Variability of Androgen-related Phenotypes in the Shionogi Mammary Carcinoma during Growth, Involution, Recurrence, and Progression to Hormonal Independence

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ABSTRACT

Several parameters of androgen action were measured in hormone-dependent Shionogi carcinoma cells during phases of growth, regression, and recurrence. In the parental C1 line under steady state conditions, dihydrotestosterone is localized exclusively in the nucleus while testosterone is confined almost entirely to the cytoplasm. After castration, the concentration of testosterone declines more rapidly than that of dihydrotestosterone. Spontaneous recurrent growth is not accompanied by significant elevation of the whole-tissue concentration of either androgen. Neither are changes observed in the concentration of cytoplasmic receptor or in the rate of uptake of androgens into the nucleus. However, relapse is associated with the appearance of a glucose-6-phosphate dehydrogenase double-enzyme phenotype and a loss of responsiveness to androgen withdrawal. The autonomous C3 variant line which is devoid of androgen-related markers is characterized by a deficiency of androgen retention by whole tissue and possibly a permeability defect of the plasma membrane. This variant tends to express a glucose-6-phosphate dehydrogenase double-enzyme phenotype. In contrast, the autonomous C4 variant line retains the ability to concentrate modest levels of testosterone in whole tissue and high levels of dihydrotestosterone in the nucleus. Although the number of nuclear binding sites is the same as that observed in the parental C1 line, the concentration of cytoplasmic receptor and the rate of nuclear uptake of androgens are relatively decreased. Expression of a glucose-6-phosphate dehydrogenase double-enzyme phenotype is less frequent than in the autonomous C3 variant line. The above results suggest that a recurrent tumor may contain hormone-sensitive cells which resume growth in an androgen-depleted environment. They also imply that progression from the androgen-dependent to the autonomous condition involves the selection and outgrowth of hormone-insensitive cells of variable phenotype.

INTRODUCTION

The recurrence of advanced carcinomas of the breast, endometrium, and prostate is inevitable in the majority of patients despite manifestations of encouraging responses to primary therapy based on surgery and hormonal manipulations (9, 12, 30, 37). On the one hand, uncontrollable recurrent growth can be explained by the emergence of hormone-resistant cells from an initial mixed population consisting of both hormone-sensitive and -insensitive cells. On the other hand, progression of a single clone of hormone-dependent cells might result in the outgrowth of resistant cells through stepwise and irreversible changes in certain biological properties (3, 17, 23, 31). Although progression may give rise to considerable phenotypic heterogeneity, it is not clear whether the process is related to multicellular origin of carcinoma, somatic mutation, or adaptational change (11, 23). Evidence from animal experiments generally supports the idea that the progression of carcinomas of the breast (36) and prostate (23, 24) is due to selection of hormone-resistant clones.

Since the androgen-related phenotypes of dependent and autonomous variants of the Shionogi mammary carcinoma are considerably different (5), we assumed that sequential phenotypic screening of the tumor during states of growth, regression, and recurrence might yield chronological evidence concerning the etiology of progression. Our results suggest that progression is a complex process involving selection of resistant clones and adaptation of surviving hormone-sensitive cells.

MATERIALS AND METHODS

Shionogi Mammary Carcinoma. Variant lines of the SC-115 androgen-dependent mouse mammary carcinoma may be grouped into at least 4 different classes as described in a previous report (5). Class 1 (C1) tumors are androgen dependent and positive for cytoplasmic binding, nuclear uptake, and displaceable nuclear binding. The dependent tumors used in the present study have progressed to a stage which is characterized by a high percentage of recurrence following castration. Class 2 (C2) tumors are autonomous and positive for cytoplasmic binding and nuclear uptake but have relatively less displaceable nuclear binding. Class 3 (C3) tumors are autonomous and devoid of any positive markers of hormone action. Class 4 (C4) tumors are also autonomous. They are negative for cytoplasmic binding and nuclear uptake but positive for displaceable nuclear binding. Depending upon conditions of propagation, the tumor may be diploid or aneuploid but retains a karyotype that is compatible with the presence of 2 X chromosomes (5). Abdominal castrations were performed using ether. At appropriate times, experimental animals were anesthetized and killed by decapitation; the tumors (2 to 3 g or 1 to 7 days after castration) were dissected free of s.c. tissue and weighed.

