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ESTROGEN AND PROGESTERONE RECEPTORS IN MAMMARY TUMORS INDUCED IN RATS BY SIMULTANEOUS ADMINISTRATION OF 17β-ESTRADIOL AND PROGESTERONE

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SUMMARY

Mammary tumors were promoted in male rats of the Wistar WAG strain by continuous and simultaneous administration of 17β-estradiol and progesterone. Tumor induction and growth were dependent on estradiol and on progesterone. Their histological features were comparable with those of human breast cancers. Hormone receptors were present in tumor cells. Estradiol receptor was found in 95% of them, at a higher level in nuclei than in cytosol. Progesterone receptor was present in 75% of tumors. In all cases, the level of androgen receptor was low.

INTRODUCTION

In 1961, Folca *et al.* observed that the uptake of tritiated hexestrol by neoplastic mammary tissue was higher in women who favourably responded to adrenalectomy than in others [1]. On the basis of this observation, estimations of the estradiol receptor (ER) in human mammary carcinomas was started in 1970–71 by Korenman[2] and Jensen[3]. Several works were then devoted to the problems raised by the presence of ER and of progesterone receptor (PgR) in breast cancers (for review see Refs [4, 5, 6]) and the present time it seems obvious that most of them are dependent on hormones.

This fact led several laboratories to study the hormone-dependence of experimental mammary tumours induced in animals by carcinogenic chemicals such as *N*-nitrosomethylurea [7] or urethan [8, 9], or dimethylbenz(a)anthracene [10–18]. Mammary tumors induced by these chemical agents did not necessarily offer the same histological and biological features as human cancers. Thus, it was of interest to search for a biological model as close as possible to human mammary tumors.

In 1961, Riviere *et al.* succeeded in inducing mammary tumors in rats by simultaneous administration of diethylstilbestrol dipropionate and progesterone [19, 20]. Identical results could be obtained in golden hamsters by prolonged administration of the association estrogen-progesterone [21, 22]. These tumors were attractive because they were induced by sexual hormones, and because their histological appearance was similar to that of human mammary cancers. However, at that time the presence of hormonal receptors in these tumors was not investigated.

The purpose of the present work was to demonstrate that mammary tumors induced in rats by simultaneous administration of estradiol and progesterone, were dependent on hormones.

MATERIALS AND METHODS

Chemicals

[6,7-^{3}H]-Estradiol (SA 60 Ci/mmol) was purchased from the Radiochemical Centre (Amersham). [17α-methyl-^{3}H]-R5020(Promegestone, SA 87 Ci/mmol), [17α-methyl-^{3}H]-R1881 (Methyltrienolone, SA 87 Ci/mmol), and the corresponding unlabeled compounds were from New England Nuclear Chemicals. Diethylstilbestrol and Bacitracin were from Sigma. Other reagents were of analytical grade.

Animals

90-Day old male rats of the Wistar WAG strain were used. On day 1 they received one pellet of estradiol (20 mg) and one of progesterone (100 mg) grafted in the dorsal region. They were housed at 22°C, and received food and water *ad libitum*. Eleven or twelve months later they were killed by decapitation and mammary tumors were dissected, frozen in liquid nitrogen and kept at −80°C until use (never more than 1 week). The remaining parts of pellets were removed and weighted.

Experimental procedures

Frozen tumors were first pulverized at liquid nitrogen temperature, then homogenized in 5 mM phos-

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phate buffer pH 7.2 containing 320 mM sucrose, 1 mM dithiothreitol, 1 mM Mg chloride and 0.3 mM bacitracine. Homogenates were centrifuged at 800 *g* for 10 min and supernatants at 105,000 *g* for 1 h. All present and subsequent operations were carried out at 4°C.

The 800 *g* pellets were washed once with homogenizing buffer containing 1% (v/v) Triton X100 then three times with buffer alone. Washed nuclei were recovered by centrifugation at 800 *g*. They were suspended in 5 mM phosphate buffer pH 7.4 containing 1 mM dithiothreitol and 0.3 mM bacitracine. An equal volume of 2 M NaCl solution was then added and nuclei were extracted at 0°C for 1 h. The ionic strength was then lowered to 0.4 M by addition of buffer and the medium was centrifuged at 15,000 rev./min for 15 min. Supernatants were considered as nuclear extracts.

Estradiol-receptor assay

Cytosols and nuclear extracts were used for the estimation of ER by the technique of hormone-exchange. Aliquots (0.3 ml) were incubated in the presence of tritiated estradiol (10–15 nM) alone or with a 100-fold excess of unlabeled DES at 0°C for 2 h. They were then incubated either at 0°C or at 25°C for 2 additional h. 0.6 ml of DCC suspension (0.5% activated charcoal, 0.05% Dextran, 0.1% gelatin in phosphate buffer) were added, samples were kept at 0°C for 10 min. and centrifuged at 5,000 rev./min for 10 min. Radioactivity was counted in supernatants.

