Influence of Insulin Deprivation on Growth of the 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinoma in Rats Subjected to Alloxan Diabetes and Food Restriction

Jean-Claude Heuson and Nicole Legros

SUMMARY

The present study investigates the possibility that insulin dependence, a property exhibited in organ culture by a majority of the 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinomas, might also be operating in vivo. It was found that induction of alloxan diabetes 3 or 4 weeks after 7,12-dimethylbenz(a)anthracene administration completely prevented mammary tumor formation. Moreover, induction of alloxan diabetes in tumor-bearing rats produced the rapid regression of 90% of the tumors, with a time course similar to that observed after oophorectomy or hypophysectomy. In contrast to what occurs after oophorectomy but in accordance with what is observed after hypophysectomy, administration of estradiol benzoate failed to prevent the tumor regression produced by alloxan diabetes.

Because induction of alloxan diabetes involves a considerable loss of body weight, the effect on the mammary tumors of a severe food restriction, leading to an even greater loss of body weight, was also studied. It was found that food restriction resulted in a rapid regression partially counteracted by estradiol benzoate.

Tumors regressing as a result of alloxan diabetes, those progressing (about 10%) in spite of diabetes, and those that progressed under the stimulating effect of estradiol benzoate in rats subjected to food restriction were investigated with respect to their insulin dependence in organ culture. It was observed that the tumors regressing in alloxan-diabetic rats were, by and large, markedly insulin dependent in vitro, whereas those growing despite diabetes were little- or noninsulin dependent. Eventually, most tumors that were stimulated to grow by estradiol benzoate in food-restricted rats proved very insulin dependent in vitro.

These observations suggest that the tumors that are insulin dependent in organ culture similarly have a stringent requirement for insulin to grow in vivo and that they regress after induction of alloxan diabetes as a consequence of insulin deprivation. This would explain why only tumors that are insulin independent in vitro are able to grow despite the insulin deprivation of alloxan diabetes. It is conceivable that food restriction results in tumor regression also as a consequence of a decreased rate of insulin secretion. However, this decrease would be smaller than in the diabetic state, and the available insulin would be present in amounts large enough to allow insulin-dependent tumors to grow, provided other limiting factors such as estrogens or estrogen-stimulated hormones are restored.

INTRODUCTION

In earlier publications (12, 13), the carcinogen-induced, hormone-dependent carcinoma of the rat was studied with respect to the effect of insulin on DNA synthesis in organ culture. It was shown that insulin markedly stimulated DNA synthesis and cell proliferation in a majority of these tumors whereas, in others, cell proliferation and DNA synthesis proceeded readily in the absence of insulin. These 2 types of mammary tumors, either insulin dependent or independent in culture, although different from this particular biological standpoint, were identical by histological criteria and often coexisted in the same rat. It was also shown that the stimulating effect of insulin in the insulin-dependent tumors was not mediated through an increase in glucose transport across the cell membrane and probably involved induction of enzyme systems related to DNA synthesis (14). This interpretation was substantiated in organ culture experiments in which DNA synthesis was measured, together with the soluble DNA polymerase activity (16). It was found that stimulation of DNA synthesis by insulin in the insulin-dependent tumors was not mediated through an increase in enzyme activity; moreover, in the tumors with insulin-independent DNA synthesis, enzyme activity was unaffected by insulin. In these in vitro experiments, evidence was presented which suggested that many of the insulin-dependent tumors were overresponding to insulin, leading to full activation of the DNA synthesizing process in vivo; conversely, inactivation of this process took place as a result of induction of alloxan diabetes.

These observations led us to study the influence of insulin on mammary tumor growth in vivo. The purpose of this article is to describe the inhibitory effect of alloxan diabetes...
on the formation and growth of the tumors and to correlate it with the insulin dependence of individual tumors in organ culture. The effects of restriction of food intake are also reported and discussed in relation to endocrine mechanisms. The influence of administration of insulin on mammary tumor growth in intact, oophorectomized and hypophysectomized rats will be described later (17).

MATERIALS AND METHODS

Mammary tumors were induced in random-bred, female Sprague-Dawley rats, at the age of 50 days, by a single feeding of 20 mg of DMBA dissolved in sesame oil (19).

