Influence of Insulin on Estrogen-induced Responses in the R3230AC Mammary Carcinoma

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SUMMARY

The R3230AC mammary adenocarcinoma was not dependent on insulin; tumor growth was equal to or greater in diabetic rats than in intact animals. However, tumor growth was reduced when daily doses of insulin were administered. Treatment with estrogen inhibited growth of the R3230AC carcinoma, either in diabetic rats or in intact animals simultaneously treated with insulin. The effects of insulin plus estrogen treatment appeared to be additive in causing inhibition of tumor growth. Tumors from diabetic rats showed few metabolic alterations as reflected by little or no changes in the activities of selected glycolytic enzymes, pyruvate kinase, phosphofructokinase, and hexokinase, nor any striking changes in the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, representing the pentose phosphate pathway. A modest reduction in the ratio of utilization of [1-14C]glucose to [6-14C]glucose was seen in vitro by tumors from diabetic rats. It was concluded that insulin, along with estrogen and prolactin, should be considered as a hormonal factor that influences growth of this autonomous, hormone-responsive adenocarcinoma.

INTRODUCTION

The R3230AC mammary adenocarcinoma of the Fischer rat represents an interesting mammary tumor model, since this neoplasm can respond to hormonal perturbations by displaying differentiated functions found in the mammary gland; treatment with estrogen and/or prolactin results in lactation-like changes with the accumulation of casein, lactalbumin, shorter-chain fatty acids, and lactose (10–12). Such changes have been reported for the mammary gland in vivo when the midpregnant gland was incubated with insulin, prolactin, and hydrocortisone (26).

A role for insulin in growth and metabolism of the R3230AC tumor has been questioned by studies performed in vitro. Insulin, added in vitro, had no further effect on DNA synthesis in tumor explants; addition of estradiol-17β to the incubation medium was also without significant effect on thymidine incorporation (28). However, the R3230AC tumor was shown to respond to hormonal stimuli in vitro as reflected by a significant increase in the synthesis of casein (29). It was concluded from these studies that rapid proliferation (thymidine incorporation into DNA) and differentiated cell function were compatible in this tumor.

With the few recent reports (4, 8, 9, 21) that insulin may stimulate tumor growth in vivo and that tumor growth may be inhibited by removal of endogenous insulin (diabetes), it seemed logical to initiate studies of the effects of insulin on growth of the R3230AC tumor in vivo. To this end, experiments were performed to examine growth of the R3230AC tumor in animals that either were diabetic after streptozotocin treatment or were intact and were given exogenous insulin. Further, it was pertinent to investigate the effects of estrogen treatment on tumor growth in the above animals to determine whether the presence or absence of insulin altered the anticipated response to estrogen therapy. Since a major function of insulin is to increase glucose transport across cell membranes, tissues were also assayed for enzymes of carbohydrate metabolism, enzymes of the hexose monophosphate shunt pathway (G6PD and 6PGD), as well as those enzymes considered to be key control points of glycolysis (PK, PFK, and HK). Some experiments were also performed to determine glucose utilization in vivo by tumors from intact and diabetic rats.

The data presented here indicate that the R3230AC carcinoma grew as well, if not faster, in the diabetic animal and that administration of insulin reduced tumor growth. Treatment with estrogen inhibited tumor growth, either in diabetic animals or in intact animals simultaneously receiving daily treatment of insulin; the effects of insulin plus estrogen treatment appeared to be additive in causing inhibition of tumor growth. Tumors from diabetic rats showed little or no alterations in the activities of glycolytic enzymes or enzymes of the pentose phosphate pathway, although tumor slices from diabetic rats did demonstrate a modest reduction in the ratio of utilization of [1-14C]glucose to [6-14C]glucose in vivo. These data indicate that the R3230AC tumor is not dependent on endogenous insulin.
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MATERIALS AND METHODS

R3230AC Mammary Tumor. Female Fischer rats were obtained from Charles River Breeding Laboratory, Wilmington, Mass. The animals were individually housed and fed food and water ad libitum. The mammary tumors were transplanted by a sterile trochar technique as described by Hilf et al. (12). Estradiol valerate (E. R. Squibb & Sons, Inc., New York, N. Y.), 40 mg/ml, when utilized, was dissolved in sesame oil and injected s.c. once per week, treatment (1 mg/week) being initiated on the day after tumor transplantation.