Progesterone receptor assay

PgR was estimated in cytosols containing 10% (v/v) of glycerol, using tritiated R5020 as ligand. Aliquots (0.3 ml) were incubated in the presence of increasing amounts of labeled R 5020 (10^{-10} M–10^{-8} M) with or without a 500-fold excess of unlabeled ligand. A slight excess (10-fold) of radioinert 5α-DHT was added to all samples for the saturation of androgen binding sites [23]. Samples were incubated at 0°C for 4 h, then bound fraction was isolated by DCC treatment.

Androgen receptor assay

Cytosol samples (0.3 ml) were incubated in the presence of increasing amounts of tritiated R 1881 with or without a 100-fold-excess of unlabeled ligand. Radioinert triamcinolone acetonide was added to all samples to avoid binding of R 1881 to PgR [23]. Incubations were carried out at 0°C for 20 h and bound radioactivity was isolated by DCC treatment.

Other procedures

Radioactivity was estimated by liquid scintillation counting using a Beckman spectrometer (LS 8000).

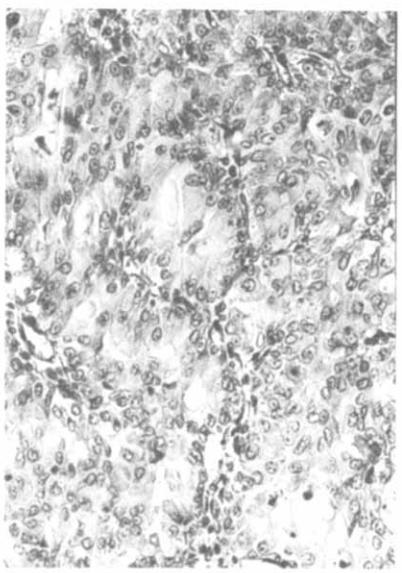
Proteins were measured by the method of Lowry *et al.* using BSA as standard [24].

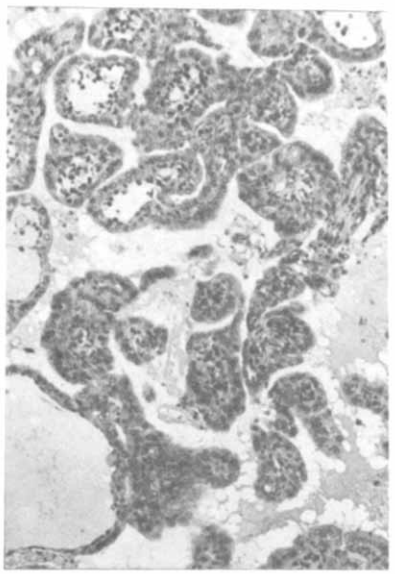
Numerical data from saturation analysis were plotted according to Scatchard[25].

RESULTS

Mammary tumors

Mammary tumors appeared during the 6th or 7th month following the graft of hormonal pellets. Their growth became faster during the next three months so that their diameter was at least 1.5 cm when rats were sacrificed. All the animals presented one if not two or three tumors. They developed generally in the middle part of the mammary line (levels 4 and 5) and their macroscopic aspect was either glandular and vascular or fibrous.





Figs 1 and 2. Two microscopic aspects of rat mammary carcinoma induced by simultaneous administration of estradiol and progesterone. H and E; × 250 and × 90.

At the light microscopic level, two types of tumors could be described:

(1) Dense and oedematous tumors populated with abnormal glandular cells. Cells were characterized by slightly basophilic cytoplasm, nuclei containing one nucleous and moderate anisokaryosis. In the epithelial bulk, pseudoacini and pseudopapillar formations were outlined, surrounded with an eosinophilic matter corresponding to oedema (Fig. 1).

(2) In a few cases, fibrous tumors with intraluminal proliferation of abnormal cells into galactophoretic ducts, and large areas of fibrous connective tissue. Terminal ducts were concerned with these proliferations, they became gigantic with large fissures and complicated splits (Fig. 2).

Thus, experimental tumors induced by natural hormones presented histological features similar to human mammary cancers.

Estradiol and progesterone uptake

At the time of sacrifice, the remaining part of pellets were removed and weighted in order to have a notion of the amount of both hormones resorbed during tumors growth. Resorption was different from one animal to another but could be evaluated on an average of 8–9 mg for estradiol and 69 mg for progesterone per rat and per one year. Thus, it appeared that a 8-fold higher amount of progesterone than of estradiol was used during tumor growth so that the mean E_2/Pg ratio during that process was 0.12. Moreover, it could be observed that the resorption of the progesterone pellet was much more regular from one rat to another than that of estradiol.

Estradiol receptor and progesterone receptor in tumor cells

Numerical data concerning ER and PgR are shown in Table 1.