Homogenization of Tissue. The tumors were minced with scissors and forced through a stainless steel wire screen (30 mesh). Fractions of tissue were collected in 50 ml of ice-cold tissue culture medium and suspended after centrifugation in 40 to 50 ml of 10 mM 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]sulfonic acid buffer (pH 7.0) containing 0.5 mM mercaptoethanol, 1.5 mM CaCl2, 0.05 mM EDTA, 5 mM MgCl2, and 0.25 M sucrose. The suspension was homogenized in a Dounce apparatus and further separated into cytosol (104,000 x g supernatant) and purified nuclear fractions as described elsewhere (5).
Radioimmunoassay. Methods for extracting and assaying the concentrations of testosterone and dihydrotestosterone in whole-tissue homogenates and samples of purified nuclei have been reported previously (10, 14).

Quantitation of Cytoplasmic Receptor. Tumor tissue was minced with scissors and suspended in a final volume of 3 ml of ice-cold phosphate-buffered saline (6.3 mM Na2HPO4·0.8 mM KH2PO4·0.154 mM NaCl, pH 7.4). [1,2-3H]Testosterone (40 Ci/mmol) was added to a final concentration of 500 nM; duplicate samples were prepared which contained a 100-fold excess of nonradioactive testosterone. After warming for 2 min in a 37° water bath, the samples were washed with phosphate-buffered saline and homogenized. Cytoplasmic receptor was recovered from the cytosol by precipitation with 80% ammonium sulfate; the final sample containing 5 to 20 mg protein was then analyzed by Sephadex G-25·G-200 dual-column chromatography. Specific binding was calculated as the difference between the amount of radioactivity in the receptor peak in the sample without competitor and the one with competitor. Details of this procedure are given in Ref. 5.

Quantitation of Displaceable Nuclear Binding. Purified nuclei were resuspended in 0.5 ml of 2-(2-hydroxy-1,1-bis(hydroxymethyl)methyl) amino)sulfonic acid buffer containing 50 mM NaCl and sonicated with three 5-sec pulses of a Bronwill Biosonic III sonicator at an intensity setting of 50. After addition of an equal volume of buffer containing 1.15 mM NaCl, the resultant solution was sonicated with three 5-sec pulses. Centrifugation of this solution at 17,000 x g for 30 min yielded a supernatant fraction of solubilized chromatin (6). One-half of the sample was incubated with 50 mM [1,2-3H]dihydrotestosterone, while the other half was incubated with both [1,2-3H]dihydrotestosterone and a 1,000-fold excess of nonradioactive dihydrotestosterone. Following incubation for 16 hr at 20°, the samples containing 5 to 15 A260 units (absorption at 260 nm in 1 n NaOH) were analyzed by Sephadex G-25·G-200 dual-column chromatography. From the difference in the amount of androgen binding between the sample without competitor and the one with competitor, an estimate of displaceable nuclear binding was obtained. The mean Kd of the binding reaction with dihydrotestosterone as ligand was 4 nM.

Measurement of Nuclear Uptake of Androgens. Tumor-bearing mice were given injections of [1,2-3H]testosterone; (200 µCi i.v.); this dose maintains the rate of uptake of androgens into the nucleus at a maximum for at least 30 min (6). After this interval, the mice were killed and the tumors were removed. Samples of purified nuclei recovered from homogenized tissue were assayed for radioactivity, and the results were expressed as dpm/nucleus.

Glucose-6-Phosphate Dehydrogenase Purification. Glucose-6-phosphate dehydrogenase was recovered from the cytosol fraction as follows. The final 104,000 x g supernatant was subjected to a fractional precipitation with ammonium sulfate which was added slowly over a period of 50 min while the temperature was controlled at 0°. The 30 to 50% fraction containing glucose-6-phosphate dehydrogenase (13) was centrifuged at 18,000 x g for 20 min to yield a protein precipitate which was frozen at 20°C. At the time of analysis, the sample was thawed and resuspended in 50 mM NaCl and then desalted by gel filtration chromatography using PD-10 Sephadex G-25. The desalted samples containing about 150 µg protein were layered on disc gels made of 7.5% acrylamide:0.18% bis, pH 8.9 and run at 4.0 mamp/gel for about 2 hr (18). The gels were stained with an enzyme-specific blue formazan mixture as described by Hunter (22). Scanning of gels was performed in a Gifford spectrophotometer at 550 nm.

