Regulation of Estrogen-binding Capacity by Insulin in 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors in Rats

Scott L. Gibson and Russell Hilf

Department of Biochemistry, University of Rochester Cancer Center, and University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

ABSTRACT

The role of insulin as a regulator of estrogen receptors (ER's) was investigated in 7,12-dimethylbenz(a)anthracene-induced mammary tumors in diabetic and insulin-treated rats. Induction of diabetes with streptozotocin produced regression of lesions classified as insulin dependent; lesions that continued to grow in diabetic rats were classified as insulin independent. Compared to tumors in intact hosts [ER, 39 ± 4 (S.E.) fmol/mg cytosol protein], regressing insulin-dependent lesions had ER levels of 8.5 ± 0.7 fmol, and insulin-independent tumors had ER levels of 24 ± 3 fmol. Treatment of diabetic rats with insulin, 8 IU/day, caused insulin-dependent regressing tumors to resume growth; these lesions had ER levels of 53 ± 10 fmol. Insulin-independent lesions in diabetic rats demonstrated two patterns after treatment with insulin; continued growth resulted in tumors having ER levels of 28 ± 11 fmol, whereas insulin-induced tumor regression resulted in tumors that demonstrated ER levels of 40 ± 6 fmol/mg cytosol protein, a value equal to the level of ER in tumors growing in intact rats. Scatchard analysis of the saturation-binding data gave linear representations, and the estimated Kd for the ER was comparable for all groups, ranging from 0.44 to 1.90 × 10⁻⁹ M. Several additional tumors were classified as demonstrating static growth. When this behavior represented a response due to insulin treatment, ER levels were elevated. Static tumors remaining static after insulin treatment demonstrated low ER levels. We conclude that (a) cessation of tumor growth after induction of diabetes resulted in reduction of ER levels, (b) treatment with insulin that resulted in an altered tumor growth was accompanied by elevated ER levels, and (c) insulin may play a role in regulation of ER independent of tumor growth.

INTRODUCTION

Considerable attention has been directed towards identification and characterization of ER's in mammary tumors of humans, and their relationship to response of breast cancer patients to hormone therapy is now established. Few patients whose breast cancer lacks cytosol ER respond to endocrine therapies, whereas more than 50% of patients whose tumors contain ER demonstrate objective responses to such treatments (5, 6). Much less emphasis has been placed on hormonal regulation of ER's in mammary tumors, or even in normal tissues, an area of research that takes on greater importance when considering the multihormonal nature of mammary gland physiology.

Several reports have appeared implicating different hormones as playing a role in the regulation of ER: prolactin (19, 22, 27, 31); progesterone (1, 17, 18, 25); estrogen itself (2, 3); insulin (9, 28); and thyroid (22). Studies in our laboratory have been directed towards the role of insulin since regression of DMBA-induced mammary tumors can occur following the induction of diabetes with alloxan (10) or streptozotocin (4, 9). In an earlier study, we observed that ER was reduced in tumors classified as insulin dependent, i.e., regressing after diabetes was induced, whereas ER levels in insulin-independent lesions were comparable to those measured in growing tumors from intact animals (9).

The purpose of the present study was to ascertain the effects of insulin administration on subsequent tumor growth and ER levels in diabetic rats bearing DMBA-induced tumors. The data presented here suggest that insulin plays a role in regulating ER independent of its effect on tumor growth, and they lend further support for our earlier proposal of a positive relationship between insulin and estrogens in mammary tumors.

MATERIALS AND METHODS

Tumor Induction. Tumors were induced in 50-day-old female Sprague-Dawley rats by intubation with DMBA as previously reported (12). Eight to 10 weeks after the last intubation, tumors appeared and were measured with calipers 2 to 3 times a week in 2 perpendicular dimensions. Tumor area was calculated as the product of the 2 perpendicular measurements.

Classification of Tumor Growth. Tumors were classified based on their growth behavior according to the following criteria: growing with an increase in area of > 20%; regressing with a decrease in area of > 20%; and static with < 20% change in area. Data were summarized as total percentage of change in tumor area from the day of hormonal manipulation until sacrifice or for each period of treatment. Change in tumor area per day for each treatment period was also calculated. Histopathological examination of all tumors indicated that the lesions were adenocarcinomas.

