific binding using Ligand.](image)

Table 1 Concentration of dihydrotestosterone in whole tissue and nucleus of parent and recurrent tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Dihydrotestosterone (pmol/mg of DNA)</th>
<th>Whole tissue</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before castration</td>
<td>1.40 ± 0.46 (14)</td>
<td>1.06 ± 0.35</td>
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<tr>
<td>After castration</td>
<td></td>
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</tr>
<tr>
<td>2-8 h</td>
<td>1.10 ± 0.16 (5)</td>
<td>0.24 ± 0.04</td>
<td>(5)</td>
</tr>
<tr>
<td>16-24 h</td>
<td>0.12 ± 0.04 (7)</td>
<td>0.38 ± 0.17</td>
<td>(4)</td>
</tr>
<tr>
<td>Recurrent</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0.22 ± 0.10 (5)</td>
<td>0.13 ± 0.03</td>
<td>(12)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Numbers in parentheses, number of tumors examined.

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N. Bruchovsky, unpublished data.
Table 2  Concentration of nuclear androgen receptor in parent and recurrent tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Nuclear androgen receptor (pmol/mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td></td>
</tr>
<tr>
<td>Before castration</td>
<td>0.65 ± 0.12 ( ^\circ ) (3) (^a)</td>
</tr>
<tr>
<td>After castration</td>
<td></td>
</tr>
<tr>
<td>2–8 h</td>
<td>0.14 ± 0.13 (4)</td>
</tr>
<tr>
<td>16–24 h</td>
<td>0.00 (3)</td>
</tr>
<tr>
<td>With testosterone replacement</td>
<td>0.72 ± 0.15 (4)</td>
</tr>
<tr>
<td>Recurrent</td>
<td></td>
</tr>
<tr>
<td>Without testosterone replacement</td>
<td>0.00 (3)</td>
</tr>
<tr>
<td>With testosterone replacement</td>
<td>0.00 (3)</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± SE.

\(^{b}\) Numbers in parentheses, number of tumors examined.

Table 3  \(5\alpha\)-Reductase activity in parent and recurrent tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>WT (g)</th>
<th>( V_{\text{max}} ) (pmol/30 min/mg of protein)</th>
<th>( K_{m} ) (nmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>2.2</td>
<td>0.0</td>
<td>156.9</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.8</td>
<td>1145.2</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.6</td>
<td>11.1</td>
<td>148.0</td>
</tr>
<tr>
<td></td>
<td>8.9</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Recurrent</td>
<td>0.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>0.0</td>
<td></td>
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<tr>
<td></td>
<td>2.0</td>
<td>0.0</td>
<td></td>
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<tr>
<td></td>
<td>3.1</td>
<td>0.0</td>
<td></td>
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<tr>
<td></td>
<td>3.2</td>
<td>0.0</td>
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<tr>
<td></td>
<td>4.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>0.0</td>
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</tr>
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</table>

Fig. 3. Relative tumorigenicity of parent and recurrent tumor cells in male mice. Single cell suspensions were prepared from parent and recurrent tumors (2 to 3 g) harvested during growth phase. The percentage of viable cells, determined by trypan blue exclusion, was greater than 90% in both cases. The preparations of single cells were serially diluted with tissue culture medium, yielding \(10^{8}\) to \(10^{9}\) cells in 0.5 ml. Groups of 10 to 60 male mice were given injections s.c. with the different cell titers, and the incidence of tumors in each group was recorded over a period of 6 to 7 mo. Points, proportion of tumor takes with parent \((O)\) or recurrent \((C)\) tumor cells; bars, 50% confidence intervals.

Fig. 4. Relative tumorigenicity of parent tumor cells in female and male mice. Single cell suspensions (greater than 90% viability) were prepared from a parent tumor \((2 to 3 g)\), and varying titers from \(10^{8}\) to \(10^{9}\) cells in 0.5 ml of tissue culture medium were injected s.c. into groups of 10 to 60 female mice. Points, proportion of tumor takes in female \((\square)\) or male \((\bigcirc)\) (reproduced from Fig. 3) mice; bars, 50% confidence intervals.