Histology. Tumors were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined by light microscopy.

Radioactive Materials. [1,2-3H]Dihydrotestosterone (40 Ci/mmol) and [1,2-3H]testosterone (40 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Purity was checked by thin-layer chromatography, and the steroids were considered acceptable only if they were 95 to 100% pure. Solutions for experimental incubations and injections were prepared as follows. Radioactive steroid in ethanol:benzene was dried under N2 and dissolved in a small volume of ethanol. Distilled H2O containing 5% (v/v) polyethylene sorbitan monopalmitate was then added to bring the steroid solution to the desired concentration. For in vivo administration, 250 µl of such a solution were injected into the tail vein of each mouse.

Radioactivity was counted in a solution containing 6 g of diphenyloxazole:75 ml of water:232 g of Bio-solv (BBS-3; Beckman Instruments, Fullerton, CA) per liter of toluene. The efficiency of counting 3H with this preparation was 33%.

Other Analytical Procedures. DNA was measured by the method of Burton (8), and protein was measured by the method of Bradford (1).

For purposes of calculation, the following recoveries were used: nuclei, 7.5 x 107 ± 0.6 x 107 (S.E.)/g tumor (n = 52); DNA content of whole tissue, 14.6 ± 0.8 µg/g tumor (n = 29); DNA content of nuclei, 15.8 ± 2.8 µg/nucleus (n = 12); A260, 5.4 x 10^-7 ± 0.4 x 10^-7 units/nucleus (n = 31); protein content, 6.3 x 10^-4 µg/cell (5).

RESULTS

Growth, Regression, and Recurrence of Androgen-dependent Shionogi Carcinoma Cells. Following the injection of 2 x 107 Shionogi carcinoma cells into adult male mice of the DDS strain, the tumor becomes palpable on the tenth day and grows with a doubling time of approximately 24 hr as shown in Chart A. Withdrawal of androgens brought about by castration induces regression of the tumor such that over a period of 5 to 10 days the bulk of tissue disappears and is not detectable by palpation. About 30 to 35 days after the transplant, recurrent...
growth is observed with a much slower doubling time of 72 hr. Earlier reactivation of the tumor can be elicited by the injection of testosterone as shown by the results in Chart 1B. The latent period before recurrent growth resumes is markedly foreshortened, and the doubling time is abbreviated 3-fold to 24 hr or less.

The histology of the Shionogi carcinoma during the 3 phases of response is shown in Figs. 1 to 6. During the growth phase (Fig. 1), the tumor has the appearance of an undifferentiated medullary mammary carcinoma. The cells have poorly defined borders and large pleomorphic nuclei often containing several small nucleoli. Mitotic figures are common. The phase of regression, illustrated in Fig. 2 is characterized by large numbers of cells undergoing lytic degeneration. The nuclei are separated from one another and take on an ill-defined smudged appearance. Mitotic figures are absent. Growth of recurrent tumors tends to be concentrated around blood vessels as shown in Fig. 3. On either side of the capillary in the center of the field viable tumor cells are seen, but further away from the vessel, the cells become spindle shaped. These cells are shown under higher magnification in Fig. 4. Being somewhat removed from the blood supply, it is possible that such cells owe their unusual appearance to hypoxia rather than to autonomous growth (27). The architecture of autonomous variant lines C3 and C4 is shown for comparison in Figs. 5 and 6, respectively. These tumors are poorly differentiated, with ill-defined cellular borders and large pleomorphic nuclei with prominent and sometimes multiple nucleoli. Mitoses are numerous in keeping with the rapid doubling time of the tumors. The autonomous variant lines thus resemble both the androgen-dependent C1 parental line depicted in Fig. 1 and the perivascular cells of the recurrent tumor shown in Fig. 3.

