Effects of Estradiol and Tamoxifen on Creatine Kinase in Rodent Mammary Carcinomas

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ABSTRACT

The effects of altered estrogenic environments on creatine kinase (CK) and adenylate kinase (AK) were studied in two rodent mammary tumor systems, R3230AC and primary 7, 12-dimethylbenz(a)anthracene (DMBA)-induced carcinomas, to determine whether response of these enzymes could be related to their hormone dependence. The hormonal perturbations studied were ovariectomy and administration of various doses of estradiol valerate or the antiestrogen tamoxifen. Total CK activity and AK activity were assessed by a spectrophotometric assay followed by electrophoretic separation of the CK isozymes to determine their relative activities. In the ovarian-independent R3230AC tumors, estrogen treatment produced a dose-related decrease in CK activity, whereas CK was not responsive in ovarian-dependent DMBA-induced tumors. Adenylate kinase activity remained unchanged regardless of the hormonal perturbation. Glucose-6-phosphate dehydrogenase and lactate dehydrogenase, which were studied for comparative purposes, were both estrogen responsive. While both estrogenic and antiestrogenic effects on enzyme activities were observed in the DMBA-induced tumors, the effect of tamoxifen in the R3230AC tumors was generally estrogenic. We conclude that the effect of estrogen on CK-BB in DMBA-induced tumors is not sufficient to be used as a biochemical marker of hormone dependence.

INTRODUCTION

ATP:creatine N-phosphotransferase (EC 2.7.3.2; CK′) is present in a wide variety of normal and neoplastic tissues, serving as an energy regulator to maintain high cellular concentrations of ATP. All three cytoplasmic isozymes, CK-MM, CK-MB, and CK-BB, are dimers. The MM and MB isozymes are associated with skeletal and cardiac muscle, respectively. The BB isozyme is found in highest activity in the brain. Although the CK-B and CK-M polypeptides have closely matched amino acid sequences (1), they differ in both their tissue specificity and their sensitivity to estrogen.

CK-BB has also been identified as the major component of the IP, a protein whose induction is a primary response of the uterus to estrogen (2). This induction can also be demonstrated in vitro, the level of CK induction correlating to estrogen binding in the uterus (3). CK-BB was reported to be estrogen responsive throughout the entire reproductive system of the immature female rat as well as in human breast tissue and tumors (4–6). Thus, induction of CK-BB offers potential as a marker of hormone responsiveness.

CK-MM has also been reported in varying amounts in neoplastic tissue. CK-MM, the predominant isozyme in skeletal muscle, has been shown to be developmentally regulated, appearing only after terminal differentiation of myoblasts has begun (7). Because of its high activity in muscle tissue, it is likely that CK-MM plays a role in contractile processes in the cell. It has been identified with the mitotic spindle movement (8) and may also influence membrane structure and cell shape through its interaction with microfilaments and intermediate fibers (9). Given the uncontrolled division of cancer cells and changes in the cell membrane associated with transformation, it would be of interest to look at relative isozyme activities as well as total CK activity in tumors.

In this report, we examined the responsiveness of CK and two other enzymes, lactate dehydrogenase and glucose-6-phosphate dehydrogenase, to a variety of estrogenic manipulations in two different rat mammary tumor systems: the estrogen-independent, responsive, transplantable R3230AC adenocarcinoma of Fischer rats and estrogen-dependent, responsive, DMBA-induced mammary tumors in Sprague-Dawley rats. The effects of ovariectomy, the administration of estradiol, or the administration of the antiestrogen, tamoxifen, were studied to ascertain whether these enzymes could be related to the hormone dependence of these tumors. In addition, the isozyme components of CK were determined in both tumor systems in ovariectomized rats, with and without administration of estrogen. The data presented here demonstrate that, while LDH and G6PD were responsive to estrogen perturbations in both tumor systems, CK was responsive in R3230AC tumors but was unresponsive in DMBA-induced dependent tumors.

