Estrogen-Prolactin Dependency in 7,12-Dimethylbenz(a)anthracene-induced Tumors

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SUMMARY

Hormonal influences on dimethylbenz(a)anthracene-induced tumor growth were investigated in detail by endocrine ablation and replacement of hormones. The majority of tumors regressed following ablation and most of them were reactivated by subsequent administrations of estrogen (0.1 to 5 µg) or prolactin (2 mg). Increasing numbers of tumors, however, were not stimulated by prolactin when administration was delayed, and a basal level of estradiol (0.01 µg) in addition to prolactin was required for reactivation of tumors. Nafoxidine hydrochloride, a competitor of estrogen at the receptor sites, arrested growth of a large portion of dimethylbenz(a)anthracene-induced tumors in intact animals but failed to retard growth of prolactin-stimulated tumors. On withdrawal of prolactin-nafoxidine, rapid regression of tumor occurred and readministration of prolactin failed to activate most of the tumors for as long as 28 days. Our results give good supporting evidence that estrogen plays a primary role in tumor growth. The interactions of prolactin and estrogen at tumor sites are necessary for regulatory events related to tumor growth.

INTRODUCTION

Estrogen and prolactin are primary hormones involved in the development and growth of rat mammary cancers induced by DMBA. Most information about the influence of prolactin in this tumor model has been derived from variation of host prolactin levels. For example, prolactin levels decreased by OVEX or ADRX, hypophysectomy (12, 14), and administration of L-dopa (15), ergot alkaloids (2, 16), or antiserum to prolactin (1) can inhibit tumor growth. On the contrary, serum prolactin levels elevated by exogenous prolactin (12, 14), phenothiazine compounds (14), or median eminence lesions (20) or during pregnancy (9) can accelerate tumor growth and form new tumor sites. Stimulation of tumors by direct estrogen action has been questioned. Since estrogen can stimulate pituitary release of prolactin, the direct role of estrogen in stimulating tumor growth has been questioned, especially when good evidence is provided from experiments showing that estrogen per se cannot reactivate tumor growth in hypophysectomized rats (14).

Several lines of information indicate, however, that estrogen participates directly at the tumor site. First, high doses of estrogen, while increasing serum prolactin, also inhibit tumor growth (11, 14). Secondly, after OVEX-ADRX, resumption of tumor growth by prolactin appears to be temporary (12). Thirdly, sustained growth resumes with estrogen replacement from ovarian isografts in OVEX rats bearing hypothalamic lesions (18). Lastly, estrogen receptor, which mediates estrogen action in normal target tissues (4), is present in tumors that respond to changes in host estrogen levels (3, 5, 10, 17).

It is evident that responses of DMBA tumors to these 2 hormones require clarification. This report attempts to distinguish the separate responses of tumors to estrogen, prolactin, and their combination. The distinction of these tumors in respect to their hormonal responses might enable further characterization of their immunological, biochemical, and detailed morphological features. Finally, this study demonstrates clearly that estrogen plays a primary role in events necessary for tumor growth.

MATERIALS AND METHODS

Tumors were induced by single intragastric feeding of 16 mg of DMBA (Sigma Chemical Co., St. Louis, Mo.) suspended in sesame seed oil to 50-day-old Sprague-Dawley rats (Holtzman Company, Madison, Wis.). In about 6 weeks, tumors started to develop and were measured with a caliper by the product of the major and minor axes of the tumor. Bilateral OVEX-ADRX were performed simultaneously via the dorsal route under light ether anesthesia. Ablated rats were given 0.9% NaCl solution to drink. Hormonal replacement therapy was performed as indicated in each experiment. Tumors included in the study were confirmed by histology as adenocarcinomas.

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RESULTS

**Estrogen Effect.** The responses of DMBA tumors to OVEX-ADRX and subsequent replacement of estrogen are illustrated in Chart 1A. Four different types of tumor responses to this mode of treatment were observed, and it was not uncommon to find a rat bearing more than 1 type of tumor. More than 90% of DMBA tumors regressed after endocrine ablation; subsequent daily administration of estrogen (0.1 to 5 μg) stimulated growth of most tumors (Group A), but some were not stimulated (Group B). Less than 10% of tumors were not influenced by this mode of therapy; some continued to grow (Group C) while others regressed spontaneously (Group D).

Differentiation of tumor responses to estrogen was tested by nafoxidine hydrochloride (U-11 100A, 1-[2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl]pyrrolidine hydrochloride; a gift from Upjohn Co., Kalamazoo, Mich.), an estrogen competitor at the estrogen receptor-binding sites (Chart 1B). Growth of most tumors was inhibited by nafoxidine hydrochloride, while about 25% of tumors in this group of animals continued to grow at their original rate.