Alloxan diabetes was produced by i.p. injection of alloxan (Eastman Organic Chemicals, Rochester, N.Y.). In the experiments studying tumor formation, alloxan was given at the dose of 15 mg/100 g body weight. In these experiments with tumor-bearing rats, the dose was 12 mg/100 g body weight; moreover, in order to reduce mortality following administration of alloxan, insulin replacement therapy in decreasing doses was started 2 days later (insulin Lente; Novo A/S, Copenhagen, Denmark). Doses were 1.25 i.u./100 g body weight daily for 1 week, then one-half that amount for an additional week. The day following cessation of insulin treatment was taken as Day 0 of diabetes. Rats were considered as diabetic when a sustained hyperglycemia (serum glucose above 300 mg/100 ml, measured by the glucose oxidase method, with Glucostat reagents; Worthington Biochemical Corp., Freehold, N.J.) was obtained.

Food restriction consisted of administration of only 5 g of the regular food pellets each morning instead of 12 g, which is the approximate amount eaten daily by such rats when fed ad libitum. The pellets, which were manufactured by Protector S.A., Brussels, Belgium, contained carbohydrates, 32%; proteins, 16.5%; fats, 4%; and vitamins and minerals. Rats on this restricted diet remained healthy for as long as 6 months of observation. Estradiol benzoate (Calbiochem, Los Angeles, Calif.) was administered i.m. at the daily dose of 6 days/week) of 5 μg, dissolved in 0.1 ml of sesame oil.

Tumor size was measured by means of a caliper and expressed in terms of "surface" by multiplying the perpendicular diameters. The total tumor surface per rat designates the sum of the surfaces of individual tumors.

Insulin dependence of the tumors in organ culture was assessed by a method described in detail elsewhere (16). It consisted of culturing tumor fragments for 48 hr in tissue culture Medium 199 (Baltimore Biological Laboratories, Baltimore, Md.), with and without added insulin (bovine, recrystallized, 22.5 i.u./mg; Calbiochem), 40 μg/ml, and of measuring the incorporation of thymidine-3H (specific activity, 1.15 Ci/m mole; Calbiochem) into DNA during the last 4 hr of culture. The results were expressed as dpm/mg fresh tissue. The tumors were designated insulin dependent when insulin produced a significant increase in thymidine-3H incorporation into DNA over the values found in the control, insulin-free cultures (p < 0.01, Student's t test on quadruplicate groups of cultured tumor tissue fragments); they were called "insulin independent" when there was no such significant increase. Insulin dependence was quantitatively estimated by the ratio between the thymidine-3H incorporation in the insulin-treated and control cultures.

Statistical analysis of the results was carried out by nonparametric methods specified in the text (25).

RESULTS

Alloxan Diabetes

Effect on Tumor Formation

In 1 experiment, 20 rats were randomized into 2 groups of 10, 3 weeks after DMBA feeding. One group served as control; alloxan diabetes was induced in the other. In a 2nd experiment, 90 rats were randomized into 2 groups of 36 and 54, respectively, 4 weeks after DMBA feeding. The 1st group served as control; alloxan diabetes was induced in the other. Each group was observed for a period of 12 weeks.

The results are given in Table 1. None of the surviving diabetic rats developed mammary tumors, whereas a majority of the controls developed 1 or several tumors. Inhibition of tumorigenesis by alloxan diabetes is highly significant in both experiments.

Effect on Existing Tumors

Diabetes Only. Thirty-seven tumor-bearing rats were subjected to alloxan diabetes 21 weeks after administration of DMBA. Fifteen became diabetic and 12 survived at the end of the 6-week period of observation. There was an average weight loss of 56 g (19% of the initial weight of 299 g). In these rats, 51 out of 56 tumors initially present (90%) decreased rapidly in size; 5 continued to grow.

The time course of tumor regression is represented in Chart 1. It is similar to that usually observed, according to our experience, in such rats after oophorectomy or hypophysectomy. Tumor regression in the diabetic rats did not seem to occur as a result of a direct effect of alloxan on tumor tissue. This interpretation is supported first by the fact that the tumors do not regress in rats that fail to become diabetic after alloxan administration and second by the observation, not detailed here, that most tumors continue to grow in diabetic rats receiving insulin replacement therapy for a period of 3 weeks.

Diabetes and Administration of Estradiol Benzoate. It is known that administration of estradiol benzoate prevents tumor regression after oophorectomy but not after hypophysectomy (23, 24). The present experiment was devised to determine whether estradiol prevents tumor regression in alloxan-diabetic rats.