ANDROGEN WITHDRAWAL EFFECTS ON STEM CELLS

...androgen independent from the start (20). Androgen-induced differentiation of stem cells gives rise to daughter cells which, in contrast, are destroyed by an active process of autophagic lysis when androgens are withdrawn (36, 37). Since the recurrent Shionogi carcinoma is androgen independent on the basis of the absence of nuclear androgen receptor and resistance to secondary androgen withdrawal, we investigated the possibility that progression is associated with a change in the relative numbers of stem cells and differentiated cells. A decreasing number of parent or recurrent tumor cells was injected into several groups of male animals, yielding the results plotted in Fig. 3. For the parent tumor, the incidence of tumor takes falls rapidly between \(10^{4}\) and \(10^{5}\) implanted cells, giving rise to an estimate of one stem cell per 4000 parent tumor cells. For the recurrent tumor, the incidence of tumor takes falls sharply between \(10^{3}\) and \(5 \times 10^{3}\) implanted cells, indicating that there is one stem cell per 200 tumor cells. The 20-fold enrichment of stem cells in the recurrent tumor is highly significant \((P < 0.001)\).

**Effect of Androgen Withdrawal on Stem Cells in Parent Tumor.** Although androgen withdrawal results in the death of large numbers of androgen-dependent Shionogi carcinoma cells (Fig. 1), the fate of stem cells which are in the minority (Fig. 3) and outwardly androgen independent is somewhat ambiguous. However, the limiting dilution assay can be used to answer the question of whether there are stem cells in the parent tumor which are killed by androgen withdrawal. The incidence of tumor takes can be compared after injecting decreasing numbers of parent tumor cells into both male and female recipients. Injection of the parent tumor cells into female mice is associated with a marked reduction in the incidence of tumor takes; this is demonstrated by the shift to the left of the sigmoidal curve that describes the relationship between the incidence of tumor takes and the number of cells implanted (Fig. 4). The change of hosts has the effect of reducing the proportion of viable stem cells from one stem cell per 4,000 tumor cells in the androgenized male to one stem cell per 370,000 tumor cells in the low-androgen environment of the female. The difference, which is highly significant \((P < 0.001)\), indicates that when the parent tumor is implanted into female animals, the resultant androgen withdrawal effect reduces the number of stem cells by a factor of about 100 or 2 logarithms.

**Effect of Androgen Withdrawal on Stem Cells in Recurrent Tumor.** The susceptibility of stem cells in the recurrent tumor to androgen withdrawal was also tested; the results are...
sent in Fig. 5. When recurrent tumor cells are injected into female animals in limiting dilutions, the incidence of tumor takes falls sigmoidally between $10^6$ and $10^8$ implanted cells; this plot yields an estimate of one stem cell per 800 tumor cells. In comparison, the estimate of stem cells obtained by injecting recurrent tumor into male recipients is one stem cell per 200 stem cells. Although this 4-fold difference is highly significant ($P < 0.001$), it is clear that the androgen withdrawal effect on recurrent tumor stem cells is small relative to the 100-fold killing of parent tumor stem cells (Fig. 4). Thus, there appears to be a subpopulation of androgen-dependent stem cells in the parent tumor which is markedly diminished after androgen withdrawal; in comparison, the stem cell compartment in the recurrent tumor is larger and characterized by a greatly increased proportion of androgen-independent stem cells.

Effect of Androgen Withdrawal on Kinetics of Tumor Growth.
To study the markedly different tumorigenicity of parent and recurrent malignancy in more detail, the time for a tumor to reach 0.5 g in weight was related to the number of cells initially implanted into a male or female host. The appearance of tumors derived from parent tumor stem cells is considerably retarded at all implanted cell dilutions in female recipient mice (Fig. 6A), consistent with a lethal effect of androgen withdrawal on a large fraction of the parental stem cell population (Fig. 4). In contrast, the time to appearance of tumors derived from recurrent tumor stem cells is almost identical in female and male recipients (Fig. 6B); the apparent small degree of retardation in female animals is in accord with the 4-fold killing effect of androgen withdrawal on this cell type (Fig. 5).

The linear-log relationship depicted in Fig. 6 may be used to obtain an estimate of the tumor-doubling time ($T_d$) since, if growth is assumed to be exponential, the slope of each graph is equal to $T_d/\log 2$. This yields a $T_d$ for the parent tumor of 12 days in male versus 9 days in female hosts, respectively. The corresponding estimates for the recurrent tumor are 5 days and 6 days; thus, the recurrent tumor grows approximately 2 times faster than the parent tumor. It is of interest that the doubling time of the parent tumor tends to be shorter in the androgen-poor environment of the female host than in the normal male animal.

