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Species-specificity of Estradiol Regulated Growth Factors in Breast Cancer

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Abstract—*Recent evidence indicates that autocrine/paracrine mechanisms may mediate the mitogenic effect of estradiol (E_2) both in human and experimental breast cancer. However, the species-specificity of E_2-regulated growth factors with regard to their biologic action has not been evaluated. To test this issue, we examined, in the soft agar clonogenic assay, the colony-stimulating activity in human breast cancers of conditioned media obtained from rat mammary carcinomas exposed to E_2 (rat E_2-CM). Of* 22 *primary human breast cancers plated in soft agar in the absence of serum,* 18 (82%) *successfully grew with a mean colony number of* 62.4 ± 9.8 (*S.E.M.*) (*range* 14–193). *Rat E_2-CM significantly stimulated colony formation in* 10/18 (56%) *human breast cancers to* 155 ± 11% (*S.E.M.*) *of control. E_2 administration* (10^{-9} M) *in these tumors had a virtually identical overall effect* (154 ± 13% *of control colony number). In the remaining eight tumors* (44%), *neither rat E_2-CM nor E_2 had, in general, a significant colony-stimulating effect. The growth-promoting action of rat E_2-CM and E_2 was not influenced by the hormone receptor status of the tumor. These results suggest that E_2-regulated growth factors may not be species-specific, at least with regard to their colony-stimulating effects in soft agar.*

INTRODUCTION

RECENT EVIDENCE indicates that autocrine/paracrine mechanisms may play an important role in supporting the growth of experimental [1, 2] and human breast cancer [3–5]. Using the hormone-responsive *N*-nitrosomethyl-urea (NMU)-induced rat mammary cancer cultured in the soft agar clonogenic assay we have recently provided evidence supporting a critical role of autocrine factors in mediating hormonally stimulated growth [6–9]. We have also shown that the same experimental system is suitable to grow primary human breast cancers and to test their hormone-responsiveness [10, 11]. Thus, we felt that the soft agar clonogenic assay would be a useful tool to test the species-specificity of hormonally regulated growth factors with regard to their ability to stimulate mammary tumor colony formation. To address this issue, we evaluated the colony-stimulating effect of conditioned media obtained from rat mammary tumors exposed to estradiol using human breast cancers grown in soft agar in the absence of serum. The effect of exogenous administration of estradiol was also simultaneously tested in the same human breast cancer specimens. By comparing in the same tumors the colony-stimulating effect of estrogen-conditioned media with that of estradiol itself, we attempted to gain insight into the contribution of autocrine/paracrine factors to the overall growth-promoting effect of estradiol in our experimental system. Finally, the effects of these treatments on colony formation was correlated with the estrogen and progesterone receptor status of the tumor.

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MATERIALS AND METHODS

Materials

Twenty-two human breast cancer specimens were obtained at the time of biopsy or mastectomy. Twenty were infiltrating ductal carcinomas, one was an invasive lobular carcinoma (Table 1, patient 1) and one was a medullary carcinoma (Table 1, patient 5). The mean age of the patients was 57 years (range: 27–86 years). Fifteen women were postmenopausal while seven were premenopausal. Estrogen receptor (ER) and progesterone receptor

Table 1. Summary of the treatment effects in the 10 human breast cancers, the growth of which in soft agar was significantly stimulated by rat E_2-CM

Patient No.	Age (years)	Menopausal status	ER (fmol/mg)	PgR (fmol/mg)	Colonies* Control	E_2-CM No.	E_2-CM % of control	C-CM No.	C-CM % of control	E_2 (10^{-9} M) No.	E_2 (10^{-9} M) % of control
1	68	Post	37	108	27 ± 0.7	45 ± 0.4	167	28 ± 0.7	104	58 ± 2.5	215
2	52	Post	42	91	59 ± 1.2	74 ± 0.7	125	59 ± 1.0	100	72 ± 2.1	122
3	60	Post	157	22	32 ± 1.2	40 ± 1.2	125	32 ± 1.2	100	39 ± 0.7	122
4	74	Post	23	73	193 ± 2.4	244 ± 5.9	126	211 ± 8.1	109	239 ± 5.2	124
5	30	Pre	0	0	88 ± 2.0	127 ± 5.1	144	80 ± 5.9	91	100 ± 5.2†	114
6	67	Post	421	57	14 ± 1.7	34 ± 1.4	243	26 ± 0.5‡	186	20 ± 0.7	143
7	67	Post	0	0	45 ± 2.9	80 ± 2.5	178	64 ± 2.0	142	89 ± 1.5	198
8	31	Pre	12	29	63 ± 1.5	97 ± 1.3	154	76 ± 3.6	121	95 ± 4.2	151
9	75	Post	660	1210	118 ± 7.8	144 ± 5.0	122	159 ± 10.0§	135	267 ± 7.0	226
10	86	Post	413	79	16 ± 3.5	27 ± 1.4	169	26 ± 1.5§	163	20 ± 1.0†	125