Host Treatment. Diabetes was induced by injection of streptozotocin as described earlier (4, 9). Treatment with insulin (protamine zinc insulin; lletin U-40; Eli Lilly and Co.) was by injection of 8 IU/day s.c. for the treatment period desired.

Estrogen-binding Capacity. Tumor-bearing rats were sacrificed by cervical dislocation, and tumors were removed, trimmed, weighed, quick-frozen in liquid nitrogen, and stored at −76°. All assays for estrogen-binding capacity were performed within 1 month from the time tissues were placed in storage. Tissues for assay were thawed on ice, weighed, minced, and homogenized (20 to 25% homogenate) using a...
Tris-EDTA buffer (0.01 M Tris-HCl-1 mM EDTA, pH 7.6) as a diluent. Homogenization was done with a Polytron homogenizer (Brinkman Instruments, Lucerne, Switzerland) using 10-sec bursts and keeping the tissue on ice to prevent overheating. To obtain the cytosol fraction, the homogenate was centrifuged at 105,000 x g at 4° for 30 min. Shell vials (0.5-dram glass; Kimble Products, Toledo, Ohio) were used for the incubation of the cytosol with estrogen. All reactions were performed in triplicate using labeled 17β-estradiol (17β-[2,4,6,7-3H]estradiol; 108 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.). Four to 10 increasing concentrations of labeled ligand were used, ranging from 0.6 to 6 nM, to obtain total binding for each cytosol. Nonspecific binding was determined by addition of 200- to 500-fold excess unlabeled diethylstilbestrol along with the labeled ligand. The cytosol (0.2 ml) was added after the steroids had been air dried in the shell vials. To prevent the steroids from sticking to the glass, all the vials were vigorously shaken after the cytosol was added. To remove free steroids after the cytosol reaction was completed, the dextrancoated charcoal method of Korenman (16) was used with minor modifications. In these assays, 1% Norit A (Pfanstie1 Chemical Co., Waukegan, Ill.) and 0.5% dextran (Schwarz/Mann, Orangeburg, N. Y.) were prepared and added to the Tris-EDTA buffer to make a charcoal suspension. The cytosol sample was incubated with the ligands for 16 to 18 hr at 0 to 4°. The reaction was stopped by the addition of 0.2 ml of the charcoal suspension. The mixture was then incubated for an additional 10 min at 0°. The mixture was then subjected to centrifugation at 1500 rpm for 10 min at 4°, and 0.2 ml of the supernatant was transferred to scintillation vials containing 10 ml of Bray’s counting fluor. Radioactivity was measured in a liquid scintillation counter (Isocap 300; Searle Diagnostics, Chicago, Ill.). Specific binding was calculated as the difference between nonspecific and total binding. The data were analyzed by the method of Scatchard (26) using linear regression analysis to obtain the best fitting straight line. Data are expressed as fmol of 17β-[3H]estradiol bound per mg cytosol protein. Protein determinations on a portion of the cytosol preparation were performed by the method of Lowry et al. (20).

RESULTS

Tumor Growth in Hormonally Modified Hosts. During these studies, a total of 116 mammary tumors was induced and followed for growth behavior. Seventy-two lesions were followed and classified for growth behavior after animals were made diabetic. In intact animals, 41 of 44 (93%) lesions demonstrated progressive growth. Response of tumors to diabetes was as follows: 36 (50%) regressed; 27 (38%) continued to grow; and 9 (12%) remained static. These results are similar to those reported earlier (4, 9, 14). Administration of insulin to diabetic tumor-bearing animals produced a full spectrum of responses in tumor growth behavior. In these experiments, of 24 carcinomas that were regressing in diabetic rats, treatment with insulin resulted in growth of 17, continued regression of 2, and static behavior of 5. On the other hand, of 19 tumors classified as growing in diabetic rats, treatment with insulin resulted in regression of 9, continued growth of 8, and static behavior of 1 lesion. Although fewer tumors could be considered as displaying static behavior in diabetic rats, administration of insulin was noted to stimulate, have no effect on, or