MATERIALS AND METHODS

Mammary Tumors. Fifty-day-old Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were intubated with a single 20-mg dose of DMBA (Eastman Organic Chemicals, Rochester, NY) dissolved in sesame oil. Rats were palpated twice weekly starting 45 days after intubation, and tumors were measured with calipers in two perpendicular dimensions. Animals were used for experiments when the largest tumor measured 2.0 cm in one dimension.

Thirty-five-day-old Fischer rats were implanted s.c. with the R3230AC mammary carcinoma by sterile trocar technique (10). One series of animals had been ovariectomized 6 days prior to implantation. Animals were always sacrificed 3 wk after tumor implantation.

Ovariectomy and Hormonal Treatment. Animals were bilaterally OVEX by standard surgical techniques. Ovariectomized Sprague-Dawley rats were given injections s.c. with estradiol valerate (Delestrogen; E. R. Squibb and Sons, Inc., Princeton, NJ) further diluted with sesame oil (Beacon Chemical Company, Inc., New York, NY), each animal receiving a dose of 2.0, 0.2, or 0.02 mg daily for 4 days beginning on the fourth day following ovariectomy. These doses were chosen because

9The terminology used here when referring to biological properties of the tumors is: hormone dependent, requiring endogenous hormones for continued growth; hormone independent, not requiring endogenous hormones for continued growth; hormone responsive, tumor growth altered in response to hormonal perturbations, such as the administration of estrogens; and hormone unresponsive (self-explanatory). The majority of DMBA-induced tumors are dependent; those lesions which do not regress after ovariectomy are termed independent. The R3230AC tumor, which grows in ovariectomized animals but whose growth is retarded by estrogen treatment, is categorized as hormone-independent responsive adenocarcinoma.
they cover the range over which the most significant enzyme responses had been seen in previous studies (11, 12). Intact Sprague-Dawley rats were given injections s.c. with tamoxifen citrate salt (a gift from Stuart Pharmaceuticals, Wilmington, DE), initially dissolved in ethanol at a concentration of 25 mg/ml and then appropriately diluted in 0.9% NaCl solution, each animal receiving a dose of 5.0 or 0.5 mg daily for 4 days.

Ovariectomized Fischer rats were given injections s.c. with estradiol valerate at doses of 1.0, 0.1, or 0.01 mg twice weekly for 2 wk, beginning 1 wk after tumor implantation. These reflect comparable doses per kg body weight to those administered to the Sprague-Dawley rats (above). Intact Fischer rats were given injections s.c. with tamoxifen citrate, at doses of 2.5, 1.5, 0.5, or 0.25 mg twice weekly for 2 wk, beginning 1 wk after implantation.

Preparation of Tissue. Excised tumors were trimmed of necrotic area, rinsed in cold 0.9% NaCl solution, and weighed. A portion of the tumor was quick frozen in liquid nitrogen and stored at −70°C until used for LDH and G6PD assays (see below). Ten % (w/v) homogenates of fresh tumor tissue were prepared in a buffer containing 0.5 M sucrose, 25 mM Tris-HCl, 2.5 mM EDTA, 2.5 mM MgCl₂, and 4 mM acetyl cysteine (pH 7.5), using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 105,000 × g, 0–4°C, for 60 min (Beckman Model L2-65B ultracentrifuge). A portion of the resulting supernatant was taken for subsequent determination (13), using bovine serum albumin as a standard. A sample (0.5 ml) of the cytosol preparation was treated with 5 mm mercaptoethanol (0.005 ml) for 10 min at room temperature and then kept at 4°C for subsequent electrophoresis (see below). The remainder of the cytosol preparation was diluted to a 5% or 2.5% solution by appropriate addition of the tissue homogenization buffer (see above) prior to performing the CK assay. Uterine tissues were prepared similarly.