Insulin treatment of R3230AC tumor-bearing animals was also begun 1 day after tumor transplantation. Isophane insulin (E. R. Squibb) was dissolved in bacteriostatic water and injected daily s.c. Three weeks after tumor transplantation, the animals were sacrificed by cervical dislocation. Tissues were removed, weighed, frozen in liquid nitrogen, and stored at −20° until assayed or were immediately utilized for tissue incubations.

Induction of Diabetes. Diabetes was induced by 1 i.v. injection of streptozotocin in animals fasted overnight. Streptozotocin (Upjohn Co., Kalamazoo, Mich.) was dissolved in 0.9% NaCl solution and was rapidly adjusted to pH 4.5 with citric acid. Fischer rats were given a dose of 40 mg/kg in a volume of approximately 0.5 ml/200 g body weight. After the treatment, animals were again allowed food and water ad libitum.

Blood glucose levels and urine glucose output were monitored in these animals in intact controls once to twice per week. A microversion of the Nelson-Somogyi (19) determination was used to measure blood glucose concentration. Urinary glucose was determined by Clinistix (Ames Co., Inc., Elkhart, Ind.). Animals were classified as diabetic if their blood glucose levels averaged 250 mg/100 ml or above and they showed greater than 0.5% glucose in their urine.

Enzyme Assays, Protein, and Nucleic Acid Determinations. Tissues were thawed and homogenized in cold 0.05 M Tris buffer, pH 7.4, with the volume of diluent added to produce a 10% homogenate (w/v). Aliquots of the homogenates were taken for determinations of nucleic acids.

Enzyme assays were performed on the supernatant of the homogenate following centrifugation at 29,250 x g for 20 min in the cold. Each of the enzyme assays was performed under identical conditions by measurement of the absorbance change at 340 nm due to the production of NADPH or the oxidation of NADH. Under these conditions (zero order kinetics) the enzyme values are comparable and are expressed as μmoles of NADPH produced per min per mg DNA or as μmoles of NADH oxidized per min per mg DNA. The enzymes were measured by the following procedures: pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), method of Susor and Rutter (25); hexokinase (ATP:α-hexose-6-phosphotransferase, EC 2.7.1.1), according to the method of Sharma et al. (24); phosphofructokinase (ATP:d-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), using the method of Ling et al. (16); glucose-6-phosphate dehydrogenase (d-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase [6-phospho-d-gluconate:NAD oxidoreductase (decarboxylating), EC 1.1.1.44], using the method of Glock and McLean (6); and α-glycerolphosphate dehydrogenase (D-glycerol-3-phosphate:NAD oxidoreductase, EC 1.1.1.8), using the method of Beisenherz et al. (2), modified by using dihydroxyacetone phosphate as substrate and measuring the oxidation of NADH.

Aliquots of the supernatants were also taken for protein determinations according to the method of Lowry et al. (17). Extraction of nucleic acids from aliquots of the initial tissue homogenates was carried out by a modification of the method of Schneider (23). RNA was measured by the orcinol reaction as described by Ceriotti (3). DNA was determined by the diphenylamine reaction according to the method of Dische (5).

Tissue Incubations. Tissue slices were prepared from tumors with the aid of a McIlwain-Buddle tissue chopper. Slices were cut 1 mm thick and weighed; 500 mg of tissue were transferred to incubation flasks. Each flask contained 4.9 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), 50 μmoles of glucose, and 0.25 μCi of either D-[1-14C]glucose (57 mCi/mmole), D-[6-14C]glucose (53.7 mCi/mmole), or uniformly labeled D-[14C]glucose (250 mCi/mmole). In addition, to some flasks, 1 IU of porcine insulin (25.4 units/mg; Eli Lilly and Co., Indianapolis, Ind.) was added. The flasks were then gassed for 20 sec with a mixture of 95% oxygen:5% CO2 and sealed with rubber serum caps; a small plastic well was suspended from the serum cap. The flasks were incubated in a 37° water bath with shaking for 1 to 3 hr. The reactions were stopped by immersing the flasks for 60 sec in boiling water. Immediately thereafter, the flasks were cooled in an ice bath. 2-Ethoxyethanol:ethanol (1:1, v/v), 0.4 ml, was injected by syringe into the suspension plastic well through the serum cap. Then, 0.5 ml of 0.2 N HCl was injected into the main compartment of the flask to liberate 14CO2. The flasks were then placed in a shaker bath for 2 hr.