Estradiol receptor Cytosol and nuclear ER were measured in 20 tumors using the technique of hormone exchange at 25°C. In cytosol, ER was found in 65% of tumors, but three values should be considered as borderlines according to standards generally established for human breast cancers ($n < 10$ fmol mg proteins). With the exception of two cases (tumors No. 16 and 17), ER levels were relatively low. In nuclei, the presence of ER was more constant than in cytosol. It was found in 16 tumors, and only one value was border line.

Progesterone receptor. PgR was found at relatively high level in 15 tumors. In one case (tumor No. 20), it was present in the absence of cytosol and nuclear ER. Dissociation constants (K_D) were similar to those found for PgR in rat uteri or human breast carcinoma (0.8–2 nM).

Androgen receptor. The androgen receptor was estimated in a restricted number of tumors. It was found in all cases, at a level generally low and constant from one to another tumor ($n = 10$ fmol/mg proteins).

DISCUSSION AND CONCLUSION

During the past years, several works were devoted to the study of hormone-dependence of breast cancers and of accurate markers of their dependence in so far as post surgical therapy was concerned [4–6, 26]. The presence of ER in tumor cells was first considered as

Table 1. Estradiol receptor in cytosol and nuclei, and progesterone receptor in cytosol from mammary tumors induced in rats by estradiol and progesterone administration (results are expressed as fmol of hormone bound per mg of proteins)

	Cytosol			Nuclei			PgR
Tumor no.	Total binding sites	Unoccupied	Occupied	Total binding sites	Unoccupied	Occupied	Total binding sites
1	23	13	10	59	23	36	96
2	23	6	17	3	0	3	93
3	6	0	6	25	0	25	56
4	10	10	0	27	18	9	22
5	26	0	26	20	5	15	10
6	7	0	7	13	2	11	5
7	13	0	13	13	7	6	157
8	23	17	6	27	0	27	29
9	19	2	17	35	35	0	291
10	7	7	0	12	12	0	161
11	0	0	0	26	7	19	121
12	0	0	0	57	45	12	81
13	0	0	0	24	0	24	91
14	0	0	0	16	6	10	205
15	0	0	0	14	0	14	99
16	186	0	186	0	0	0	0
17	83	83	0	0	0	0	0
18	0	0	0	37	31	6	0
19	10	4	6	39	9	30	0
20	0	0	0	0	0	0	58

sign of dependence, then it appeared that the simultaneous presence of PgR, considered as eventual marker of estrogen action, led to more accurate prediction of hormone-dependence [33].

For experiments related to problems of hormone-dependence, it seemed quite interesting to dispose of animals with mammary tumors as similar as possible to human tumors and possibly induced by and dependent on hormones, according to criteria of human pathology.

Mammary tumors at our disposal seemed attractive in some respects: (1) They were induced in rats of the Wistar WAG strain which did not develop spontaneous mammary cancers contrary to some other rat strains or other animal species. (2) Tumors were induced by simultaneous administration to animals of estradiol and progesterone by means of hormonal pellets grafted in the dorsal region. Under these conditions mammary tumors appeared in all animals after 7 months while cellular abnormalities could be observed under microscope after 3 months. Estradiol alone was able to induce mammary tumors but the time allowed for induction was longer and results was not so patent since some animals did not develop any tumor. (3) Suppression of hormone pellets resulted in the gradual regression and disappearance of tumors, however, growth could be promoted again by estradiol administration. (4) Tumor transplantation from one to another rat was successful when grafted animals were treated with estradiol. (5) The presence of viruses in tumor cells was not substantiated by electron microscopy examination.

Thus it appeared that tumors were dependent on both estradiol and progesterone for induction. Then, growth of primitive or of transplanted tumors was essentially dependent on estradiol, and to a lesser extent on progesterone which acted as an accelerating factor.

According to criteria outlined in human mammary pathology the hormone dependence of 20 experimental tumors was investigated by estimation of ER and PgR. Estradiol receptor was found in 19 tumors either in cytosol and nuclei, or in one or other of these subcellular components. Whether in cytosol or in nuclei a large part (61–62%) of binding sites were occupied by endogenous hormone. Generally, the level of ER was higher in nuclei than in cytosol giving evidence of correct translocation and nuclear retention of ER. When two tumors from the same animal were examined, ER was found in both tumors but their levels were somewhat different. This fact correlated with different stages of histological evolution. Progesterone receptor was found in 15 tumors. This percentage of PgR positive tumors was quite higher than that usually reported concerning human breast cancers. In so far as PgR could be considered as eventual marker of estrogen action it could be concluded that estrogen action was efficient. On the other hand PgR probably accounted for the disappearance of ER from cytosols of some tumors since it was demonstrated that PgR was able to inhibit either synthesis or replenishment of ER [27–32].

Contingent-to further investigations it seems that such mammary tumors induced by and dependent on sexual hormones could be used for the study of biological events concerning the role of hormones in tumorogenesis and neoplastic processes.

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