Stem Cell Composition of Regressing Tumors. The 100-fold lower tumorigenicity of parental stem cells in female mice (Fig. 4) implies that the overwhelming majority of stem cells in the parent tumor is androgen dependent. As confirmation, the stem cell composition of regressing tumors was measured by the limiting dilution assay 1 day and 7 days after castration of the male tumor-bearing host (Fig. 1). Three possible results were anticipated: (a) preferential survival and enrichment of androgen-independent stem cells; (b) no change in the proportion of stem cells relative to the total number of regressing tumor cells, consistent with a balanced effect on all cellular components of the tumor; (c) killing and depletion of a singular population of androgen-dependent stem cells. The proportions of androgen-dependent and androgen-independent stem cells 1 day after castration (data not shown) were identical to those before castration (Fig. 4). The results 7 days after castration when the regressing tumor was approximately 30% of its original weight are shown in Fig. 7. Injection of the regressing parent tumor cells into male hosts demonstrates a large decrease in the proportion of stem cells relative to control; this changes from one stem cell per 4,000 tumor cells before castration to one stem cell per 70,000 tumor cells 7 days after castration. Injection of the same regressing tumor cells into female hosts results in even a lower estimate of one stem cell per 2,200,000 tumor cells. Since the proportion of stem cells clearly does not increase in regressing tumors, it is unlikely that the parent tumor contains many preexisting androgen-independent stem cells. The approximate 20-fold reduction ($P < 0.001$) of androgen-dependent stem cells (assayed in male mice) in regressing tumors...
ENDOCRINE-RELATED PROGRESSION OF BREAST MALIGNANCY

Endocrine-related progression of breast malignancy was first described in detail by Foulds (17) and later by Noble (18), both of whom noted that hormone withdrawal, a procedure expected to check tumor growth, accelerated progression of hormone-dependent tumors to an independent state. This paradox is clearly demonstrated by the growth characteristics of the Shionogi mammary carcinoma in the presence and absence of androgens. As long as the tumor is transplanted successively into male hosts, the constant hormonal environment minimizes progression, and the tumor can be maintained in an androgen-dependent state over many transplant generations. Furthermore, castration of the male host at any time will precipitate the regression of the tumor in a highly reproducible fashion (Fig. 1), analogous to a type of response observed during treatment of carcinomas of the breast and prostate. Despite extensive involution of tumor tissue, androgen withdrawal fails to cure the tumor-bearing animal, and recurrence of malignancy eventually takes place in virtually all animals. The failure of castration to check the growth of transplanted recurrent tumor cells indicates that progression to an androgen-independent condition has taken place. These results are consistent with Noble’s conclusion that progression to autonomy is initiated by the withdrawal of hormone on which the growth of tumor is dependent and is not the result of regression per se (18).

Our findings indicate that progression of the Shionogi carcinoma cannot be ascribed to the incomplete suppression of androgenic mechanisms within the cell. Following castration of male tumor-bearing animals, the mean whole tissue and nuclear concentrations of dihydrotestosterone are 9% and 36%, respectively, of normal, compared with 16% and 12%, respectively, of normal, in the recurrent tumor. These results, together with the observation that the recurrent tumor is devoid of 5α-reductase activity, indicate that the recurrent malignancy neither metabolizes testosterone nor accumulates dihydrotestosterone (Table 1). Also in keeping with an androgen-independent state, no androgen receptors are present in the recurrent tumor.
ANDROGEN WITHDRAWAL EFFECTS ON STEM CELLS

cells (Table 2). Such a deficiency is also observed in parent tumor cells 16 to 24 h after castration. In parent tumor cells, the effects of castration are rapidly reversed with the administration of testosterone which induces the reappearance of nuclear androgen receptor almost immediately. In contrast, the recurrent tumor cells fail to respond in similar fashion, and no androgen receptor is regenerated in the nuclear compartment. Thus, recurrent tumor cells lack the molecular mechanisms that would enable them to respond to androgens.