At the end of this time the rubber serum caps were carefully removed from the flasks and the plastic wells were cut from their stems, such that the wells and their contents were deposited into a scintillation vial containing 10 ml of scintillation fluor [4 g of Omnifluor per liter of toluene:2-ethoxyethanol (2:1, v/v)]. The trapped 14CO2 was measured in an Isocap, Model 300 scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Quench corrections were made so that the incorporated radioactivity could be expressed as dpm.

Remaining tissue fragments were separated from the incubation media by centrifugation. Fatty acids were extracted with hexane from the tissues by the method of Abraham et al. (1) after the tissue slices were heated for 24 hr in 5 N KOH at 65°. Aliquots of the hexane extract were
deposited into a scintillation vial, containing 10 ml of scintillation fluor (4 g of Omnifluor per liter of toluene), and radioactivity was determined in a Nuclear-Chicago scintillation counter.

Glucose uptake by the tissue slices was determined utilizing a glucose oxidase method (Worthington Biochemical Corp., Freehold, N. J.).

Analysis of Data. All values are expressed as the mean ± S.E. Significance was determined by the Student t test. A probability value of 0.05 or less was considered to be significant.

RESULTS

Effect of Diabetes and Estrogen on Tumor Growth. The data in Table 1 summarize the effects of diabetes and administration of estradiol valerate on growth of the R3230AC carcinoma. The absence of endogenous insulin did not reduce tumor growth; indeed, on the basis of a modest decrease in total body weight of the diabetic rats, tumor weight at the end of the experimental period appeared to be greater in the diabetic rats than in the intact animals (average tumor weight in diabetic rats, 2.15 g/100 g body weight versus 1.27 g/100 g body weight for intact animals). Administration of estradiol valerate, 1 mg/week, to intact animals produced a significant decrease in tumor weight (approximately 50% inhibition), whereas the same dose of estrogen produced a greater decrease in tumor weight in the diabetic rat (about 75% inhibition versus the untreated diabetic animal). However, there was a somewhat greater toxicity in the diabetic animals receiving estrogen, as reflected by a significantly reduced body weight. Even so, the reduction of tumor growth represented 72% when expressed per unit of body weight (0.60 g tumor/100 g body weight in the estrogen-treated diabetic rats versus 2.15 g tumor/100 g body weight for the diabetic rats). As shown in Table 1, uterotrophic responses were obtained in intact and diabetic rats treated with estrogen and the increases in uterine weight were comparable, even though the weight of the uterus from diabetic rats was significantly reduced compared with that obtained in intact animals. Blood glucose levels of diabetic rats were elevated by 300%.

Effect of Insulin on Tumor Growth in Intact Rats. Also shown in Table 1 is the effect of daily administration of insulin on the growth of the R3230AC tumor in intact animals. At a dose level of 2 IU/day, insulin treatment significantly inhibited growth of the neoplasm while increasing body weight gain. When animals received both hormones (daily injections of insulin and weekly injections of estradiol valerate), tumor growth was decreased to a greater extent than resulting from either hormone treatment alone. It appeared that the effects of insulin and estrogen were additive, each hormone producing approximately 50% inhibition of tumor growth, and the combined hormone treatment resulted in an 80% inhibition of neoplastic growth. No deleterious effect on body weight was seen in animals receiving estradiol valerate plus insulin treatment, although the uterotrophic response to the estrogen treatment was somewhat reduced by the daily injections of insulin. These data suggest that insulin does not stimulate growth of the R3230AC adenocarcinoma in vivo; rather, insulin (or hypoglycemia) inhibited tumor growth. The effect was additive to the inhibition of tumor growth produced by estrogen therapy.