Creatine Kinase Assay. CK and AK activities were measured spectrophotometrically by a slight modification of the method of Shatton et al. (14), by following the formation of NADPH at 340 nm with a Gilford Model 2400 spectrophotometer (Gilford Instruments, Oberlin, OH). The assay mixture, in a total volume of 0.4 ml, contained 0.01 ml diluted tissue supernatant solution, 20 mM creatine phosphate, 50 mM Tris-histidine buffer (pH 6.7), 10 mM MgCl₂, 0.5 mM glucose, 8 mM acetyl cysteine, 0.75 mM NADP, 0.3 units hexokinase, and 0.5 units G6PD. The reaction was started by the addition of ADP (2.0 mM acetyl cysteine, 0.75 mM NADP, 0.3 units hexokinase, and 0.5 units G6PD). The reaction was started by the addition of ADP (2.0 mM). At the same time, an identical amount of the diluted cytosol preparation was assayed, as above, in the absence of creatine phosphate, which provided a measurement of the AK activity. CK activity, as reported here, represents the amount of NADPH produced in the presence of creatine phosphate minus the amount generated in the absence of creatine phosphate, i.e., AK, at room temperature. The reaction conditions were such that, after 1 min, the change in absorbance at 340 nm was linear with time for at least 3 to 5 min. Enzyme activities are expressed as units (μmol NADPH/min)/mg cytosol protein.

Creatine Kinase Isozyme Electrophoresis. Electrophoresis of CK isozymes was performed on 0.4% agarose (Sigma) gels with a polyester backing (FMC Corporation, Rockland, MA). Following electrophoresis, gels were incubated, as suggested, in staining reagents obtained from Sigma Diagnostics (St. Louis, MO). The basis for detection of the isozymes is the same as that for the photometric assay of CK. However, on the gel, production of NADPH was quantified by the precipitation of TNBT-formazan through its reaction with phenazine methosulfate. Ten % supernatants were treated with 5 mM mercaptoethanol (from a 1 mM stock in 0.2 M KOH) as advised by Sigma, although duplicate samples, with and without mercaptoethanol treatment, showed no significant difference.

Treated samples were loaded (0.001 ml) onto gels, which had been preequilibrated in a solution containing 50 units hexokinase and 40 units (G6PD) in 0.1 mM bis-tris/acetate (pH 6.9) for at least 1 h. Gels were run with a 0.1 mM bis-tris/acetate buffer (pH 6.9) at 90 V, for 45 min, in a cold room, and then stained at 37°C in a solution containing 0.5 mmol glucose, 0.05 mmol ADP, 0.25 mmol MgCl₂, 0.825 mmol AMP, 0.546 mmol phenazine methosulfate, 0.75 mol creatine phosphokinase, 50 units hexokinase, 40 units G6PD, 0.05 mmol NADP, and 0.009 mmol TNBT in a total volume of 25 ml 0.1 mM bis-tris/acetate buffer (pH 6.7). The isozymes were visualized by the purple precipitate (TNBT-formazan). After 30 min, the gels were removed and destained in a solution containing 5% glacial acetic acid, 25% distilled deionized H₂O, and 70% methanol for 30 min. Gels were then stored in distilled deionized H₂O at 4°C until scanned with a laser densitometer (LKB 2222-010 Ultrascan XL laser densitometer; Bromma, Sweden) to determine the band intensities of each isozyme and their relative proportions. Activities of the isozymes were calculated as the percentage of total CK activity determined by the photometric assay.

AK (1.4 units) was used as a negative control on each gel to demonstrate the effectiveness of AMP as an inhibitor and to show that only CK-MM was staining on the gel. In addition, a sample of CPK Isotrol (Sigma Chemical Co., St. Louis, MO), a freeze-dried preparation containing rabbit tissue CK in a bovine serum base, was run on each gel to identify the isozymes.

Other Enzyme Assays. For LDH and G6PD assays, frozen tumor tissue was thawed and rapidly homogenized in 0.5 M Tris buffer (pH 7.4) to produce a 10% (w/v) homogenate. Homogenates were centrifuged at 29,000 × g, 0–4°C, for 20 min (Beckman Model J-21 centrifuge). The resultant supernatant was used for enzyme assays. G6PD activity was determined by the method of Glock and McLean (15). LDH activity was determined by the Henry et al. (16) modification of the Wroblewski procedure. Both assays were monitored at 340 nm in a Gilford Model 2400 spectrophotometer, and the change in absorbance was related, in the case of G6PD, to the formation of NADPH from NADP and, in the case of LDH, to the oxidation of NADH to NAD. Reaction conditions were such that zero order kinetics was achieved for at least 4 to 5 min.