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Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal state</th>
<th>No. of tumors</th>
<th>Tumor response</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Intact</td>
<td>18</td>
<td>Growth</td>
<td>+238 ± 78</td>
</tr>
<tr>
<td>B</td>
<td>Diabetic</td>
<td>18</td>
<td>Regression</td>
<td>-41 ± 8.7</td>
</tr>
<tr>
<td>C</td>
<td>Diabetic</td>
<td>18</td>
<td>Growth</td>
<td>+49 ± 22</td>
</tr>
<tr>
<td>D</td>
<td>Diabetic</td>
<td>18</td>
<td>Regression</td>
<td>-90 ± 42</td>
</tr>
<tr>
<td>E</td>
<td>Diabetic</td>
<td>18</td>
<td>Growth</td>
<td>+19 ± 30</td>
</tr>
<tr>
<td>F</td>
<td>Diabetic</td>
<td>18</td>
<td>Regression</td>
<td>-71 ± 35</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
decrease the size of the tumor. Thus, regardless of the characteristics of tumor growth in the diabetic animal, daily administration of 8 IU of insulin could cause tumor growth behavior classifiable as growing, static, or regressing.

Unfortunately, not all tumors were deemed satisfactory for biochemical assays for the following reasons: ulceration and/or necrosis of the lesion; insufficient tissue due to almost complete regression; and serious debilitation or death of the host. Because of these considerations, we opted to summarize and report the growth characteristics and observation periods for only those lesions on which ER assays were performed (Table 1). It should be noted that when these data are combined for only those lesions on which ER assays were performed and report the growth characteristics and observation periods, the results of assays for ER in these tumors are shown in Table 2. Tumors regressing in diabetic rats demonstrated a significant reduction in estrogen-binding capacity compared to ER in tumors from intact rats. Resumption of tumor growth in response to insulin treatment was accompanied by a return of ER levels to those observed in intact animals. Insulin-independent tumors, i.e., growing in diabetic rats, showed a slight but significant (p < 0.05) reduction in ER compared to tumors from intact rats. The insulin-independent tumors that showed no response to insulin treatment had ER levels similar to those of insulin-independent tumors of diabetic rats. However, those insulin-independent tumors that regressed after insulin treatment displayed ER levels equal to those in tumors of intact rats and not significantly different from ER levels in the insulin-dependent lesions stimulated to grow by insulin treatment. Thus, despite the opposite behavior of tumor growth in these latter 2 groups, insulin treatment stimulated estrogen-binding capacity.

Representative plots of binding data analyzed according to the method of Scatchard are summarized in Table 2 and shown in Chart 2. The calculated dissociation constant, Kd, appeared to be similar among all tumors although there was the suggestion that the average Kd was lower in diabetes-independent tumors that regressed after insulin treatment. Thus, although the amount of binding differed significantly among treatment groups, the estimated affinity constants for estradiol binding were in the same range.

Tumors that displayed static (<20% change) behavior during some phase of this study were not included in Table 2. Because such tumor behavior was fewer in number, a summary is given in Table 3. It is interesting to note that the ER level in the one static tumor that failed to respond to insulin therapy was comparable to that found in static tumors reported earlier (9), whereas the ER level was elevated in the static lesion that responded to insulin therapy with growth. Two lesions initially

### Table 2

**Effect of diabetes and insulin treatment on ER's in DMBA-induced mammary tumors**

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal state</th>
<th>No. Treatment</th>
<th>Tumor response</th>
<th>Estrogen-binding capacity (fmol/mg protein)</th>
<th>Kd (x 10^-8 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Intact</td>
<td>18 None</td>
<td>Growth</td>
<td>39.4 ± 3.6</td>
<td>15−80</td>
</tr>
<tr>
<td>B</td>
<td>Diabetic</td>
<td>6 None</td>
<td>Growth</td>
<td>24.2 ± 2.9</td>
<td>16−35</td>
</tr>
<tr>
<td>C</td>
<td>Diabetic</td>
<td>6 None</td>
<td>Regression</td>
<td>8.5 ± 0.7</td>
<td>6−11</td>
</tr>
<tr>
<td>D</td>
<td>Diabetic</td>
<td>12 Insulin</td>
<td>Regression</td>
<td>52.8 ± 9.9</td>
<td>14−118</td>
</tr>
<tr>
<td>E</td>
<td>Diabetic</td>
<td>8 Insulin</td>
<td>Growth</td>
<td>39.9 ± 6.0</td>
<td>19−71</td>
</tr>
<tr>
<td>F</td>
<td>Diabetic</td>
<td>4 Insulin</td>
<td>Growth</td>
<td>27.5 ± 10.5</td>
<td>11−58</td>
</tr>
</tbody>
</table>