**Prolactin Effect.** Rats bearing DMBA tumors that regressed after OVEX-ADRX were divided randomly into 2 groups. At 7 or 11 days following ablation, rats were given 2 mg of prolactin (ovine prolactin, NIH-P-Sb, obtained through the courtesy of the National Institute of Arthritis and Metabolic Diseases) daily (Chart 2, A and B). Some tumors were stimulated by prolactin while others were not. Regression rates of prolactin-resistant tumors were much reduced while animals were on prolactin. In other experiments, when prolactin was injected slightly earlier or later, similar tumor responses were observed. However, when prolactin was given immediately following endocrine ablation or soon thereafter (2 or 3 days), most tumors were stimulated (Chart 2C). On removal of prolactin, sizes of these prolactin-stimulated tumors decreased rapidly. New tumors that developed on prolactin therapy also responded to prolactin withdrawal (Chart 2D). Readministration of prolactin again activated tumor growth. Although 2 mg of prolactin was an effective dose, other doses of prolactin as low as 0.5 mg were able to stimulate some tumors.

In order to clarify whether tumors that were stimulated by prolactin were independent of estrogen during their growth phase, nafoxidine hydrochloride was injected into rats bearing prolactin-stimulated tumors. Administration of nafoxidine hydrochloride (10 to 50 μg) failed to retard the rapid tumor growth that was initiated by prolactin (Chart 3A). Removal of both prolactin and nafoxidine hydrochloride resulted in prompt regression of tumors. Subsequent prolactin therapy could not reactivate these tumors. When this mode of hormonal manipulation was repeated in another experiment (Chart 3B), daily readministration of prolactin for up to 28 days failed to activate 4 out of 5 tumors. It appears that prolactin-stimulated tumors were independent of estrogen in the growth phase but that they might require estrogen during certain early steps in cell differentiation prior to prolactin actions.

**Combined Prolactin and Estrogen Effect.** In order to investigate whether estrogen is required for growth of prolactin-resistant tumors, rats bearing such tumors were given a low level of estradiol (0.01 μg) in addition to prolactin (Chart 4A). Rapid growth was observed after an
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Chart 2. Prolactin effect. The size of a tumor was derived from the product of the 2 major axes measured with a caliper. Relative growth rate of a tumor was calculated by comparing the size to that at time of OVEX-ADRX (OA), designated as 100%. In A and B, stimulated and nonstimulated tumors were grouped separately in calculating for mean ± S.D. from the number of tumors shown in parentheses. Prolactin (2 mg/day/rat) was injected s.c. on the 7th or 11th day after ablation, as shown. C, tumor growth responses to same daily dose of prolactin (P) and subsequent withdrawal (W). Dotted line, growth rate (mean ± S.D.) of 4 tumors where rats were given injections of prolactin immediately after ablation: solid line, 7 tumors where rats were given injections 2 days after ablation. Each line in D represents the individual growth pattern of tumors of rats receiving same treatment. OVEX-ADRX induced rapid regression of all tumors. During the 1st course of prolactin injection (4th to 15th day after endocrine ablation), all tumors were stimulated and new tumors developed. Rapid regression of tumors on discontinuation of prolactin was followed by activation of tumor growth when prolactin was readministered.

initial lag period of about 4 days on prolactin-estrogen treatment (Group B). This low level of estrogen was ineffective in stimulating tumor growth by itself (Group A). In contrast, accelerated growth with a shorter or no lag period was observed when animals were first primed with estradiol (0.01 μg) and then given prolactin-estradiol (Chart 4C). In another experiment (Chart 4B), rats were initially given injections of a combined dose of prolactin-estrogen at 11 days after ablation. All tumors were stimulated immediately by this regimen without a lag period. These results again indicate that estrogen may be required for certain early events necessary for prolactin-stimulated tumor growth.

When prolactin and estradiol were injected sequentially (Chart 4D), the OVEX-ADRX-responsive tumor, which failed to be stimulated by prolactin, also failed to be sustained by low levels of estradiol. On withdrawal of prolactin, however, a rapid decrease of tumor size was observed. Combination of these 2 hormones stimulated rapid growth of this tumor with subsequent regression or reactivation following removal or addition of these 2 hormones, respectively. Similarly, the stimulated tumor during the 1st course of prolactin treatment also responded to subsequent estrogen and prolactin manipulation.

To determine whether lower prolactin levels in combination with estrogen may stimulate this tumor type, rats were
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Chart 3. Effect of prolactin and nafoxidine. Each line shows the clinical course of a tumor. Other designations were the same as described in Chart 2.