Effect of Insulin on Tumor Growth in Diabetic Rats. On the basis of the foregoing results, in which tumor growth was increased in the diabetic animal, experiments were conducted to determine the effects of various doses of insulin on tumor growth in the diabetic rat. These results are summarized in Table 2. The lowest dose of insulin injected, 0.5 IU/day, caused an increase in growth of the R3230AC carcinoma in diabetic animals; as the dose of insulin was increased, tumor growth was not stimulated further. The tumor weight in the diabetic rats receiving insulin, 2.0 IU/day, was the same as that found in intact, untreated control animals. Thus, a small amount of insulin may produce stimulation of tumor growth in vivo, but tumor growth was not further stimulated as the dose of insulin was increased. The results are reminiscent of the effects of estrogen on mammary tumor growth; low doses stimulate tumor growth whereas large doses cause significant inhibition of neoplastic growth (13). Uterine weight, which was decreased in diabetic animals, was increased in rats treated

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment administered</th>
<th>Estradiol valerate (mg/wk)</th>
<th>Insulin (IU/day)</th>
<th>Body wt (g)</th>
<th>Tumor wt (g)</th>
<th>Uterine wt (mg)</th>
<th>Blood glucose (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.0</td>
<td>139 ± 3e</td>
<td></td>
<td></td>
<td>1.77 ± 0.18</td>
<td>306 ± 14</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.0</td>
<td>122 ± 3a</td>
<td></td>
<td></td>
<td>0.88 ± 0.15</td>
<td>766 ± 33</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.0</td>
<td>115 ± 3x</td>
<td></td>
<td></td>
<td>2.47 ± 0.44</td>
<td>157 ± 10</td>
<td>434 ± 18</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.0</td>
<td>96 ± 4b,c,d</td>
<td></td>
<td></td>
<td>0.57 ± 0.08e</td>
<td>462 ± 32</td>
<td>436 ± 20</td>
</tr>
<tr>
<td>Intact</td>
<td>2.0</td>
<td>160 ± 6e</td>
<td></td>
<td>0.99 ± 0.29</td>
<td>313 ± 62</td>
<td>114 ± 3</td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>2.0</td>
<td>135 ± 4e</td>
<td></td>
<td>0.30 ± 0.15x</td>
<td>618 ± 52</td>
<td>104 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

- Mean ± S.E. number of animals ranged from 5 to 26 per group.
- Significant difference (p < 0.05) from intact control.
- Significant difference (p < 0.05) from diabetic.
- Significant difference (p < 0.05) from control (intact) + estrogen.
- Significant difference (p < 0.05) from control (intact) + insulin.
with the highest dose of insulin.

Activities of Enzymes of Carbohydrate Metabolism in Tumors of Animals Treated with Estrogen and/or Insulin.

To ascertain whether alterations in carbohydrate metabolism occurred in tumors from animals treated with estrogen and/or insulin, samples were obtained and assayed for the activities of G6PD, 6PGD, PK, PFK, and HK as potential indicators of oxidative and anaerobic glucose metabolism. As shown in Table 3, administration of estradiol valerate caused an increase in G6PD activity and a decrease in PK and PFK activities in the R3230AC tumor, results which confirm our earlier reports. Tumors from diabetic rats displayed reductions in PK and HK activities; administration of estradiol to the diabetic rats did not produce a significant increase in G6PD activity, although the activities of PK and PFK were significantly lower than in tumors of control animals. Administration of insulin to intact tumor-bearing animals produced decreases in the activities of G6PD and PK. When estradiol valerate was injected along with insulin, the estrogen-induced increase in G6PD activity was observed but no further significant decreases in PK or PFK activities were seen as expected after estrogen treatment alone. These data indicate that tumor growth retardation was often accompanied by a reduction in PK activity, but no clear cut parallel behavior of PFK or HK was observed. In addition, the ability of estrogen to increase G6PD activity in the tumor may require the presence of insulin; high levels of glucose in the blood did not influence G6PD activity in the tumor.