Presentation of the Data. Data are presented as mean (range) or mean ± SE and analyzed according to Student’s t test; P ≤ 0.05 was considered to be significant.

RESULTS

Enzyme Activities and Response to Treatment in R3230AC Transplantable Tumors. Enzyme activities in R3230AC mammary carcinomas from OVEX or intact rats were not significantly different (Table 1). Treatment of tumor-bearing OVEX rats with estradiol (0.01 mg, 0.1, or 1 mg twice weekly for 2 wk) produced a dose-related decrease in CK activity in these tumors (40, 56, and 83% decreases, respectively). AK activity in these tumors was unresponsive to estrogen administration. Tumor LDH activity was significantly decreased (60%) with estrogen treatment compared to OVEX controls, whereas G6PD activity in these tumors was significantly increased for each estrogen dose administered, the two lower doses providing the greater increases (157, 151, and 85%, respectively). When OVEX rats were treated with 2 μg estradiol plus 200 μg progesterone twice weekly for 2 wk, no significant change in tumor CK activity was observed; however, AK and G6PD activities were significantly increased, and LDH activity was significantly decreased (data not shown).

The isozyme composition of CK activity was determined for a limited number of the R3230AC tumors. CK-MM activity, which varied from 0.000 to 0.389 units/mg cytosol protein, was found in 60% (6 of 10) of the tumors from untreated intact or OVEX animals; in 5 of 6 samples, however, the MM isozyme contributed less than 20% of the total CK activity. In contrast, CK-BB activity was present in every tumor examined. CK-BB activity from these untreated animals (0.055 ± 0.004, n = 10) was consistent with total CK activities seen in other intact and OVEX rats, suggesting that earlier samples not analyzed for isozymes likely contained little MM isozyme. In OVEX animals treated with estradiol (0.01 mg, 0.1 mg, or 1 mg twice weekly for 2 wk), only 13% (2 of 16) of the tumors contained CK-MM. When CK-BB activities on gels from the estradiol-treated rats
Results from int
ESTROGEN AND CREATINE KINASE IN MAMMARY TUMORS

Table 2 Effects of estradiol and tamoxifen on enzyme activities on DMBA-induced mammary tumors in the Sprague-Dawley rat

<table>
<thead>
<tr>
<th>Host status</th>
<th>Treatment</th>
<th>Enzyme activities (units/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CK</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>None</td>
<td>0.028 ± 0.005$^a$ (12)$^b$</td>
</tr>
<tr>
<td></td>
<td>Estradiol, 0.02 mg</td>
<td>0.049 ± 0.008$^a$ (6)</td>
</tr>
<tr>
<td></td>
<td>Estradiol, 0.2 mg</td>
<td>0.061 ± 0.009$^a$ (6)</td>
</tr>
<tr>
<td></td>
<td>Estradiol, 2.0 mg</td>
<td>0.042 ± 0.012 (6)</td>
</tr>
</tbody>
</table>

| Intact      | None      | 0.050 ± 0.007 (11) | 0.092 ± 0.009 (16) | 0.815 ± 0.048 (8) | 0.043 ± 0.005 (14) |
|             | Tamoxifen, 0.5 mg | 0.074 ± 0.009$^a$ (9) | 0.080 ± 0.010 (13) | 1.500 ± 0.13$^a$ (12) | 0.028 ± 0.004$^a$ (12) |
|             | Tamoxifen, 5 mg | 0.056 ± 0.010 (7) | 0.084 ± 0.016 (13) | 1.340 ± 0.14$^a$ (6) | 0.025 ± 0.004$^a$ (7) |

* Mean ± SE.
$^a$ Numbers in parentheses, number of tumors.
$^c$ Statistically significant difference from the respective control; P < 0.05.