Concentration of Androgens. Since the results in Chart 1B indicate that a significant number of hormone-dependent cells survive regression, it is possible that the recurrent growth described in Chart 1A might simply represent renewed androgen-dependent proliferation, with the adrenal glands providing the source of hormone. To measure the effects of hormone withdrawal by castration, the concentrations of testosterone and dihydrotestosterone were measured during the 3 phases of growth of the Shionogi carcinoma. The results are presented in Table 1. The mean concentration of testosterone in whole tissue is 1.9 pmol/mg DNA, and surprisingly, the nuclear compartment is virtually devoid of testosterone. The whole-tissue concentration is equivalent to 28 nm, virtually identical to the 10 to 20 nm plasma concentration of testosterone in male mice of the DDS\(^3\) and other strains (15, 29). This correspondence of values suggests that either the tumor is highly vascular or that testosterone diffuses freely into the cytoplasm of the malignant cells. The mean concentration of dihydrotestosterone in whole tissue is 1.3 pmol/mg DNA, nearly identical to the mean concentration in the nucleus, 1.4 pmol/mg DNA (t test, p > 0.05). These results indicate that all of the intracellular dihydrotestosterone is concentrated in the nucleus and that the cytoplasm is devoid of dihydrotestosterone. Castration brings about a reduction in the whole tissue concentration of testosterone to trace levels in the regressing tumors, and the concentration remains low in the recurrent tumors as well. Owing to the nuclear localization of dihydrotestosterone, the whole-tissue concentration was not measured in regressing or recurrent tumors. However, the concentration of dihydrotestosterone in the nucleus also falls after castration but does not decline as rapidly as does the whole tissue concentration of testosterone; in fact, regressing tumors are characterized by a relatively high concentration of dihydrotestosterone. The concentration of dihydrotestosterone in recurrent tumors is only about 15% of that observed in the initial growing tumors (t test, p < 0.05).

The exclusive nuclear localization of dihydrotestosterone in tumors was an unexpected finding since the results of previous work based on tracer studies with \(^{3H}\)testosterone indicated that in short-term pulse studies very little conversion of testosterone takes place. More than 85% of the intranuclear androgen is in the form of unmetabolized testosterone and only 3% in the form of dihydrotestosterone (4). In contrast, the data in Table 1 implies that under physiological conditions, the nucleus selectively concentrates dihydrotestosterone.

Concentration of Binding Sites. The concentrations of binding sites were next measured in cytoplasmic and nuclear fractions of the Shionogi carcinoma in the different proliferative states. The results of sequential measurements of the cellular content of androgen receptors are listed in Table 2. Whereas the concentration of binding sites in the cytoplasm does not change during growth regression and recurrence of the Shionogi carcinoma (t test, p > 0.05), there is a 50 to 70% reduction in the mean number of nuclear binding sites for dihydrotestosterone in the regressing and recurrent tumors (t test, p < 0.01).

Nuclear Uptake of Androgens. Since the rate of uptake into the nucleus varies as much as 47-fold between dependent and autonomous variants of the Shionogi carcinoma (6), we assumed that a major decrease in this parameter might identify an autonomous condition of a recurrent tumor. Accordingly, tumor-bearing

<table>
<thead>
<tr>
<th>Condition</th>
<th>Testosterone (pmol/mg DNA)</th>
<th>Dihydrotestosterone (pmol/mg DNA)</th>
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<tbody>
<tr>
<td></td>
<td>Whole tissue</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Growth</td>
<td>1.9 ± 0.9(^a) (13)(^b)</td>
<td>0.1 ± 0.1 (6)</td>
</tr>
<tr>
<td>Regression</td>
<td>&lt;0.1 (6)</td>
<td>&lt;0.1 (8)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>0.1 ± 0.1 (3)</td>
<td>0.2 ± 0.1 (3)</td>
</tr>
</tbody>
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\(^a\) Mean ± S.E.  
\(^b\) Numbers in parentheses, number of tumors examined.

\(8\) N. Bruchovsky, unpublished data.
ing animals were given injections of [1,2-3H]testosterone (200 μCi), and 30 min later, the amount of radioactivity incorporated into purified nuclei was determined. The rate of uptake of androgens expressed as dpm per 30 min per nucleus is 3.2 × 10^{-4} ± 0.6 × 10^{-4} (n = 7), 7.2 × 10^{-4} ± 1.5 × 10^{-4} (n = 8), and 12.3 × 10^{-4} ± 2.1 × 10^{-4} (n = 8) during tumor growth, regression, and recurrence, respectively. By comparison, the autonomous C3 and C4 lines described later in this report demonstrate mean rates of uptake of only 0.5 to 0.6 × 10^{-4} dpm/30 min/nucleus. Thus, recurrent tumors are characterized by a rate of uptake which is more typical of androgen-dependent cells than of autonomous ones.