Effect of Insulin on Enzyme Activities in Tumors from Diabetic Rats. In the liver, insulin has been shown to increase the activities of the enzymes in the pentose pathway and of the enzymes considered to be control points of glycolysis. To determine whether this response occurred in the R3230AC mammary tumor, diabetic animals were used and various doses of insulin were administered for 21 days prior to sacrifice. Tumors were assayed for G6PD, 6PGD, PK, PFK, and HK activities and the data are presented in Table 4. The tumor in the diabetic rat showed a modest decrease in the activities of PK and HK but not the other enzymes. Administration of a small dose of insulin, 0.5 IU/day, resulted in an increase in the activities of all of the enzymes except HK when compared to tumors from untreated diabetic rats. In tumors of animals receiving 0.5 IU insulin, the activities of G6PD, 6PGD, and PFK attained values that were significantly higher than those found in tumors of intact rats; this suggested that the tumor in the diabetic rat may have become more sensitive to the effects of insulin on carbohydrate metabolism. The elevation in enzyme activities accompanied the increased growth rate of the carcinoma in these diabetic animals receiving the lowest dose of insulin used. However, as the dose of insulin

Table 2
The effect of various doses of insulin on R3230AC tumor weight and uterine weight

<table>
<thead>
<tr>
<th>Animal</th>
<th>Daily dose of insulin (IU)</th>
<th>Body wt (g)</th>
<th>Tumor wt (g)</th>
<th>Uterine wt (mg)</th>
<th>Blood glucose (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (34)*</td>
<td>0</td>
<td>139 ± 3</td>
<td>1.77 ± 0.18*</td>
<td>306 ± 14</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Diabetic (23)</td>
<td>0</td>
<td>115 ± 3*</td>
<td>2.47 ± 0.44</td>
<td>157 ± 10*</td>
<td>434 ± 18*</td>
</tr>
<tr>
<td>Diabetic (8)</td>
<td>0.5</td>
<td>124 ± 7</td>
<td>3.32 ± 0.50*</td>
<td>175 ± 22</td>
<td>402 ± 19*</td>
</tr>
<tr>
<td>Diabetic (8)</td>
<td>1.0</td>
<td>131 ± 4*</td>
<td>2.39 ± 0.34</td>
<td>192 ± 24*</td>
<td>345 ± 20*</td>
</tr>
<tr>
<td>Diabetic (10)</td>
<td>2.0</td>
<td>130 ± 5*</td>
<td>1.93 ± 0.32</td>
<td>200 ± 10*</td>
<td>331 ± 20*</td>
</tr>
</tbody>
</table>

a Numbers in parentheses, number of animals per group.
b Mean ± S.E.
c Significant difference (p < 0.05) from the control.
d Significant difference (p < 0.05) from the diabetic.

Table 3
Effects of insulin and estrogen on enzyme activities in R3230AC tumors from intact and diabetic rats

<table>
<thead>
<tr>
<th>Treatment administered</th>
<th>Estradiol valerate (mg/wk)</th>
<th>Insulin (IU/day)</th>
<th>G6PD</th>
<th>6PGD</th>
<th>PK</th>
<th>PFK</th>
<th>HK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact control</td>
<td>0.413 ± 0.034*</td>
<td>0.867 ± 0.067</td>
<td>19.42 ± 1.11*</td>
<td>0.382 ± 0.041</td>
<td>0.181 ± 0.031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>1.0</td>
<td>0.815 ± 0.103*</td>
<td>0.913 ± 0.068</td>
<td>15.90 ± 1.10*</td>
<td>0.253 ± 0.019*</td>
<td>0.194 ± 0.017</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.442 ± 0.047</td>
<td>0.830 ± 0.079</td>
<td>14.64 ± 1.11*</td>
<td>0.408 ± 0.054</td>
<td>0.144 ± 0.012*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.0</td>
<td>0.598 ± 0.104</td>
<td>1.000 ± 0.199</td>
<td>12.36 ± 1.76*</td>
<td>0.197 ± 0.029*</td>
<td>0.242 ± 0.052</td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>2.0</td>
<td>0.213 ± 0.040*</td>
<td>0.757 ± 0.073</td>
<td>12.68 ± 1.19*</td>
<td>0.259 ± 0.052</td>
<td>0.174 ± 0.012</td>
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</tr>
<tr>
<td>Intact</td>
<td>1.0</td>
<td>0.570 ± 0.151*</td>
<td>0.957 ± 0.143</td>
<td>13.42 ± 1.42*</td>
<td>0.237 ± 0.088</td>
<td>0.230 ± 0.008*</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E.; number of tumors assayed ranged from 5 to 20 per experimental group.
b Significant difference (p < 0.05) from intact control.
c Significant difference (p < 0.05) from diabetic control.
d Significant difference (p < 0.05) from insulin-treated intact animals.
administered was increased, no further increase in enzyme activities was observed; rather, the enzyme activity profile resembled that found in tumors from intact or diabetic rats. Thus, when tumor growth rates were comparable, the enzyme activity profiles were quite similar.