Rats bearing prolactin-stimulated tumors were selected out for these experiments. In A, injection of nafoxidine, first with 10 μg and then with 50 μg, did not retard the growth rate of tumors stimulated by prolactin. On withdrawal of prolactin rapid regression of tumors occurred. The same dose of prolactin (P) readministered to rats for seven days was unable to reactivate tumor growth. In B, rats were similarly treated with prolactin followed by 2 mg of prolactin and 50 μg of nafoxidine (P/PU). Nafoxidine was unable to inhibit rapid growth of tumors induced by prolactin. On prolactin withdrawal, rapid regression of tumors occurred. Readministration of prolactin for up to 28 days could not reactivate tumor growth in all 4 tumors identified as adenocarcinomas. One that was stimulated (O) was histologically identified to contain essentially fibroadenoma.

that breast cancers contain heterogeneous cell population within a single tumor and among different tumors. Using DMBA tumors as a model in the investigation of these problems in human breast cancers is advantageous because of their many similarities (3). DMBA tumors, like human breast cancers, are heterogeneous in cell population. Our results show that DMBA tumors responded to hormonal manipulation differently and, in addition, the degree of responses also varied. Most tumors regressed after OVEX or OVEX-ADRX. Estrogen activated ablation-responsive tumors but did not influence the growth of tumors that showed signs of regression before ablation of the host. Prolactin stimulated growth of some ablation-responsive tumors but required estrogen, in addition, to stimulate others. Different types of hormonal-responsive tumors were present occasionally in the same animal.

DISCUSSION

For more than 2 decades hormonal therapy has been an effective means of palliation for disseminated human breast cancer. About one-third of these patients respond objectively. However, many uncertainties still remain in the selection of patients who may benefit from hormonal manipulation and in the modality of therapy, e.g., ADRX versus hypophysectomy and addition versus removal of hormones. It is not uncommon to find in the same patient that tumors respond differently to a single mode of hormonal therapy. After OVEX-ADRX, for example, progression of metastases in the liver may be accompanied by regression of all metastases in other regions of the body. Moreover, the degree of measurable responses also varies. This phenomenon is consistent with histological findings that breast cancers contain heterogeneous cell population within a single tumor and among different tumors.

Finally, it was observed that a small number of tumors were not stimulated by estrogen-prolactin (Chart 5B). Whether this group belongs to the estrogen-independent tumor type (Chart 1, Group B) is uncertain since lack of response might also be contributed by delayed usage of effective levels of estrogen (0.01 μg) and prolactin (2 mg).

Given an injection of 0.5 mg prolactin and 0.01 μg of estradiol 18 days after ablation (Chart 5A). Since higher levels of estrogen can stimulate more prolactin secretion, this 0.01 μg of estradiol dose was retained. No tumor stimulation was observed with this combination of hormones. When the level of prolactin was increased to 2 mg, this new combination stimulated rapid tumor growth. A lag period of 3 or 4 days was again observed before stimulation occurred.

It is conceivable that the rate of tumor growth in response to a particular hormone (e.g., prolactin) might depend on the number of prolactin-dependent cells within it. A tumor containing only prolactin-dependent cells will increase in size faster than one that contains one-half this cell population, as tumor growth in this case is dependent on a larger number of dividing cells rather than on the rate of cell division. Furthermore, tumor growth is a result of many preceding events during cell preparation for functional integrity and cell differentiation. With such variations, certain cell types may flourish while others remain in a quiescent state during different hormonal manipulations of the host. It is not certain from our experiments whether the estrogen-prolactin dependent tumors were of the same cell types as those of prolactin-stimulated tumor or whether their differences in response were simply due to variations in the degree of cell differentiation.
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Chart 4. Effect of prolactin and estrogen. Measurement of tumor growth was as described in Chart 2. In A, after OVEX-ADRX (OA) rats bearing regressed tumors were randomly divided for treatment. Group A animals were given s.c. injections of 0.9% NaCl solution and estradiol as shown. Group B animals were those with tumors that were not stimulated by prolactin treatment for 15 days. When estradiol was injected in addition to prolactin, all tumors were stimulated after a lag period of about 4 days. (Rats bearing prolactin-stimulated tumors were not included in this treatment.) In B, 11 days after OVEX-ADRX (OA), rats bearing regressed tumors were given injections of a combination of 0.01 μg of estradiol and 2 mg of prolactin (E + P). All tumors were stimulated without a lag period. The chart illustrates only the growth pattern of 3 different tumors, ranging from fast to slow growing before treatment, which responded similarly to estradiol stimulation. Injection of this combination of hormones at other time points after ablation had similar effect. C, growth pattern of 2 tumors on long-term subphysiological dose of estradiol and subsequent addition of prolactin (2 mg/day/rat). No stimulation occurred with the former treatment, while immediate response to the latter treatment was observed. In D, 2 days after ablation (OA), rats were treated with 2 mg of prolactin (P) for 11 days and withdrawal for 3 days. Injection of 0.01 μg of estradiol (E) and combination of prolactin and estradiol were followed by withdrawal of hormones and readministration of hormones. Only 2 rats survived at the termination of this treatment. The chart shows the growth pattern of 2 tumors to this hormonal regimen.