*Data represent actual colony number (mean ± S.E.M. of three replicate dishes). In each of these human tumors rat E_2-CM significantly stimulated colony formation over control ($P < 0.05$, Newman–Keul test).
†P = NS vs. control. In the remaining eight tumors the colony-stimulating effect of E_2 was significant ($P < 0.05$ vs. control).
‡$P < 0.05$ vs. control and E_2-CM.
§$P < 0.05$ vs. control.

(PGR) measurements were performed in all tumors. Seventeen patients were ER+ PGR+, one patient was ER− PGR+, and four patients were ER− PGR−. The rat mammary tumors used to generate our conditioned media were obtained with a single injection of NMU (Sigma Chemical Co., St. Louis, MO) (5 mg/100 g body/wt) in female Sprague–Dawley rats as previously described [12].

Experimental design and methods

a. *Preparation of the rat mammary tumor conditioned media.* Single cell suspensions obtained from freshly excised NMU-induced mammary tumors were plated in soft agar in the presence or in the absence of E_2 (10^{-9}M) under serum-free media conditions. The details of our culture technique, which is a slight modification of the methods described by Hamburger and Salmon [13] and Von Hoff *et al.* [14] have been extensively reported in recent publications [15, 16]. Conditioned media from E_2-treated and control dishes were obtained by adding 2 ml of Hank's balanced salt solution/dish 2 days after plating and removing it 24 h later. This method has been found to be effective in collecting secretory products of cells plated in soft agar [14, 17, 18]. E_2-CM and C-CM were then concentrated on ice using a 3500 molecular weight cut-off dialysis membrane (Spectra-Por/6) with aquacide (sodium salt of carboxymethyl cellulose). The concentrated media were then treated with dextran-coated charcoal (0.25% charcoal and 0.0025% DEAE-dextran) and then kept frozen at −70°C until used in the experiments. Since the stability with time of the colony-stimulating effect of the conditioned media in our system is not known, a new set of media was prepared on a weekly basis. Thus, a recently prepared set was always available whenever we received a human breast cancer specimen. In addition to collecting the conditioned media we verified, in each case, that E_2 had exerted its known colony-stimulating effect in this system as we have previously reported [19]. Each set of conditioned media was routinely checked for the contaminating presence of estradiol measured by RIA [20]. In each case E_2 levels were found to be less than 10 pg/ml. Since the samples were at least 10 times more concentrated than their final concentrations in our experiments described below, the amount of contaminating E_2 was actually less than 1 pg/ml. Thus, we can reasonably exclude any contribution from contaminating E_2 to the observed effects of the rat conditioned media on human mammary cancer colony formation. Estradiol (Sigma Chemical Co., St. Louis, MO) was initially dissolved in 100% ethanol and then added to both layers of the medium at the desired concentration.

b. *Evaluation of the effects of the rat mammary tumor conditioned media on human breast cancer growth in soft agar.* Each human tumor was plated in triplicate 35 mm Petri dishes in the absence of serum under the following experimental conditions: (1) control, no treatment, (2) rat E_2-CM, (3) rat C-CM and (4) E_2 10^{-9} M. Between 100,000 and 500,000 cells/dish were plated depending on the size and the cellularity of the specimens received. Obviously, each tumor was plated at the same cell density under the different experimental conditions. Immediately after plating, the dishes were inspected to exclude the presence of clumps which could erroneously be

scored as colonies formed during the incubation time. We have previously consistently demonstrated the feasibility of growing experimental [16, 19] as well as human [10, 11] breast cancers under serum-free medium conditions in the soft agar clonogenic assay. The number of colonies (aggregates of more than 50 cells with a mean square diameter >85 μm) formed 6 days after plating was used to evaluate the effect of our treatments on colony formation. The validity of this parameter as an index of tumor growth has been supported by its correlation with incorporation of tritiated thymidine into DNA [21]. Simultaneously with each human tumor, an NMU rat mammary tumor was plated under identical culture conditions. This served as a positive control since we have observed that, in this system, both E_2 [19] and E_2-CM [7] consistently stimulate colony formation. An aliquot of the same human tumor sample used for plating was also assayed for ER and PGR. The measurement of these receptors was performed according to standard techniques routinely used in our laboratory [22, 23].

Statistical analysis

The treatment effects on colony formation were individually evaluated for each human tumor tested by analysis of variance using the Newman–Keul test [24].