Diabetes was induced in tumor-bearing rats 11 to 26 weeks after DMBA administration. On Day 0 of diabetes, 34 rats were paired according to total tumor surface and allocated at random into 2 groups, one group serving as control and receiving i.m. injections of sesame oil only, the other receiving estradiol benzoate dissolved in sesame oil; at the end of 6 weeks, there were 13 and 12 survivors, respectively. In both groups, more than 90% of the tumors regressed. The changes
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Table I

Effect of alloxan diabetes on DMBA mammary carcinogenesis

Alloxan diabetes was induced 3 and 4 weeks (Experiments 1 and 2, respectively) after DMBA administration. Period of observation: 12 weeks after DMBA.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Initial no. of rats</th>
<th>No. of surviving rats</th>
<th>No. of tumor-bearing rats</th>
<th>Total no. of tumors</th>
<th>Mean no. of tumors per tumor-bearing rat</th>
<th>Mean body wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>23</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetes</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>36</td>
<td>33</td>
<td>25</td>
<td>64</td>
<td>2.6</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>Diabetes</td>
<td>54</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>182</td>
</tr>
</tbody>
</table>

*a p < 0.02, Fisher's exact probability test.

*b p < 0.001 (same test).

Effect of Caloric Restriction and of Concomitant Administration of Estradiol Benzoate in Tumor-bearing Rats

As reported under “Diabetes Only,” the alloxan-diabetic rats lost a considerable amount of body weight, would produce the same effects on tumor growth as did the diabetic state.

Twelve to 14 weeks after DMBA, 33 tumor-bearing rats were arrayed by order of increasing total tumor surface and were randomized within each successive lot of 3 into the following treatment groups: Group 1, control; Group 2, a group subjected to food restriction; and Group 3, a group subjected to food restriction and receiving estradiol benzoate i.m. in sesame oil (Groups 1 and 2 received sesame oil only). After 6 weeks, the mean weight changes were as follows: Group 1, from 273 to 301 g (+10%); Group 2, from 273 to 174 g (−36%); and Group 3, from 265 to 163 g (−38%). The

Chart 1. Time course of tumor regression after induction of alloxan diabetes. Each curve represents the total surface of the regressing tumors in each of 12 rats.

in the mean total tumor surface per rat were identical in both groups (Chart 2). It is concluded that there was no detectable effect ascribable to estradiol benzoate and that this hormone was totally unable to prevent tumor regression due to the diabetic state.

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Chart 2. Tumor regression after induction of alloxan diabetes in 2 groups of match-paired rats. One of the groups received daily injections of estradiol benzoate, 5 μg. Period of observation from Day 0 of diabetes: 6 weeks. Mean total tumor surface per rat: ■, at start (Day 0); ■, after 6 weeks. The statistical significance of tumor regression was estimated by the Wilcoxon test on the paired differences.
final weights in Groups 2 and 3 were 42 and 46% lower, respectively, than that in the control group.

The effects of these experimental conditions on tumor size are represented in Chart 3. Food restriction produced a marked and highly significant decrease in tumor surface, contrasting with the normal increase observed in the control group. In the 3rd group, subjected to both food restriction and estrogen administration, there was no significant change in tumor surface during the period of observation. When comparing Groups 2 and 3 with respect to changes in tumor surface during the observation period, we found that apparently estradiol benzoate significantly counteracted the effect of food restriction (p < 0.025; Wilcoxon test on match-paired differences; one-tailed); however, this effect was only partial, as demonstrated by comparison of Group 3 with the control group (p < 0.005; Wilcoxon test on match-paired differences; 2-tailed). Prevention of tumor regression by estradiol benzoate under conditions of food restriction is also apparent from analysis of the growth pattern of individual tumors in each rat. Growing or newly formed tumors were found in only 1 of 11 rats in Group 2, whereas such tumors were found in 8 out of 10 rats in Group 3; this difference is highly significant (p < 0.002; Fisher's exact probability test; 1-tailed).

Correlation between Tumor Growth Pattern in Vivo and Response to Insulin in Organ Culture

The observations described under "Alloxan Diabetes" and "Effect of Caloric Restriction and of Concomitant Administration of Estradiol Benzoate in Tumor-bearing Rats," demonstrate that only a small number of mammary tumors were able to grow in alloxan-diabetic rats and that this number was not increased by the concomitant administration of estrogens; on the other hand, in rats subjected to food restriction, growing tumors occurred almost entirely as a consequence of estrogenic stimulation. The present experiment investigates in organ culture the insulin dependence of tumors either regressing or growing in diabetic rats and of tumors growing under the stimulating effect of estradiol benzoate in rats subjected to food restriction.