Table 3 CK isozyme and AK activities of DMBA-induced tumors from ovariectomized Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Tumor characteristic</th>
<th>Enzyme</th>
<th>Control (OVEX)</th>
<th>Estrogen treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tumors</td>
<td>CK-BB</td>
<td>0.145 ± 0.05$^a$ (26)$^a$</td>
<td>0.139 ± 0.006 (26)</td>
</tr>
<tr>
<td></td>
<td>CK-MM</td>
<td>0.110 (0.000-0.643)$^a$ (26)</td>
<td>0.145 (0.000-1.465) (26)</td>
</tr>
<tr>
<td></td>
<td>AK</td>
<td>0.116 ± 0.011 (26)</td>
<td>0.144 ± 0.029 (25)</td>
</tr>
<tr>
<td>Tumors without MM</td>
<td>CK-BB</td>
<td>0.142 ± 0.004 (13)</td>
<td>0.132 ± 0.008 (15)</td>
</tr>
<tr>
<td></td>
<td>AK</td>
<td>0.099 ± 0.005 (13)</td>
<td>0.085 ± 0.005 (15)</td>
</tr>
<tr>
<td>Tumors with MM</td>
<td>CK-BB</td>
<td>0.152 ± 0.008 (13)</td>
<td>0.156 ± 0.0015 (9)</td>
</tr>
<tr>
<td></td>
<td>AK</td>
<td>0.134 (0.049-0.322) (13)</td>
<td>0.267 (0.072-0.363) (9)</td>
</tr>
<tr>
<td>Dependent tumors</td>
<td>CK-BB</td>
<td>0.147 ± 0.007 (16)</td>
<td>0.138 ± 0.008 (16)</td>
</tr>
<tr>
<td></td>
<td>CK-MM</td>
<td>0.160 (0.000-0.643) (16)</td>
<td>0.189 (0.000-1.465) (16)</td>
</tr>
<tr>
<td></td>
<td>AK</td>
<td>0.127 ± 0.017 (16)</td>
<td>0.158 ± 0.099 (16)</td>
</tr>
<tr>
<td>Independent tumors</td>
<td>CK-BB</td>
<td>0.143 ± 0.006 (10)</td>
<td>0.141 ± 0.017 (9)</td>
</tr>
<tr>
<td></td>
<td>CK-MM</td>
<td>0.030 (0.000-0.124) (10)</td>
<td>0.067 (0.000-0.286) (9)</td>
</tr>
<tr>
<td></td>
<td>AK</td>
<td>0.098 ± 0.007 (10)</td>
<td>0.116 ± 0.026 (9)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
$^a$ Numbers in parentheses, number of tumors.
$^c$ Numbers in brackets, mean with a range of values.

reagents than to the tumors themselves. Although the sources of the reagents were not changed, we noted that this coincided with the use of a different lot of phosphocreatine. Nevertheless, the trends observed were comparable.

CK-BB activity in these estrogen-treated, DMBA-induced tumors was essentially unchanged from the OVEX control for both dependent and independent tumors. Also, there were no significant differences in the amount of CK-BB in tumors which contained MM versus those that lacked CK-MM. CK-MM activity, in those tumors displaying the isozyme, was highly variable with no obvious relationship to estrogen treatment, tumor growth, size, or hormone dependence. Tumors from 54% (14 of 26) of the OVEX animals and 50% (13 of 26) of the estrogen-treated animals displayed the MM isozyme. A slightly higher percentage of dependent tumors (with or without estrogen treatment, 54 and 44%) contained the MM isozyme compared to independent tumors (40 and 33%, respectively). CK-MM had a higher average activity in dependent tumors from both the treated and untreated animals (Table 3).

AK activity in those tumors without CK-MM activity was unaffected by estrogen treatment compared to the OVEX controls (Table 3). There was, however, an elevated AK activity in those tumors possessing CK-MM, whether the OVEX host was untreated or received exogenous estrogens. A plot of AK activity for each tumor containing MM isozyme versus CK-MM activity

Fig. 2. Relationship of CK-MM activity to AK activity for individual DMBA-induced tumors from ovariectomized Sprague-Dawley rats, both with and without estrogen treatment. Enzyme activities were measured as described in "Materials and Methods." Intercepts, slopes, and correlation coefficients are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activities (units/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK-BB</td>
</tr>
<tr>
<td>None</td>
<td>0.31 ± 0.02$^a$ (13)$^b$</td>
</tr>
<tr>
<td>Estradiol, 0.65 ± 0.04 (16)</td>
<td>0.966 ± 0.077$^a$ (16)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
$^a$ Numbers in parentheses, number of tumors.
$^c$ Statistically significant difference from the respective control (no estradiol treatment); P < 0.001.