Effect of Diabetes on Glucose Utilization by R3230AC Carcinoma in Vitro. Although the alterations in enzyme activities were modest in tumors from diabetic rats, it was important to ascertain whether utilization of glucose substrate by tumor tissue was influenced. To study this, tissue slices of tumors from intact and diabetic rats were incubated with glucose labeled in various positions, and the production of $^{14}$CO$_2$ and incorporation of label into fatty acids were measured. Glucose uptake was similar by tumor slices from intact and diabetic rats. After 1 hr of incubation, no differences in the production of $^{14}$CO$_2$ from labeled glucose substrate were seen when comparing tumors from intact and diabetic animals (data not shown). At 3 hr (Table 5), the production of $^{14}$CO$_2$ from [1-$^{14}$C]glucose by tumor slices from diabetic animals appeared to be lower than that observed for tumors from control animals, and this was reflected by a significant decrease in the ratio (C-1:C-6) of $^{14}$CO$_2$ produced by tumors from diabetic animals. There was no difference in $^{14}$CO$_2$ production from [6-$^{14}$C]glucose or from uniformly labeled glucose, indicating that tumors from diabetic rats did not demonstrate alterations in anaerobic glycolytic pathways. Insulin added in vitro did not markedly affect $^{14}$CO$_2$ production by tumor slices from diabetic rats, although utilization of [1-$^{14}$C]glucose was reduced in the presence of insulin in vitro. Incorporation of label into fatty acids appeared to be decreased in tumor slices from diabetic rats, although only the fatty acids extracted from tissue slices incubated with uniformly labeled glucose were found to be significantly reduced. Insulin added in vitro did not reverse these differences.

From these experiments it was possible to determine the contribution of the pentose cycle to glucose metabolism in the R3230AC tumor. One method of calculation, proposed by Katz et al. (14), involved both $^{14}$CO$_2$ yields and radioactivity in fatty acids. Calculated by this method, the pentose phosphate contribution to the metabolism of glucose was 3.8% in tumors from the intact animals, whereas in tumors from diabetic animals this percentage was decreased to 2.1%. These data imply that tumors from diabetic rats

<table>
<thead>
<tr>
<th>Animal and treatment* (IU/day)</th>
<th>PK</th>
<th>HK</th>
<th>PFK</th>
<th>6PGD</th>
<th>G6PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (23)</td>
<td>19.42 ± 1.11a</td>
<td>0.181 ± 0.013</td>
<td>0.382 ± 0.041</td>
<td>0.867 ± 0.067</td>
<td>0.413 ± 0.034</td>
</tr>
<tr>
<td>Diabetic (15)</td>
<td>14.64 ± 1.11a</td>
<td>0.144 ± 0.012a</td>
<td>0.408 ± 0.054</td>
<td>0.820 ± 0.079</td>
<td>0.442 ± 0.047</td>
</tr>
<tr>
<td>Diabetic + 0.5 IU insulin (8)</td>
<td>20.24 ± 1.98a</td>
<td>0.170 ± 0.026</td>
<td>0.588 ± 0.064a</td>
<td>1.064 ± 0.069a</td>
<td>0.579 ± 0.046a</td>
</tr>
<tr>
<td>Diabetic + 1.0 IU insulin (8)</td>
<td>17.32 ± 2.10a</td>
<td>0.127 ± 0.023a</td>
<td>0.399 ± 0.034</td>
<td>0.774 ± 0.041</td>
<td>0.457 ± 0.045</td>
</tr>
<tr>
<td>Diabetic + 2.0 IU insulin (10)</td>
<td>16.61 ± 1.81a</td>
<td>0.168 ± 0.038</td>
<td>0.442 ± 0.061</td>
<td>0.774 ± 0.044</td>
<td>0.368 ± 0.032</td>
</tr>
</tbody>
</table>

* Activity expressed as μmoles NADH oxidized per min per mg DNA or μmoles NADPH produced per min per mg DNA.