Table 2 shows that most tumors regressing in diabetic rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Course of tumors in vivo</th>
<th>No. of tumors studied</th>
<th>Insulin independent</th>
<th>Insulin dependent</th>
<th>Thymidine-3H/DNA ratio, insulin/control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>1. Regressive</td>
<td>13</td>
<td>3</td>
<td>10 (1.8 to 8.5)</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2. Progressive</td>
<td>8</td>
<td>6</td>
<td>2 (1.7 and 1.8)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Food restriction + estradiol benzoate</td>
<td>3. Progressive</td>
<td>7</td>
<td>1</td>
<td>6 (1.9 to 5.2)</td>
<td>3.4 ± 0.58</td>
</tr>
</tbody>
</table>

* a The differences of distribution into insulin-dependent and -independent categories are significant at the level p = 0.06 (Fisher exact probability test; 2-tailed), (a) between tumors with in vivo Courses 1 and 2, and (b) between those with in vivo Courses 2 and 3.

b Nos. in parentheses, range of magnitude of insulin dependence.

c Mean values ± S.E. for all tumors. The differences are significant (p < 0.002; Mann-Whitney U test; 2-tailed) between tumors with in vivo Courses 1 and 2 and between those with in vivo Courses 2 and 3.
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are highly insulin dependent in organ culture, while those growing despite the diabetic state are little- or noninsulin dependent. In contrast to the tumors growing in diabetic rats, most of those growing under estradiol stimulation in rats subjected to food restriction are markedly insulin dependent in organ culture.

DISCUSSION

These results indicate that induction of alloxan diabetes produced a rapid regression, similar to that observed after oophorectomy or hypophysectomy, of a majority of the DMBA-induced mammary carcinomas of the rat. Evidence was also presented suggesting that the observed regression was not due to a direct effect of alloxan on the tumor tissue, but rather to the destruction of the pancreatic β-cells and ensuing diabetes.

The influence of alloxan diabetes on tumor growth has been little studied. Garvie (6) showed that it decreases the growth rate and metastasizing capacity of Walker carcinosarcoma 256, transplantation in alloxan-diabetic rats of the Zaj-Dela ascitic was quite small, amounting to a 50% decrease in growth rate, as compared with the massive regression observed here. Conversely, Wieser et al. (27) reported that successive transplantation in alloxan-diabetic rats of the Zaj-Dela ascitic hepatoma progressively increased its growth rate over that in untreated controls. These data indicate that the strong inhibitory effect of alloxan diabetes on the rat mammary tumor is far from being a general phenomenon in cancer research.

Several mechanisms may be proposed to explain this inhibitory effect. A likely one is that endogenous insulin is required to sustain cell proliferation in the rat mammary tumor and that insulin deprivation in alloxan diabetes decreases the rate of cell proliferation and results in tumor regression. This explanation is suggested by our previous demonstration that insulin displays such properties in organ culture; under these conditions, insulin stimulates DNA synthesis and cell proliferation in the epithelial component of the mammary tumor tissue (12, 13), probably by induction of enzyme systems involved in the process of DNA synthesis (14, 16). Furthermore, there is evidence derived from organ culture experiments suggesting that these enzyme systems are “overresponsive” to insulin in vivo in many of the tumors and that they are inactivated by alloxan diabetes (16). The experiments reported here are consistent with this explanation. Most tumors regressing in alloxan-diabetic rats are insulin dependent when assayed in organ culture. It is true that there were apparent exceptions. Three tumors regressing in diabetic rats were classified here as insulin independent; however, as described elsewhere (16), 2 of them were slightly but significantly insulin dependent by criteria other than those used here, and the 3rd had an extremely low rate of DNA synthesis, suggesting that the explanted tissue was not thriving well in culture. It is therefore possible that all tumors regressing in diabetic rats are indeed insulin dependent. Conversely, it was found that all tumors growing in diabetic rats were little- or noninsulin dependent in culture. The concordance between the in vivo and in vitro findings suggests that the mechanisms of insulin dependence of the mammary tumors, as described in organ culture, are also operating in vivo. This would account for the present observations. Tumors that are insulin dependent in organ culture regress as a result of the insulin deprivation of alloxan diabetes, whereas only those that are insulin independent in vitro are able to grow under these conditions.

To our knowledge, Walker carcinosarcoma and Zaj-Dela hepatoma, which have been investigated in regard to the effect of alloxan diabetes on growth, as mentioned above (6, 27), have not been studied with respect to insulin response in vitro. Yet, the hepatoma which was reported to grow faster in alloxan diabetic animals is an ascitic tumor and ascitic tumors in general are known for their extremely high rate of glycolysis (5). It is therefore conceivable that the enhancing effect of diabetes on the hepatoma was a result of hyperglycemia.