* Statistical analysis for differences in estrogen-binding capacities between various groups showed the following: p < 0.01 for A versus C; C versus D; and C versus E, p < 0.05 for A versus B and B versus E.
* Mean ± S.E.
* Mean ± S.D.
regressing became static during administration of insulin. Their ER levels were considerably higher than those found in regressing tumors but not as high as those found in insulin-dependent tumors stimulated to grow by insulin treatment. In the last case, an insulin-independent lesion became static after insulin treatment. The ER level in this tumor was similar to those found in lesions that were caused to regress by administration of insulin. The general picture emerging from these anecdotal cases is that the response to insulin treatment, i.e., change in growth behavior, was accompanied by an increase in ER.

One additional case is worthy of comment. This lesion was classified as regressing in a diabetic rat and continued to decrease in size during insulin treatment. Analysis of this lesion revealed it to contain an ER level of 36 fmol/mg cytosol protein. Thus, the ER level appeared to be higher after insulin treatment even though this insulin-dependent lesion continued to regress.

DISCUSSION

Because it is evident that receptors are mediators of hormone action and because data have been presented suggesting a proportional relationship between quantity of ER and response rate in women with breast cancer (6, 21), studies that are directed towards regulation of receptors could ultimately have application to improved therapy. Such studies are certainly appropriate in breast cancer since its multihormonal basis requires a clarification of the roles of several hormones in stimulation and inhibition of tumor growth. Several investigators have reported that prolactin may regulate ER (19, 27, 31), that estrogens influence prolactin binding (23, 29), and that progesterone may alter estrogen binding (1, 18, 25).

The data presented here on DMBA-induced mammary tumors confirm and extend our earlier findings (9) that insulin may play a regulatory role in ER status. This conclusion was reached on the basis of quantitative assessment of estrogen-binding capacity in tumors from diabetic rats and in lesions examined after administration of insulin. In insulin-dependent tumors, i.e., regression in diabetic rats, the significantly reduced ER levels were observed to return to control (growing tumors in intact rats) levels concomitant with tumor growth resulting from insulin treatment. A most interesting result was that obtained in insulin-induced tumor regression of lesions that initially continued to grow in diabetic rats (insulin independent). ER levels in these responsive carcinomas were also increased after insulin treatment. These data taken together with the other results strongly suggest that lesions responding to insulin by a change in growth behavior also demonstrate an increase in ER. Such results indicate a separation of ER from growth processes and imply a more direct regulatory role of insulin on estrogen-binding capacity. Because the binding affinity for estradiol was similar in all of the tissues examined, we interpret changes in ER as reflecting more or less receptor molecules available for ligand interaction.

However, an effect of insulin on ER levels in the absence of a change in growth behavior cannot be completely excluded. Indeed, some of the data presented here could be interpreted as being consistent with an increased ER resulting from insulin treatment independent of a change in tumor behavior. Additional tumors demonstrating such growth characteristics will have to be obtained and analyzed to support this proposal. If DMBA-induced tumors contain a heterogeneous cell population, i.e., both with and without cytosol ER, it is also conceivable that the insulin treatment-induced regression could have resulted from a selective destruction of cells lacking cytosol ER. The resulting lesion would be relatively enriched in ER-containing components and present with higher ER values.