Expression of Glucose-6-Phosphate Dehydrogenase. Since the Shionogi carcinoma is derived from female mammary tissue, the transplantable malignant cells contain 2 X chromosomes (26). This affords the possibility of examining the tumor for mosaicism of the X-linked marker, glucose-6-phosphate dehydrogenase. A double-enzyme phenotype might be expected in tumors of multicellular origin although other interpretations would have to be considered (16, 21). The results presented in Charts 2 and 3 and Table 3 indicate that growing and regressing parental C1 cells are characterized almost exclusively by a single-enzyme phenotype. In contrast, a double-enzyme phenotype occurs with increasing frequency in the recurrent tumor and in the parental C1 line after an interval of progression spanning 10 transplant generations. However, as can be seen from the tracing in Chart 2E, the fast-moving form of glucose-6-phosphate dehydrogenase in the progressed cells is present only in a small amount compared to the quantity detected in recurrent tumors growing in either male (Chart 2D) or female hosts (Chart 3A). The expression of the fast-moving form is reduced after the recurrent tumor has been passaged in a male host (Chart 3B). Castration of the male host tends to amplify the amount of this component slightly (Chart 3C) even though the passed tumor fails to involute (data not shown). It is to be noted that no evidence of glucose-6-phosphate dehydrogenase polymorphism is observed in the control liver tissue which is characterized by a single-enzyme phenotype in both female and male animals (Chart 2A). Thus, only in recurrent or progressed tumors is the double-enzyme phenotype expressed with regularity. Also, manipulation of the androgen environment appears to have a greater effect on the expression of the fast-moving variant.

Autonomous C3 Shionogi Carcinoma Cells. The concentration of testosterone in whole tissue of the autonomous C3 tumor in male animals is 0.1 the concentration in cells of the androgen-dependent parental C1 line (Table 4); only trace amounts of testosterone are found in the nuclear compartment. The whole-tissue and nuclear preparations obtained from tumors growing in female animals are virtually devoid of testosterone. The extremely low concentration of testosterone in this class of tumors suggests either that the tumor is relatively avascular or that there is deficient transport of testosterone across the plasma membrane. The concentration of dihydrotestosterone in autonomous C3 cells is at least as high if not higher than the concentration of testosterone, and this androgen appears to be concentrated entirely in the nuclear compartment. In view of the low concentration of testosterone in the whole tissue, especially in that

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cytoplasm (dpm/mg protein)</th>
<th>Nucleus (dpm/A260)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>3500 ± 900* (3)</td>
<td>1500 ± 100 (4)</td>
</tr>
<tr>
<td>Regression</td>
<td>4000 ± 1000 (3)</td>
<td>500 ± 200 (4)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>2900 ± 500 (3)</td>
<td>700 ± 200 (4)</td>
</tr>
</tbody>
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* Mean ± S.E. Numbers in parentheses, number of tumors analyzed.
Although neither testosterone nor dihydrotestosterone is expressed as a double-enzyme phenotype in 90% of the tumors.

Dehydrogenase is expressed as a double-enzyme phenotype in dihydrotestosterone by radioimmunoassay. Results are normalized on the basis of tumors during phases of growth, regression, and relapsing disease promotes the emergence of a recurrent tumor the phenotype of which does not differ markedly from that of the parental tumor except for the acquisition of a fast-moving electrophoretic variant of glucose-6-phosphate dehydrogenase. Although neither testosterone nor dihydrotestosterone reaccumulate in the recurrent tumor to normal levels, several phenotypic indices of hormonal responsiveness persist. These include the presence of cytoplasmic androgen receptor, a reduced but nevertheless detectable concentration of binding sites in the nucleus, and the ability to incorporate androgens into the nucleus at a rapid rate. 