The emergence of the androgen-independent recurrent carcinoma is associated with a major change in the proportion of stem cells in the overall tumor cell population. The evidence for this shift in cellular composition is summarized schematically in Fig. 9. In the parent androgen-dependent tumor, one in 4000 cells is tumorigenic; this implies 12 divisions of the immediate progeny of each stem cell to establish the final stem/non-stem cell ratio in the tumor. Androgen withdrawal triggers a mean 90% regression of tumor mass (Fig. 1), eliminating the majority of nonstem progeny cells. In addition, since the incidence of tumor takes decreases almost 100-fold when parent tumor stem cells are injected into female hosts (Fig. 4), it is assumed that androgen withdrawal ultimately reduces the proportion of stem cells in the regressed tumor to 1% of the number in the parent tumor. However, in view of the data presented in Figs. 1 and 7, the surviving fraction of stem cells is consistent with a somewhat greater cell kill of 2 to 3 logarithms. Progression of surviving tumor cells is associated with the development of an androgen-independent recurrent carcinoma in which the proportion of stem cells increases 20-fold (Fig. 3) or 2000% over the fraction measured in the parent tumor. For each stem cell in the recurrent tumor, there are approximately 200 nonstem descendents. Thus, clonal expansion of the progeny of an androgen-independent stem cell is possible but appears to be limited to 8 divisions. On the basis of a similar incidence of tumor takes in male and female animals, a 2-fold faster doubling time and no response to castration, the recurrent tumor and its stem cell compartment are clearly androgen independent. In fact, the striking 20-fold increase in the relative proportion of all stem cells in the recurrent tumor is overshadowed by a much greater increase in the proportion of androgen-independent stem cells. Owing to the very small contribution of the latter to the combined pool of stem cells in the parent tumor, there is at least a 500-fold difference (1/370,000 versus 1/800) in the number of androgen-independent stem cells between parent and recurrent tumors; since the proportion of androgen-independent stem cells in the parent may be as small as 1/2,200,000 (Fig. 7), the actual difference might be much larger than our data suggest. Thus, androgen withdrawal therapy eventually results in a massive increase in the proportion of androgen-independent stem cells in the previously androgen-dependent malignancy.

The origin of androgen-independent stem cells is unclear. The concept that such cells already coexist with androgen-dependent cells in the parent tumor is offset by two findings. (a) There is no evidence of selective survival of androgen-independent stem cells in regressing tumors, and (b) the number of androgen-independent stem cells appears to be exceptionally small. This is shown by the extremely low incidence of tumor takes when regressing cells are implanted into female mice. It remains possible that the survival of a small number of uniformly androgen-dependent stem cells is the end result of an adaptive process involving the expression of androgen-inhibited protooncogenes such as c-fos and c-myc (42-44) and the secondary repression by induced nonandrogenic factors of the androgen-repressed cell death gene, trpm-2 (44, 45). Thus progression may involve the nascent synthesis of transcripts coding for growth factors that substitute for androgens in stimulating the division and differentiation of surviving parent stem cells. In support of this alternative concept, Nomura et al. (46) have recently isolated an autocrine acidic fibroblast growth factor from androgen-independent Shionogi carcinoma cells grown in culture.

In addition to the killing effect of androgen withdrawal, the stem cell compartment may be depleted by cell loss through tissue necrosis, detachment, migration, random extinction, and differentiation. In fact, the absence of androgen-induced differentiation after castration may explain why the recurrent Shionogi carcinoma contains such a high proportion of androgen-independent stem cells. It is also possible that the faster doubling time of the recurrent tumor from the time of implantation to palpation (0.5 g) is related to less frequent differentiation of stem cells.

For technical reasons, female mice were used in place of castrated male mice in some of our experiments. Although the results presented in Fig. 7 unequivocally demonstrate that stem cells are androgen dependent, the question arises of whether estrogens or other growth factors in females could add to or subtract from the effects of androgen withdrawal alone. In previous experiments, we found little or no difference in the average incidence of tumors in female and castrated male mice implanted with a similar number of cells. This varied between 0% and 15% over observation periods of 30 to 60 days and was increased only by the administration of androgen (4, 47). The absence of any female-specific effect is also supported by the finding that the hormonal environment of the female host does not result in lengthening of the doubling time (Fig. 6); any retardation of tumorigenesis appears to be related only to the initial androgen-dependent killing of stem cells. In fact, since the growth of the Shionogi carcinoma is characterized by a similar doubling time in male and female mice, it is very unlikely that estrogens or other growth factors in the female play an important role in determining the outcome of androgen-withdrawal therapy. This is consistent with the observation that the Shionogi carcinoma does not express the gene for the estrogen receptor; in contrast, mRNA transcripts for the androgen receptor are readily detectable by Northern analysis.4

Analysis of the DNA content of the parent and recurrent tumors by flow cytometry demonstrated that the Shionogi carcinoma is composed of two subpopulations: one diploid and the other aneuploid. Androgen withdrawal favors the elimination of the diploid subpopulation and, from this observation, we infer that aneuploid cells are more adaptable than diploid

Fig. 9. Summary of effects of androgen withdrawal on the stem cell composition of the Shionogi carcinoma.
ANDROGEN WITHDRAWAL EFFECTS ON STEM CELLS

cells. Thus, predominantly aneuploid tumors contain a greater number of potentially androgen-independent stem cells. In conclusion, the probability of development of androgen-independent stem cells within the Shionogi carcinoma is greatly increased in an androgen-depleted environment and appears to be linked to the cessation of androgen-induced differentiation of stem cells in the parent tumor.

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Effects of Androgen Withdrawal on the Stem Cell Composition of the Shionogi Carcinoma

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