Table 5

Influence of insulin on glucose metabolism by the R3230AC tumor in vitro

<table>
<thead>
<tr>
<th>Animal</th>
<th>Insulin added in vitro (IU)</th>
<th>% of initial radioactivity recovered during the 3-hr incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C-I</td>
</tr>
<tr>
<td>$^{14}$CO$_2$ evolved from glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (8)</td>
<td>0</td>
<td>9.73 ± 0.83a</td>
</tr>
<tr>
<td>Diabetic (8)</td>
<td>0</td>
<td>7.39 ± 1.09a</td>
</tr>
<tr>
<td>Diabetic (4)</td>
<td>1.0</td>
<td>6.06 ± 1.05a</td>
</tr>
</tbody>
</table>

$^{14}$C in fatty acids from glucose

<table>
<thead>
<tr>
<th>Animal</th>
<th>Insulin added in vitro (IU)</th>
<th>% of initial radioactivity recovered during the 3-hr incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C-I</td>
</tr>
<tr>
<td>Control (6)</td>
<td>0</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>Diabetic (7)</td>
<td>0</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>Diabetic (4)</td>
<td>1.0</td>
<td>0.20 ± 0.05a</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of samples per group.

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showed a decrease in pentose phosphate metabolism, but little or no effect was seen for glycolytic pathway metabolism.

DISCUSSION

The data presented here indicate that the R3230AC mammary adenocarcinoma is not dependent on endogenous insulin secretion, as tumor growth proceeded as well in the diabetic animal as in the intact animal. These results agree with the earlier conclusion of Turkington and Hilf (28) on the basis of studies of tumor explants in vitro. However, the administration of exogenous insulin to intact animals or to diabetic animals resulted in a decrease in growth of the tumor in vivo. It would appear that insulin may play a role analogous to that of estrogens in which administration of low doses may stimulate tumor growth, whereas treatment with pharmacological doses of estrogens produces inhibition of tumor growth (13). Whether such effects of insulin are direct or indirect, through alteration of metabolism of the host, cannot be concluded at this stage of experimentation.

Reports by other investigators on the effects of insulin deprivation and tumor growth generally indicated that mammary tumors were decreased in growth in diabetic animals. For example, a majority of DMBA-induced mammary tumors regressed after the animal was made diabetic (4, 8, 9) and the growth of a transplantable mammary adenocarcinoma was decreased in diabetic C3H mice (21). With the exception of our studies with DMBA-induced tumors (4), considerable body weight loss accompanied the retardation of neoplastic growth and it was difficult to assess the effects of insulin deprivation as separate from nutritional restriction. In the data presented here, the R3230AC tumor in diabetic rats grew as well as tumors in intact animals and the modest difference in body weight gain seen in the diabetic animals was not deleterious to neoplastic growth. Indeed, in diabetic rats receiving the lowest dose of exogenous insulin, 0.5 IU/day, the final body weight averaged about 10% less than that seen in diabetic animals, although the final tumor weight of these diabetic insulin-treated animals was almost double that obtained in intact animals. While there were no significant alterations in RNA, DNA, or protein content in the tumors of diabetic animals receiving the lowest dose of insulin, the activities of the glycolytic enzymes in these tumors were equal to (PK and HK) or elevated above (PFK) the activities of the glycolytic enzymes in tumors from intact animals and tumors from untreated diabetic rats. Similarly, the activities of G6PD and 6PGD were significantly increased in tumors of diabetic rats that received 0.5 IU insulin per day. From these results, one might propose that the enzyme activities were reflecting increased metabolic activity and growth.

Although reports (15, 22) in the literature attribute increased G6PD activity in mammary glands in vitro to the presence of insulin in the incubation medium, this has been questioned by a recent report showing that high concentrations of glucose in the medium could stimulate G6PD activity in the absence of insulin (7). While a direct comparison of these data with the experiments performed here is not possible, G6PD activity in tumors of diabetic rats was comparable to that found in intact animals even though the circulating level of glucose in the diabetic rat was 3 to 4 times that found in the intact animal. In the diabetic animals receiving low levels of insulin, 0.5 IU/day, but still showing high blood glucose levels, an increase in G6PD activity was observed in the tumors, suggesting that insulin may have played a role in regulation of G6PD activity. Since no further effect on G6PD activity was seen when higher levels of insulin were administered, a simple relationship between blood glucose levels, insulin, and G6PD activity in tumors is not apparent. However, it is possible that growth of the R3230AC tumor may be dependent on circulating levels of glucose; preliminary studies indicate that glucose enters this tumor by a simple diffusion mechanism (J. T. Harmon and R. Hilf, unpublished observations). Such a mechanism would not require insulin to aid substrate entry into the tumor; thus tumor growth could be influenced by substrate availability, i.e., glucose levels in blood. Hence, the diabetic host demonstrating elevated levels of blood glucose would demonstrate greatest tumor growth. Administration of exogenous insulin would lead to hypoglycemia and reduced tumor growth. Experiments to test this proposal are in progress.