RESULTS

Basal Growth

Eighteen of 22 (82%) primary human breast cancers successfully grew in soft agar under serum-free media conditions. The average number of colonies formed by these tumors was 62.4 ± 9.8 (S.E.M.) (range: 14–193). The four tumors that did not grow were infiltrating ductal carcinomas, removed from three postmenopausal and one premenopausal women. All were estrogen and progesterone receptor positive.

Overall effects of rat mammary tumor conditioned media and estradiol on human breast cancer growth in soft agar

E_2-CM significantly stimulated colony formation in 10 of 18 (56%) human breast cancers to 155 ± 11% (S.E.M.) of control (Table 1). Rat C-CM simultaneously tested, had no effect in five human tumors (Table 1, patients 1–5) while having a significant colony-stimulating effect in the remaining five (Table 1, patients 6–10). Such colony-stimulating effect was significantly less than that observed with rat E_2-CM in three tumors (patients 6–8) while it was similar in the other two (patients 9 and 10). The overall colony-stimulating effect of E_2 in these 10 tumors was virtually identical to that of rat E_2-CM (154 ± 13% of control). The effect of E_2 was statistically significant in all but two tumors (Table 1, patients 5 and 10). Compared to rat E_2-CM the colony-stimulating effect of E_2 was significantly higher ($P < 0.05$) in three tumors (Table 1, patients 1, 7 and 9) and significantly lower ($P < 0.05$) in two (Table 1, patients 5 and 6).

In eight of 18 tumors (44%) rat E_2-CM had no colony-stimulating effect (Table 2). Administration of E_2 had a remarkably similar lack of growth-promoting action. In only one of these eight tumors (Table 2, patient 6) E_2 had a modest, although significant, colony-stimulating effect.

Influence of the hormone receptor status on the treatment effects

Rat E_2-CM significantly stimulated colony formation in eight of 13 (62%) ER and PGR positive human tumors and in two of five (40%) receptor negative mammary cancers. The overall effects of the rat conditioned media and E_2 in the 13 receptor positive and five receptor negative tumors is illustrated in Fig. 1. It can be seen that the colony-stimulating effects of the treatments used were similar in the receptor positive and negative tumors.

Table 2. Summary of the treatment effects in the eight human breast cancers, the growth of which in soft agar was not significantly stimulated by rat E_2-CM

Patient No.	Age (years)	Menopausal status	ER (fmol/mg)	PgR (fmol/mg)	Colonies* Control	E_2-CM No.	E_2-CM % of control	C-CM No.	C-CM % of control	E_2 (10^{-9} M) No.	E_2 (10^{-9} M) % of control
1	71	Post	95	77	59 ± 4.2	58 ± 1.4	98	53 ± 3.3	90	56 ± 2.0	95
2	40	Pre	0	22	28 ± 2.9	32 ± 2.2	114	28 ± 2.9	100	36 ± 3.3	129
3	60	Post	11	81	62 ± 3.1	70 ± 5.0	113	57 ± 3.1	92	65 ± 3.0	105
4	71	Post	787	273	33 ± 1.4	32 ± 1.7	97	31 ± 1.2	94	35 ± 3.4	106
5	28	Pre	24	24	60 ± 6.1	52 ± 1.7	87	47 ± 2.1	78	49 ± 0.8	82
6	35	Pre	0	0	88 ± 7.0	86 ± 7.0	98	75 ± 1.4	85	107 ± 2.8†	122
7	64	Post	39	18	83 ± 3.1	82 ± 2.1	99	78 ± 3.5	94	73 ±1.8	88
8	27	Pre	0	0	56 ± 2.2	55 ± 1.5	98	56 ± 2.9	100	57 ± 2.9	102

*Data are expressed as in Table 1.

†$P < 0.05$ vs. control and E_2-CM. In the remaining seven tumors E_2 had no significant effect on colony formation.

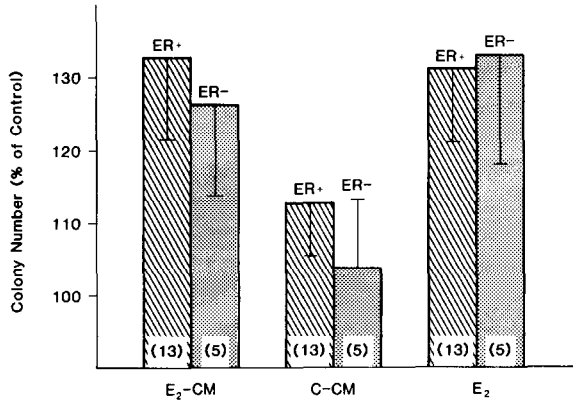

Fig. 1. Influence of the estrogen receptor status on the effects of the rat conditioned media and E_2 on human breast cancer growth in soft agar. Data represent means ± S.E.M. The number of human tumors tested is shown in parentheses. The 13 ER+ tumors were also PGR+. Of the five ER− tumors, four were also PGR− while one was low positive (22 fmol/mg cytosol protein).

