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PRELIMINARY NOTES

ESTROGEN PRODUCTION BY AN ESTABLISHED CELL LINE FROM PULMONARY SMALL CELL ANAPLASTIC CARCINOMA

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SUMMARY

Radioimmunoassays demonstrated the presence of estradiol-17β in growth medium from DMS-44, a continuous cell line obtained from pulmonary small cell anaplastic carcinoma; other steroids such as testosterone and cortisol were not present in measurable amounts. To study further the ability of DMS-44 cells to produce estrogens, [^{3}H]-testosterone or [^{3}H]-androstenedione was incubated with cells for 24 h. [^{3}H]-estrone and [^{3}H]-estradiol-17β were isolated from the incubation medium by extraction, partition and thin layer chromatography. The two estrogens were identified by chromatography after addition of authentic [^{14}C]-estrone to the fractions presumed to contain [^{3}H]-estrone and authentic [^{14}C]-estradiol-17β to that of [^{3}H]-estradiol. Estradiol-17β was favored over estrone as the main estrogen formed both from testosterone and androstenedione. The estradiol-17β fraction was repeatedly chromatographed in several thin layer chromatographic systems, reaching constant Tritium: Carbon-14 ratio. These findings, together with the radioimmunoassay data, are considered conclusive evidence for the formation of estradiol-17β by small cell carcinoma cell lines *in vitro*.

Ectopic production of corticotropin and other peptide hormones by non-endocrine tumor tissues such as small cell anaplastic carcinomas has been well established [1]. The possibility that estrogens or other steroids could also be produced by such tumor tissues has been raised, but not seriously considered [2].

In this report, we will present our findings that an established small cell anaplastic tumor cell line can indeed produce estrogens in addition to peptide hormones [3].

In our laboratory, continuous tumor cell lines from primary or metastatic small cell anaplastic carcinoma of the lung have been established *in vitro* [3, 4]. In initial studies where the growth media from a number of such cell lines were screened at our request by an outside laboratory, significant amounts of estradiol-17β were found in addition to a variety of peptide hormones [5]. The presence of estradiol-17β in duplicate aliquots of the medium of one cell line, DMS-44, has been confirmed in our laboratory using a radioimmunoassay based on the method by Mikhail *et al.*[6] *with* [^{3}H]-estradiol-17β (Table 1). Radioimmunoassays for cortisol [7] and testosterone [8] failed to demonstrate the production of either of these hormones by cultured cells. We could not demonstrate estradiol by radioimmunoassay in the growth medium of a fetal lung cell line (WI-38, Microbiological Associates, Walkersville, Maryland), and since the incubated medium alone did not contain any estradiol, it seems that estradiol-17β is synthesized by small cell carcinoma cells *in vitro*. In the following we will present direct evidence that DMS-44 cells synthesize estrogens. The continuous cell line, DMS-44, was established from a male patient with small cell anaplastic carcinoma of the lung [3].

For estrogen biosynthesis to take place, DMS-44 cells would require the enzymes responsible for the aromatization of the A-ring of the steroid molecule [9]. To demonstrate this pathway, experiments were carried out with approximately 1 μCi of [^{3}H]-testosterone or [^{3}H]-androstenedione (all steroids were obtained from New England Nuclear, Boston, Massachusetts, and freshly purified by chromatography) incubated for 24 h with cell cultures, otherwise under the same conditions as in Table 1. Following the incubations, the steroids were extracted from the incubation medium and processed according to current techniques in our laboratory [10, 11]. The extracts were chromatographed in the thin layer systems described in Table 2, that allow simultaneous separation of estrone, estradiol-17β and estriol as well as testosterone and androstenedione. Unlabeled estrogens were added to the extracts and the steroid spots were located by ultraviolet light scanning. The steroids were eluted following tentative identification, so that fractions representing testosterone, androstenedione, estrone and estradiol-17β could be counted for radioactivity. The radioactivity incorporated into the estrogens clearly showed that both testosterone and androstenedione favored the formation of estradiol-17β over estrone; between 75 and 83% of the radioactivity found in the combined estrogen fraction from both precursors, was incorporated into estradiol-17β, indicating active aromatizing enzymes as well as 17β-hydroxy-steroid dehydrogenase in these cultures. To the fraction representing estradiol-17β was added [^{14}C]-estradiol-17β, and it was analyzed by isotope ratios following acetylation and chromatography as described in Table 2 [12, 13].