Our experimental data demonstrated clearly that ablation-responsive tumors stimulated by prolactin per se 7 or 11 days after OVEX-ADRX were independent of estrogen during the growth phase. Nafoxidine hydrochloride, which inhibited most DMBA tumor growth, presumably by competing at estrogen receptor sites, was unable to retard this prolactin-induced growth. Rapid tumor regression occurred on removal of both prolactin and nafoxidine hydrochloride. Subsequent readministration of prolactin failed to activate most of these tumors. These results indicate that nafoxidine hydrochloride impairs certain estrogen-required steps that are responsible for subsequent tumor growth. Unless these events are restored by estrogen, tumor growth cannot occur. It is likely that estrogen might induce the synthesis or activation of certain enzymes responsible for tumor metabolism. For example, recent evidence from other laboratories (3) and our own (B. S. Leung, unpublished data) showed that estrogen increases activities of many enzymes in the glycolytic pathways. If these metabolizing enzymes are still present during prolactin stimulation, tumors may grow in the absence of estrogen. Estrogen might also be responsible for inducing the synthesis of tumor growth-stimulating factors, the presence of which in DMBA tumors is not yet known. However, a growth-stimulating factor for other mammary tumors was recently reported (13).

The hypothesis that estrogen exerts its influence on tumor growth by stimulating pituitary release of prolactin and not by acting directly at the tumor site (14) cannot be supported by our present findings. It is well demonstrated in this
Chart 5. Hormonal treatment of rats bearing OVEX-ADRX (OA) impeded tumors is indicated on top of each figure. A. growth rate (mean ± S.D.) of 5 tumors from each group. Combination of prolactin (2 mg) and estradiol (0.01 µg) is an effective dose for stimulating tumors. B. growth patterns of 5 separate tumors to hormonal treatment. An increase of prolactin from 0.5 µg to 2 mg along with estradiol did not stimulate these tumors.

investigation that estrogen is an absolute necessity at the tumor site. First, as previously mentioned, prolactin failed to reactivate tumor growth when blocked by antiestrogen. Secondly, prolactin could not promote growth of some tumors 7 or 11 days after OVEX-ADRX when the estrogen effect had subsided. If animals were supplied daily with a low dose of estrogen in addition to prolactin, all tumors were reactivated (Chart 4). This subphysiological dose of estrogen was unable to stimulate tumor growth by itself and is unlikely to cause a marked increase in host prolactin levels. Since these tumors exhibited a lag period before stimulation with a combination of prolactin and estrogen occurred, it is conceivable that cell preparation for functional events due to estrogen might be taking place. Indeed, when the ablated host was first given an injection of 0.01 µg of estrogen, shorter or no delay of growth was observed with subsequent estrogen-prolactin administration. Finally, the number of tumors in ablated animals that responded to prolactin stimulation appears to be dependent on the time of prolactin initiation. Consequently, most hormone-responsive tumors were stimulated by prolactin immediately or soon after ablation. As time was lengthened, however, a good portion of tumors required both estrogen and prolactin. These results further indicate that estrogen directly affects cellular events prior to and/or during cell division.

The ability of low estrogen levels in stimulating prolactin-treated tumors has important clinical implications. It is often observed that patients who had initial benefit from OVEX-ADRX often experience short remission (6). In view of our present finding and the persistent low estrogen levels in these ablated patients, consequent tumor exacerbation is not surprising.

Although the influence of these 2 hormones on tumor growth has been demonstrated, the intimate relationship of these hormones in causing tumor growth is not understood. Since estrogen mediates its action by first binding to its receptor (4), it was investigated if prolactin might potentiate estrogen expression by interacting at this level. We reported recently that prolactin in vitro stimulates specific-estrogen binding in DMBA tumors which contain estrogen receptor and are responsive to hormonal manipulation (17). The in vivo effect of prolactin on estrogen receptor in DMBA tumors was recently reported elsewhere (8, 19). Such stimulatory action of prolactin on estrogen receptor binding capacity seems to be common to all estrogen target tissues so far investigated (7). Whether this phenomenon relates to the actual cellular events leading to growth of tumors remains to be answered.

In conclusion, we have demonstrated that the majority of DMBA tumors are responsive to OVEX-ADRX. Some of these tumors can be stimulated by prolactin, others by prolactin-estrogen, but a small number were unresponsive to both hormones applied exogenously. The participation of estrogen in situ is mandatory of tumor growth.

REFERENCES

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