Fig. 3. Relationship of LDH activity to G6PD activity for individual DMBA-induced tumors from ovariectomized Sprague-Dawley rats, both with and without estrogen treatment. Enzyme activities were measured as described in "Materials and Methods." Intercepts, slopes, and correlation coefficients are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activities (units/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK-MM</td>
</tr>
<tr>
<td></td>
<td>0.09 ± 0.02$^a$ (13)$^b$</td>
</tr>
<tr>
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<td>0.966 ± 0.077$^a$ (16)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
$^a$ Numbers in parentheses, number of tumors.
$^c$ Statistically significant difference from the respective control (no estradiol treatment); P < 0.001.
suggestions the possibility that the 2 enzymes may be coregulated (see Fig. 2).

Enzyme Activities and Hormone Response in the Rat Uterus. To determine the effect of estrogen administration on a normal estrogen-responsive tissue, we assessed CK isozyme activity, AK activity, and wet weight of uteri from tumor-bearing OVEX rats treated with estradiol valerate. The short time period between ovariectomy and sacrifice, i.e., 4 days, which was selected based on consideration of tumor behavior, was probably insufficient to allow the circulating ovarian hormones to reach a nadir and may be responsible for the variations found in the CK activities; CK activity was reported to cycle in the rat uterus (17). Such variations were not observed for uterine AK activity. Uterine weight, as expected, was approximately doubled in OVEX animals treated with estrogen. Uterine AK activity was not altered by estrogen treatment, but CK-BB was increased (123%) in uteri from OVEX animals treated with estrogen. The BB isozyme was the only CK species present in the uterus.

**DISCUSSION**

The data presented here indicate that CK-BB activity was inhibited by estrogen treatment in R3230AC tumors, but in both the hormonally dependent and independent DMBA-induced tumors, CK-BB was apparently unresponsive to such treatment. It is interesting that Kaye et al. (6), studying the response of CK-BB to estrogen treatment in vitro, found a significant number of human breast carcinomas demonstrating a decrease in CK activity. It is not known whether such a result correlates to clinical responses of those patients to hormone therapy.

Our results for DMBA-induced tumors, however, do not agree with previous reports, which were obtained prior to the identification of the IP as CK-BB. Mairesse et al. (18) reported a 12 to 30% increase in IP in 10 DMBA-induced tumors from intact and OVEX animals after in vitro exposure of tumor slices to estrogens. Their results in vivo, however, were less definitive because of the higher background. Kaye et al. (19) also reported an increase in IP in 6 of 8 tumors after estradiol administration, although these tumors displayed a large range of IP activities, the lowest being approximately one-fourth the highest. The increases seen in the tumors were modest compared to those occurring in the uterus. Unfortunately, no interassay standard deviations were offered to evaluate their significance. We examined CK-BB activity in more than 50 tumors, half of which were from animals receiving estrogen treatment, using a spectrophotometric assay for total CK followed by electrophoretic separation to determine the relative activity of each isoenzyme. Our results show a relatively narrow range of CK-BB activity in DMBA-induced tumors; differences in host status (intact versus OVEX) may account for some of this discrepancy. In general, we observed no significant increase in CK-BB in response to estrogen treatment, even when the data were analyzed according to hormone dependence of tumors or the presence/absence of CK-MM. Although the variances in total CK activity probably reflected the wide range of values seen for CK-MM, we expected an overall increase in the BB isozyme in the tumors from animals treated with estrogen if CK-BB were, in fact, stimulated. Because this was not seen, we must conclude that the response of CK-BB to estrogen treatment in vivo in the DMBA-induced tumors does not provide a consistent measure of hormone dependence.