The studies performed on glucose utilization in vitro indicated that little alteration in glycolysis occurred in tumors from diabetic rats and only a modest decrease in utilization of [1-14C]glucose was observed in tumors growing in diabetic rats. Thus, the data obtained from enzyme activity assays did not correlate completely with the findings in vitro as had been anticipated from our earlier findings with DMBA-induced tumors (4). These results reemphasize the need to seek correlations between enzyme measurements, conducted under optimum assay conditions, and actual flow through of substrate in intact cells. The report (1) that insulin stimulated 14CO2 production from [1-14C]glucose in mammary glands suggests that the hormone exerted its effect primarily on the pentose pathway; removal of insulin by induction of diabetes might result in a decrease in this pathway of glucose utilization.

The results observed from the effects of estrogen treatment on growth and metabolism of the R3230AC tumor indicate that this neoplasm was capable of responding to administration of the steroid hormone in the presence or absence of insulin. Indeed, when both estrogen and insulin were administered simultaneously, growth of the tumor was markedly inhibited and the effects of the 2 hormones appeared to be additive. Such results suggest that the 2 hormones may act by different mechanisms, a proposal earlier suggested by Heuson et al. (9) in their experiments on DMBA-induced tumors. The results reported here in vivo differ from the data reported for the effects of 17beta-estradiol and insulin on DNA synthesis in vitro (28), pointing out that the metabolic consequences of hormonal treatment in vivo may be far reaching and not readily duplicated by studies under artificial conditions in vitro. Administration of estrogen alone or with insulin to intact animals resulted in elevations in G6PD activity in the tumor, but estrogen did not significantly elevate G6PD in the tumor of the diabetic rat. Further, although estrogen
treatment resulted in a decrease in PK and PFK activities in tumors of intact animals, only PFK activity was further reduced by estrogen treatment of diabetic rats (PK activity appeared to be lower but the level of significance was not at the 95% confidence limits). Thus, the metabolic responses to estrogen appeared to be compromised in the diabetic rat, even though the ability of estrogen to inhibit tumor growth was equal to or greater than that seen in the intact animal. The report (20) that 12 of 23 patients with breast cancer demonstrated diabetic-type glucose tolerance curves, suggesting decreased pancreatic insulin reserve, may be pertinent to a further consideration of responses of mammary tumors and insulin status of the host.

Finally, it would appear that insulin may be another hormone to which the R3230AC tumor responds in a pattern similar to that in earlier reports on estrogen and prolactin (11). The R3230AC tumor is autonomous; its growth is not dependent on endogenous levels of estrogen, prolactin, or insulin. However, growth of this carcinoma can be significantly decreased by administration of estrogen or insulin or by increasing the circulating levels of prolactin (10). The R3230AC tumor may represent an experimental model comparable to those recurring breast cancers in women that arise after ablation of hormone-producing organs. The characteristics of such tumors may be quite different from those possessed by the primary lesion, which may be hormone dependent. An experimental model of hormone-dependent tumors is the lesions that arise after administration of DMBA; these are tumors that regress after removal of endogenous estrogen, prolactin, or insulin. Comparative studies of the R3230AC tumor and of DMBA-induced tumors for biochemical defects may shed light on the differences between hormone dependence and autonomy. Data obtained by comparing activities of glycolytic enzymes, utilization of glucose in vitro, and levels of estrogen and prolactin receptors (18, 27, 30) have demonstrated the existence of quantitative differences in these biochemical characteristics between these 2 tumor systems, and these approaches are currently being examined to seek correlations with clinical course of breast cancer patients.

REFERENCES


Influence of Insulin on Estrogen-induced Responses in the R3230AC Mammary Carcinoma

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