One might object that diabetes has an adverse effect on the whole organism and that the resultant tumor inhibition, which spares only the most “aggressive” tumors, could possibly be devoid of any specificity. It is true that some tissues, particularly the muscular and adipose tissues, are subject to a considerable reduction in mass as a result of the insulin deprivation of diabetes. However, this is not a general phenomenon. The diabetic liver, for example, is increased in size and in amount of DNA, relative to body weight. Moreover, it is able to respond almost normally to the stimulus of partial hepatectomy (28). The bowels are much hypertrophied, and glucose transfer through their walls is markedly increased under alloxan-diabetic conditions (4). The normal mammary tissue has also been investigated with respect to the effect of diabetes. Kumaresan and Turner (20) showed that the amount of DNA in this organ relative to body weight is slightly but not significantly decreased in oophorectomized, alloxan-diabetic female rats receiving estradiol benzoate and progesterone. Ahren and Angervall (2) observed, under the same experimental conditions, a normal ductal growth but a marked reduction in lobuloalveolar development; conversely, in castrated, alloxan-diabetic male rats, testosterone produced a massive lobuloalveolar development, considerably greater than in the nondiabetic controls (3). These studies show that insulin deficiency of alloxan diabetes does not lead indiscriminately to atrophy of all tissues; rather, it favors the development of some tissues, among which is the normal differentiated mammary tissue under certain circumstances.

One might also claim that mammary tumor regression in alloxan-diabetic rats could result merely from the concomitant body weight loss. Indeed, it was shown in this paper that severe food restriction produced a rapid regression of most tumors. This observation was not unexpected and had been described by others (9, 10). It is well known that food restriction, resulting in relative or absolute body weight loss, decreases the growth rate of several experimental tumors (8, 26). It would appear, however, that the inhibitory effect of food restriction, like that of alloxan diabetes, is much more intense on the carcinogen-induced rat mammary carcinoma than on any other experimental tumor. As for diabetes, several mechanisms might be proposed to explain this effect. One is of particular interest in this discussion. It is known that fasting (7, 21), as well as prolonged food restriction (11), decreases the base level of insulinemia and the insulin response to
glucose administration. It is quite conceivable that food restriction in our rats produced such effects and that the latter in turn could represent 1 factor leading to tumor regression. On the other hand, in alloxan diabetes, it is unlikely that weight loss was the sole factor responsible for tumor regression. Against such interpretation is the fact that estradiol benzoate partially counteracted the effect of severe food restriction on the tumors but did not at all counteract the effect of alloxan diabetes, although the latter induced a much smaller loss of body weight than did food restriction. Moreover, the tumors growing under estrogen stimulation in rats subjected to food restriction were insulin dependent, when studied in organ culture, whereas those growing in diabetic rats were insulin independent.

It is probable that the inhibitory effect of alloxan diabetes and of food restriction on mammary tumors involves intricate factors but that insulin deprivation may be a common one under both experimental conditions. The difference between these 2 conditions could then simply be quantitative, insulin deprivation being less pronounced when a result of food restriction than when a result of alloxan diabetes. In the case of alloxan diabetes, insulin deprivation would be sufficiently intense to make it impossible for insulin-dependent tumors to grow whereas, under conditions of food restriction, enough insulin would be available to allow the growth of insulin-dependent tumors, provided other limiting factors, such as estrogens or estrogen-stimulated hormones [prolactin? (24)], are restored.

These observations lead to the conclusion that the rat mammary tumors, when insulin dependent in organ culture, have a stringent requirement for insulin in order to grow in vivo. In this respect, insulin plays a part similar to that of pituitary prolactin (18, 22, 24), although the mechanisms involved are probably different. Absolute requirement for hormones seems to be a characteristic of the tumor tissue, as opposed to its normal counterpart. In the normal mammary tissue, ductal growth and lobuloalveolar development can take place in diabetic and in hypophysectomized rats, provided steroids and, in the case of hypophysectomized rats, insulin are administered (1–3). Therefore, paradoxically speaking, mammary tumors seem to be more hormone dependent than the corresponding normal tissue, although they are less sensitive to other inhibitory control factors. With regard to insulin, this conclusion is consistent with the interpretation derived from previously reported experiments (16) that the rat mammary tumor seems to be overresponsive to insulin.

REFERENCES

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