Consistent with the elevated concentration of dihydrotestosterone in the nuclear compartment of autonomous C4 tumors in male hosts, the concentration of nuclear binding sites at 1900 ± 500 (n = 3) is identical (t test, p > 0.05) to the value observed in the androgen-dependent parental C1 line (Table 2).

The quantity of cytoplasmic receptor in tumors propagated in female hosts, 400 ± 100 (n = 8) dpm/mg protein is not different than the amount in autonomous C3 cells. Neither is the rate of uptake of androgens at 0.5 x 10^-4 ± 0.1 x 10^-4 (n = 10) dpm/30 min/nucleus significantly different (t test, p > 0.05). These findings confirm that both autonomous C3 and C4 tumors are deficient in cytoplasmic binding and nuclear uptake.

Glucose-6-phosphate dehydrogenase is expressed as a double-enzyme phenotype in only 50% of the autonomous C4 tumors (Chart 3D; Table 3).

The results of this investigation provide new information about the androgen dependence of the Shionogi carcinoma. The proliferation of the tumor is stimulated by both testosterone and dihydrotestosterone (2, 20), and on the basis of these and other studies (25, 28, 34, 39), it has been inferred that dihydrotestosterone is responsible for the mitotic activity induced by testosterone. However, the Shionogi carcinoma contains little 5α-reductase, and consequently, in experiments in which the metabolism of exogenous radioactive testosterone has been studied either in vivo (2, 4, 39) or in vitro (20), the formation of dihydrotestosterone has been slight. The data reported in Table 1 indicate that under steady state conditions, as opposed to acute short-term experiments, selective accumulation of dihydrotestosterone by the nucleus takes place. The exclusive nuclear localization of dihydrotestosterone in dependent Shionogi carcinoma cells directly confirms that dihydrotestosterone is the active intracellular androgen in this target tissue (7).

Progression of the Shionogi carcinoma through successive phases of growth, regression, and relapsing disease promotes the emergence of a recurrent tumor the phenotype of which does not differ markedly from that of the parental tumor except for the acquisition of a fast-moving electrophoretic variant of glucose-6-phosphate dehydrogenase. Although neither testosterone nor dihydrotestosterone reaccumulate in the recurrent tumor to normal levels, several phenotypic indices of hormonal responsiveness persist. These include the presence of cytoplasmic androgen receptor, a reduced but nevertheless detectable concentration of binding sites in the nucleus, and the ability to incorporate androgens into the nucleus at a rapid rate. Comparison of the phenotype of recurrent tumors to those of several variant lines of autonomous Shionogi carcinoma cells (5) suggests that the recurrent growth corresponds to the C2 variant line of autonomous tumors. The chief alteration observed in this autonomous carcinoma.
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variant is a reduction in the concentration of nuclear binding sites. We have not determined whether this is a reversible change, and it remains possible that the reduction in the amount of nuclear binding is simply a reflection of androgen withdrawal. This would be consistent with the observation that the recurrent tumor is composed in part of cells which respond to androgenic stimulation, as the growth rate of this tumor is accelerated 3- to 4-fold with androgen replacement (Chart 1B).

The phenotype of the recurrent tumor differs markedly from the phenotypes of the C3 and C4 autonomous tumors. C3 autonomous cells, in addition to lacking cytoplasmic receptor, nuclear binding sites, and ability to take up androgens into the nucleus, are characterized by a deficiency of androgen retention by whole tissue. This would be consistent with a change in plasma membrane composition affecting permeability to steroids (19). Another unusual feature of the C3 variant is that it almost invariably displays the glucose-6-phosphate dehydrogenase double-enzyme phenotype.

The phenotype of the C4 autonomous tumor is different from that of the recurrent tumor and the C3 autonomous tumor. The C4 variant retains the ability to concentrate modest levels of testosterone in whole tissue; in male animals, the concentration of testosterone appears to be sufficient to afford a relatively high concentration of dihydrotestosterone in the nucleus. Parallelizing the latter, the concentration of nuclear binding sites is the same as that observed in the parental androgen-dependent tumor. On the other hand, the concentration of cytoplasmic receptor and the rate of nuclear uptake of androgens are noticeably decreased. The C4 autonomous tumor also differs from the C3 variant in that the glucose-6-phosphate dehydrogenase double-enzyme phenotype is not expressed as frequently.