DISCUSSION

Despite considerable evidence suggesting a major role of autocrine/paracrine factors as mediators of human and experimental breast cancer growth [1–5], the issue of species-specificity with regard to the biological activity of hormonally regulated growth factors has not been previously addressed. The availability of an *in vitro* culture system which allows the growth of both primary human and experimental mammary tumors under serum-free media conditions provided us with the opportunity to approach this question. In previous experiments we had shown that conditioned media obtained from NMU rat mammary tumors exposed to estradiol [7] and prolactin [8, 9] were able to stimulate the growth of this experimental mammary tumor model in the soft agar clonogenic assay. Those results provided support for the contention that autocrine/paracrine factors are important mediators of hormonally induced tumor growth in our system. The present experiments were designed to test whether conditioned media obtained from estradiol-exposed rat mammary tumors would be able to stimulate the growth of human breast cancers thus testing whether hormonally regulated growth factors are species-specific at least with regard to their colony-stimulating effect in soft agar. Our data provide evidence for a lack of species-specificity in this regard since a significant fraction of human breast cancers (56%) was stimulated to grow in soft agar by rat E_2-CM. It is conceivable that in this as well as our previous reports [7–9], we may actually have underestimated the biologic activity of the conditioned media since treatment of the media with dextran-coated charcoal may have partially removed the growth factors. On the other hand, such treatment is necessary in order to remove the contaminating presence of even small amounts of estradiol which would confound the interpretation of our results. Our biologic data are in agreement with the available information on the biochemical characterization of growth factors produced by experimental and human breast cancers. Zwiebel *et al.* [1] detected high molecular weight TGFα-like growth factors in acid ethanol extracts of NMU-induced rat mammary tumors. These authors found similar growth factors in tumor extracts and conditioned media obtained from another hormone-responsive experimental breast cancer, i.e. the 7,12-dimethyl-benza(a)anthracene-induced rat mammary tumor [1]. Recently, high molecular weight TGFα-like growth factors have been found to be secreted by a variety of human breast cancer cell lines [25]. Furthermore, in the hormone-responsive cell lines TGFα-like activity has been found to be highly estrogen inducible [25]. Taken together, these data indicate that hormonally regulated growth factors, potentially involved in the growth of hormone-responsive human and experimental mammary tumors, may be similar in nature.

The correlation between the colony-stimulating effect, or lack of it, of E_2-CM and E_2 observed in our experiments (Tables 1 and 2) deserves some comment. Although autocrine/paracrine factors are considered to be important mediators of estradiol effects on breast cancer cell proliferation, the relative contribution of these 'distal messages' to the overall estrogen effect is not fully established. Our experiments testing simultaneously the colony-stimulating effects of E_2 and E_2-CM partially address this issue. The similarity of the growth-promoting action of these two treatments indicate that, in our experimental system, autocrine/paracrine factors play a predominant role in estradiol-stimulated growth of hormone-responsive breast cancer. If E_2, in fact, would promote tumor growth predominantly through alternative mechanisms, one would anticipate that a significant fraction of tumors not stimulated by E_2-CM (i.e. the E_2-regulated growth factors) would be, instead, stimulated to grow by E_2 itself. However, this was not observed in our experiments as shown in Table 2, where E_2 essentially failed to stimulate colony formation in tumors that were unresponsive to E_2-CM. Our data also do not support the concept that hormone-independent breast cancer cells are stimulated by growth factors produced by hormone-responsive cells under estradiol influence. If this were the case, one would anticipate that a significant fraction of tumors that are insensitive to E_2 would be stimulated instead by E_2-CM (i.e. estradiol-regulated growth factors). This, however, again was not observed since tumors which were insensitive to E_2 administration were, in general, also similarly insensitive to E_2-CM.

Finally, we observed that the colony-stimulating effects of the E_2-CM and E_2 were not influenced by

the hormone receptor status of the tumor. This finding is in agreement with our previous observation that a significant fraction of estrogen receptor negative human breast cancers are stimulated to grow in soft agar by the addition of estradiol [10] and also prolactin [11]. Thus, it appears that tumors defined as receptor negative on the basis of the biochemical assay probably contain clones of hormone-responsive cells as recently indicated by assays that employ immunohistochemical methods [26]. Our findings, in this regard, are also in agreement with the recent observation that administration of estrogens to women with breast cancer as a means of synchronizing tumor growth has been found to stimulate DNA synthesis both in receptor positive and receptor negative tumors [27, 28].

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