Table 1. Estradiol content in medium from small cell anaplastic carcinoma culture cells

Sample*	Estradiol-17β (pg/ml)
DMS 44 (10^6 cells/ml) RPMI 1640/20% FCS (incubated medium control)	337 ± 13†
	4‡

* Medium samples were collected from cultures of the small cell tumor cell line, after 4 days incubation in 10 ml RPMI 1640 with 20% heat inactivated fetal calf serum, at 37°C in 5% CO_2 and air. † Mean of 4 determinations with S.D. Radioimmunoassay reagents from Wien Laboratories, Inc., Succasunna, New Jersey. ‡ Indistinguishable from zero.

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Table 2. Radiochemical homogeneity of estradiol-17β formed in small cell tumor cultures*

Step No.†	Steroid derivative	Thin layer chromatography systems	Total ^3H (c.p.m.)	$^3H/^{14}C$
1.	Free estradiol-17β	Methylene chloride: Acetone, 95:5	36,014	10.49
2.	Estradiol-17β diacetate	Chloroform 100%	28,170	10.02
3.	Estradiol-17β diacetate	Methylene chloride: ether (saturated)‡ 95:5	11,664	2.0
4.	Estradiol-17β diacetate	Chloroform 100%	9,919	2.15
5.	Estradiol-17β diacetate	Methylene chloride: ether (saturated) 95:5	8,078	2.27
6.	Estradiol-17β diacetate	Chloroform 100%	5,319	2.30

* Incubated in 5 ml of medium (see Table 1), about 1.2×10^6 cells per ml. The precursor was [1,2,6,7,-^{3}H]-testosterone with a specific activity of 250 μCi per 0.85 μg. For identification was used [4-^{14}C]-estradiol-17β, specific activity 10 μCi per 49 μg in an amount of about 1/20 to 1/10 of the activity originally present in the [^{3}H]-estrogen fraction. † Numbered from the last chromatogram prior to acetylation. ‡ A saturated system uses a lining of filter paper on all sides of the chromatography tank. Before use the tank is equilibrated with the solvents for 3 h.

During these procedures, after an initial change due to purification and removal of components of somewhat similar polarity, the fraction reached a constant isotope ratio ($^3H/^{14}C$) providing conclusive evidence that the steroid formed by the small cell tumor cultures was indeed estradiol-17β, i.e. identical to [^{14}C]-estradiol-17β. Table 2 represents an incubation where estradiol-17β was formed from [^{3}H]-testosterone. The amount of radioactivity from the labeled precursor incorporated into the finally purified estradiol-17β was 3.2%. Similar evidence was obtained for the formation of estrone, as well as estrone and estradiol-17β from [^{3}H]-androstenedione. In other experiments, estrogen methyl ethers were formed as derivatives during purification and t.l.c., and the estrogens formed from the cell cultures were found to be identical to authentic estrone and estradiol-17β, based solely on their chromatographic mobilities.

To test to what degree estrogen biosynthesis might be due to the medium and to possible normal cells in the cultures, testosterone and androstenedione were incubated in normal male skin fibroblast cultures (El-San, American Type Culture Collection, Rockville, Maryland) and in medium alone to serve as negative controls, and in a human choriocarcinoma cell line, BeWo [14], as a positive control. Both incubations were carried out with approx. the same numbers of cells per ml. as those used above. The human choriocarcinoma cell line, BeWo, is composed of malignant trophoblastic cells, capable of synthesizing and secreting human chorionic gonadotropin, human placental lactogen and steroid hormones such as estrogens and progesterone [15]. In incubations identical to the DMS-44 incubations with [^{3}H] labeled androgens, medium alone and the fibroblast cultures showed no significant conversion to estrone and estradiol-17β, and essentially all the radioactivity was recovered in the androgen precursor. The BeWo cells converted virtually all the androgen to estradiol-17β. These control studies showed that (a) the conditions of the incubations were favorable to the enzymatic reactions required for steroid aromatization; (b) the aromatization observed in DMS-44 cultures appeared to be specifically related to the lung tumor cells.

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