Although CK-MM in DMBA-induced tumors displayed higher activities in response to estrogen treatment, the proportion of tumors with the MM form was comparable irrespective of treatment. Shatton et al. (14) reported that, in 8 DMBA-induced tumors assayed, 4 to 27% of the total CK activity was MM isozyme, while we found tumors with up to 88%. The presence of MM was not correlated to any other characteristic that we measured: hormone-dependence; growth rate; or size. Shatton et al. (14) also reported that mammary glands from pregnant rats had 81 to 93% MM while the lactating gland had only 61%. In comparison, the normal human breast contains predominantly the BB isozyme, while human breast tumors had anywhere from 100% BB to 100% MM with a tendency towards higher percentages of BB (5, 6, 20, 21). It would appear from the data presented here that DMBA-induced tumors resemble the isozyme composition of human tumors more closely than surmised previously.

The isozyme composition of CK has also been studied in the GR mouse mammary tumor. Kaye et al. (17) demonstrated an increase in CK activity in those tumors as they progress towards hormone independence, with a trend towards a greater proportion in the MM isozyme. However, those tumors contain a greater proportion of MM at the time of their induction. In contrast, in DMBA-induced tumors, a large percentage of tumors contained only the BB isozyme and did not display an elevation in either isozyme in the hormone-independent versus -dependent tumors.

Although the estrogenic manipulations used here had no effect on CK-MM, there appeared to be a correlation between AK activity and CK-MM in those DMBA-induced tumors displaying CK-MM (see Fig. 2). This could be an artifact of the coupled assay system for CK and AK, since AK and CK-MM have similar electrophoretic mobility. However, to preclude this, we increased the concentration of AMP, a competitive inhibitor of AK, from 0.125 mM to 1.0 mM in the staining reaction; this higher level of AMP was found to be an effective inhibitor of AK without selectively inhibiting either one of the CK isozymes (22). A separate assay for both CK and AK would be desirable to determine whether these two enzymes are truly coregulated.

AK activity in neoplasms and uteri was not found to be estrogen responsive. Shatton et al. (14) also observed little difference in the AK activity in the various normal and neoplastic tissues. On the other hand, LDH and G6PD both responded to hormonal perturbations. In accordance with previous reports (23, 24), we observed a decrease in LDH activity in DMBA-induced tumors following ovariectomy and an increase in LDH activity following estrogen administration. In the R3230AC tumors, we observed no change in the LDH activity following ovariectomy, but a decrease in LDH activity following estrogen administration. The latter differs from an earlier report (12), in which LDH was stimulated by estrogen administration; differences in host status (intact versus ovariectomized host used here) may account for the apparent discrepancy. Confirming earlier reports (23–26), a decrease in G6PD activity following ovariectomy and an increase with estrogen administration were observed in both tumor systems (10, 12, 27, 28). However, since no differences in the estrogen-induced response of G6PD were seen between hormone-independent and -dependent tumors, we infer that this parameter may reflect the more general state of hormone responsiveness rather than the more discrete property of hormone dependence.

We examined the effects of the antiestrogen, tamoxifen, on enzyme activities in both tumor systems, since tamoxifen has been reported to have antitumor effects (29–31). If tamoxifen
inhibited the effects of endogenous estrogens, a decrease in the activity of any enzyme induced by estrogen would be anticipated. Our results, however, showed that for some end points, the data were compatible with estrogenic effects, and for others, there appeared to be antiestrogenic effects. Interestingly, tamoxifen has been found to exert both agonist and antagonist effects (29), results attributable to the observation that the tamoxifen-receptor complex can be found in the nucleus (29, 31–33).

We also investigated the effects of estrogen on CK-BB activity in the uteri of tumor-bearing animals. Our results show an increase of 123% in CK-BB, which concurs with past reports (3, 17). This demonstrates that CK-BB in the DMBA-induced carcinomas is not responsive to estrogen at the same dose which causes a specific enhancement of this isozyme in a normal target tissue.

We conclude that neither the presence of CK-BB nor its response to estrogenic perturbations was sufficient to represent a biochemical marker of hormone dependence in DMBA-induced tumors. However, induction of CK-BB, as well as LDH and G6PD, by estrogens may possibly serve as a marker of hormone responsiveness in human breast tumors.

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