At this time, the specificity of the insulin-ER relationship is not known. Smith et al. (29) have also shown that prolactin binding was reduced in DMBA-induced tumors from diabetic rats. In contrast, a comparison of the activities of some selected enzymes in growing and diabetic regressing tumors indicated that only some enzymes showed decreased activities while others were unchanged (4). Thus, although it is well known that insulin stimulates the uptake and incorporation of amino acids into protein in muscle, adipose tissue, and liver, a somewhat more specific role of insulin on certain proteins is also known, e.g., induction of glucokinase in liver. Furthermore, the role of insulin in ER regulation was seen also in the R3230AC transplatable mammary tumor. Growth of this neoplasm was faster in diabetic rats, but ER levels were reduced to 25% of that found in tumors from intact rats (28). Preliminary data from short-term culture of R3230AC cells indicated that higher ER levels were observed when insulin was added to the culture medium compared to those levels in cells grown in the absence of insulin supplementation (28). Should these suggestions regarding insulin's role in regulating ER apply to the human

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Table 3

<table>
<thead>
<tr>
<th>Tumor response to Insulin treatment</th>
<th>Estrogen-binding capacity (fmol/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static (4)</td>
<td>8.0 ± 0.4</td>
</tr>
<tr>
<td>Static (1)</td>
<td>10</td>
</tr>
<tr>
<td>Static (1) Growing</td>
<td>56</td>
</tr>
<tr>
<td>Regressing (2) Static</td>
<td>29, 24</td>
</tr>
<tr>
<td>Growing (1)</td>
<td>48</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of animals.

a Mean ± S.E.

b Growth rate, +50%/day.
disease, one could consider utilization of insulin as an agent to potentiate estrogen therapy. Such an effect may have occurred in the breast cancer patients studied by Rhomberg (24). He observed that a higher proportion of diabetic patients (whose diabetes was being controlled by medication or insulin) responded to hormonal therapy than did the nondiabetic patients with comparable disease.

Consideration must be given to the permissive or indirect role of insulin when seeking explanations for tumor growth behavior in animals made diabetic. Because DMBA-induced tumors are known to be ovarian and prolactin dependent (13), regression of lesions could arise from reduced circulating levels of ovarian and/or pituitary hormones secondary to induction of the diabetic state. Further, administration of insulin and subsequent tumor growth could occur if administration of insulin stimulated, or at least returned to normal, secretion of ovarian and/or pituitary hormones. Although attention has been directed towards pregnancy in the diabetic woman, the causes for the observed reproductive failure often associated with diabetes are poorly understood. Although much of that literature is not germane to this discussion, a few reports are noteworthy. Kirchick et al. (15), noting anovulation in alloxa diabetic rats, could not attribute this to lack of ovarian responsiveness but rather to a loss of the luteinizing hormone surge. Indeed, serum estradiol levels were comparable in intact and diabetic rats. Interestingly, they consider that their results are consistent with a decreased sensitivity to estradiol in diabetic rats. The work of Gentry et al. (8) who reported that nuclear uptake of tritiated estradiol in hypothalamus and pituitary was reduced in diabetic rats supported this contention. The clinical literature is also replete with data on hormonal alterations in diabetics. However, it appears that estrogen levels were equal to or somewhat higher in postmenopausal diabetics than in postmenopausal patients demonstrating normal glucose tolerance (7). A similar situation is recorded for prolactin levels (30). Thus, it would appear that an explanation for changes in ER in diabetic rats based solely on decreased estrogens and/or prolactin may be too simplistic at this time.

Finally, there were some subtle differences in tumor growth behavior in response to alteration of the insulin milieu of the host. Growth rates of lesions classified as insulin independent were not as rapid as those rates for lesions growing in intact hosts. When insulin was administered, these lesions demonstrated an enhanced growth rate. It would appear that insulin was needed for maximal tumor growth either directly or indirectly through improved nutritional status of the host. On the other hand, the rates of regression seen in diabetic hosts for insulin-dependent lesions due to removal of endogenous insulin were less rapid than were the regressions observed in those insulin-independent lesions that responded to insulin therapy. One explanation for these observations may be attributed to the more gradual declining of insulin levels in the diabetic in contrast to the diabetic host receiving exogenous insulin on a daily basis. The fact that removal of insulin or its administration can cause tumor regression resembles to some extent the well known and paradoxical effects of estrogen removal or administration (11). Additional experiments will be necessary to elucidate the interrelation of insulin with estrogens as to whether direct or indirect mechanisms are responsible for altered tumor growth (14).

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