Multiple forms of glucose-6-phosphate dehydrogenase have been observed in normal, preneoplastic, and carcinomatous mammary tissue of mice by Hilf et al. (21). These investigators noted that pregnancy and lactation caused a shift in the electrophoretic pattern with increased formation of a fast-moving variant at the expense of a slow-moving variant. Whether the variant forms in the Shionogi carcinoma correspond to any of the forms described by Hilf et al. (21) is difficult to judge since the hormonal and physiological conditions are considerably different. The Shionogi carcinoma is dependent on androgens rather than on estrogens, and the multiple-enzyme pattern is not seen in the unprogressed parental C1 line. It is of interest that glucose-6-phosphate dehydrogenase activity can be inhibited by androgens possessing a 5α-androstan configuration (32, 33), but owing to the large K values, i.e., in the μM range (32), it is doubtful that the relatively small intracellular concentration of dihydrotestosterone could directly influence glucose-6-phosphate dehydrogenase activity in the Shionogi carcinoma.

Shifts in the pattern of molecular forms of glucose-6-phosphate dehydrogenase may occur in some tissues owing to alterations in the concentration of glutathione (38). However, glutathione reductase activity per se does not appear to determine the molecular form of glucose-6-phosphate dehydrogenase in a changing hormonal environment (21). Furthermore, since the fast-moving variant of the Shionogi carcinoma appears during progression (after 10 transplant generations) of the parental C1 line (Table 3) in the absence of any hormonal change, it is unlikely that our results can be explained by hormone-dependent fluctuations in glutathione levels.

Expression of the fast-moving variant of glucose-6-phosphate dehydrogenase occurs more often under conditions which might be expected to favor the enrichment of androgen-insensitive cells. This effect is observed in tumors that recur in castrated male hosts (Chart 2D), in recurrent tumors passaged in female hosts (Chart 2A), and in parental C1 tumors after several transplant generations (Chart 2E). Expression of the fast-moving variant is also a constant feature of the autonomous C3 line but is less certain in the autonomous C4 line (Table 3). The latter difference is harmonious with the idea that the autonomous lines may contain variable numbers of at least 2 resistant cell populations, one characterized by the slow-moving variant of glucose-6-phosphate dehydrogenase, the other by the fast-moving variant. The possibility that the fast-moving variant is a marker for resistant cells is also supported by the observation that under conditions which would favor the growth of androgen-stimulated cells, a relative reduction in the amount of the variant enzyme takes place (Chart 3B).

Although it is tempting to attribute the foregoing observations to X-linked mosaicism of the glucose-6-phosphate dehydrogenase marker, this interpretation is complicated by the absence of enzyme polymorphism in female liver cells (Chart 2A) and the fact that no autonomous variant lines expressing only the fast-moving variant have been isolated. As discussed by Fialkow (18), the interpretation of double-enzyme phenotypes would have to take into consideration the possibility of normal cell admixture, the ratio of isoenzymes in the normal tissue from which the neoplasm originates, and activity of both X chromosomes in a single cell. Generally speaking, our results would also be compatible with the latter possibility assuming that the activity of one of the X chromosomes is normally suppressed in the presence of androgens during the initial growth phase of the tumor. When proliferation of the recurrent tumor resumes in the absence of androgens, the activity of both X chromosomes would become apparent. Our failure to detect the double-enzyme phenotype in androgen-withdrawn regressing cells might be explained by the rapid decline in synthesis of scarce and abundant classes of mRNA sequences during tumor involution.4 Whatever the basis for the expression of the fast-moving variant of glucose-6-phosphate dehydrogenase, it is clear that the isoenzyme is strongly associated with the emergence of androgen-insensitive cells.

Thus, early progression of the androgen-dependent Shionogi carcinoma cells is indicated by a drop in the number of nuclear androgen-binding sites, the appearance of the fast-moving variant of glucose-6-phosphate dehydrogenase, and the loss of responsiveness to androgen withdrawal. Such changes would be consistent with a multicellular origin of the carcinoma and clonal selection, or alternatively, the irreversible adaptation of androgen-dependent cells associated with the expression of both X chromosomes.

ACKNOWLEDGMENTS

We thank Cynthia Wells for assistance in the preparation of the manuscript.

REFERENCES


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