Changes in ovary after clomiphene citrate administration (Mean ± SD)

Time of sacrifice (month)	Mean wt of ovary/100 g b.wt (mg)			Percentage of nature oocytes			Diameter of nature oocytes			Percentage of atretic oocytes		
	Control	25 µg clomid	50 µg clomid	Control	25 µg clomid	50 µg clomid	Control	25 µg clomid	50 µg clomid	Control	25 µg clomid	50 µg clomid
15 days (February)	625±205	575 ±175	345 ±141	8.5	17*	30*	225±35	325 ±56	355 ±29	4	5*	6.5**
30 days (February)	650±225	1675 ±345	1595 ±150	15	29*	45*	226±42	495 ±52	475 ±35	5	7*	5**
45 days (March)	1155±175	2625*±305	3695**±225	17	39*	60*	322±58	575*±45	625**±72	3	7.5*	7.2**
60 days (March)	1485±275	2048*±125	5725**±250	16	55*	75*	370±52	625*±41	905**±86	6	6.0*	9.5**
75 days (April)	2095±315	3275*±276	8925**±275	13	85*	105**	395±39	972*±32	1025**±75	4	5*	12.5**
90 days (April)	2548±325	4525*±700	9845**±310	20	98*	125**	325±21	1195*±85	1315**±55	5	7.5*	14.5**

* $p<0.05$ Significant increase c.f. corresponding controls. ** $p<0.05$ Significant increase c.f. corresponding controls and 25 µg administrations.

'gravid' fishes, while in this study it is shown that Clomid can be effectively used during the regressed or 'off-season' and the fishes can be made to spawn about 4 months in advance of their normal breeding period (table).

1 The authors wish to express their gratitude to Richardson Merrel Co. for supplying the sample of clomiphene citrate, and Dr K.N. Udupa, Institute of Medical Sciences, Banaras Hindu University, Varanasi for facilities.

2 S. Roy, V.B. Mahesh and R.B. Greenblatt, Acta endocr. *47*, 669 (1964).
3 A.P. Labhsetwar, J. Endocr. *47*, 481 (1970).
4 S. Roy, R.B. Greenblatt and V.B. Mahesh, Fert. Steril. *15*, 310 (1964).
5 S. Roy, R.B. Greenblatt, V.B. Mahesh and E.C. Tungok, Fert. Steril. *14*, 575 (1963).
6 N. Kase, A. Mrouch and L.E. Olson, Am. J. Obstet. Gynec. *98*, 1037 (1967).
7 R.W. Kristner, Am. J. Obs. Gyn. *92*, 380 (1965).
8 M.J. Whitelaw, L.R. Grams and W.J. Stamm, Am. J. Obstet. Gynec. *90*, 355 (1964).
9 O.W. Smith, G.W. Smith and R.W. Kistner, J. Am. med. Ass. *184*, 878 (1964).
10 G.M. Riley and T.N. Evans, Am. J. Obstet. Gynec. *89*, 97 (1964).
11 D. Charles, T. Klein, S.F. Lunn and J.A. Loraine, J. Obstet. Gynec. Br. Commonw. *76*, 1100 (1969).
12 S. Pandey and W.S. Hoar, Can. J. Zool. *50*, 1679 (1972).
13 D. Khosa, Thesis, Banaras Hindu University 1970.

Radioautographic demonstration of dihydrotestosterone receptor in cultured human fibroblasts

H. Ozasa, T. Tominaga, T. Nishimura and T. Takeda[1,2]

Department of Obstetrics and Gynecology, Faculty of Medicine, Kyoto University, and Department of Pathology, Chest Disease Research Institute, Kyoto University, Sakyo-ku, Kyoto 606 (Japan), 11 December 1979

Summary. Radioautographically, we obtained direct evidence for the localization of ^{3}H-dihydrotestosterone binding sites in cultured human fibroblasts derived from vulvar skin.

Evidence from biochemical studies indicates the presence of dihydrotestosterone (DHT) receptors in cultured fibroblasts derived from human skin[3]. To our knowledge, DHT receptors in fibroblasts have not been morphologically demonstrated. Using a radioautographic method developed by Weiller et al.[4], we attempted to obtain direct evidence for the intracellular localization of DHT binding sites in cultured human fibroblasts derived from vulvar skin. In the cytosols of the cells the presence of 8 S receptors was also examined by sucrose gradient analysis done in parallel studies.

Materials and methods. 1,2,4,5,6,7-^{3}H dihydrotestosterone, 123 Ci/mmole was purchased from New England Nuclear. Non-radioactive steroids were from Sigma. Nuclear emulsion (Sakura NR-M11), developer (Konidol X), and fixative (Konifix) were obtained from Konishiroku, Co.

Fibroblasts were grown from vulvar skin taken at delivery from a normal gravid woman after obtaining her consent. Cultures were maintained in a nutrient medium consisting of Eagle's minimum essential medium supplemented with 50 µg/ml of ascorbic acid and 10% fetal calf serum. Fibroblasts had undergone 5–10 passages at the time of the experiments. For 48 h preceding experiments, fibroblasts were treated with a medium containing charcoal-treated serum in place of the regular serum. Charcoal-treated serum was prepared according to the method of Griffin et al.[5].

Sucrose gradient analysis. Fibroblasts grown to confluence were collected with a rubber policeman, suspended in 2–3 ml of cold TED buffer (10 mM Tris-HCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol), and homogenized in a glass-glass homogenizer. The cytosol fraction was obtained after centrifugation at 105,000 × g for 60 min and incubated with 5 nM ^{3}H-DHT with or without the addition of a 500-fold excess of unlabeled hormone for 2 h at 0–4 °C. The incubate was layered on to a linear 5–20% sucrose gradient

prepared in 5 ml nitrocellulose tubes. A small volume of the ^{14}C-marker protein (bovine γ-globulin) was also layered on the gradient. The gradients were centrifuged at 178,000 × g at 4 °C for 18 h. 2-drop fractions were collected by puncture of the bottom of the tube and counted in a liquid scientillator using Bray's solution[6]. Sedimentation coefficient was determined according to the method of Martin and Ames[7].

Radioautography. Fibroblasts were grown on 12 × 35 mm glass coverslips and used for experiments before they reached confluence. ^{3}H-DHT radioautography was carried out essentially in accordance with the method described by Weiller et al.[4].

Statistical significance. Student's t-test was used to estimate the statistical significance.

Results and discussion. Cytosol prepared from fibroblasts used in this study contained a small but readily measurable peak of high affinity ^{3}H-DHT binding that sediments at approximately 8 S (figure 1). This sedimentation profile was much the same as that already reported, thus supporting the presence of DHT receptors documented in biochemical studies[3,5].

An autoradiogram of these fibroblasts obtained after a 3-h incubation at 37 °C with 0.5 nM ^{3}H-DHT is presented in figure 2,a. While the labeling is distributed over both the cytoplasm and the nucleus, it is rather concentrated over the nuclei. The labeling over the cytoplasm unquestionably reflects cytoplasmic uptake of DHT, already documented in biochemical studies. Although layers of cytoplasm present over and below the nucleus should be taken into account, this nuclear distribution of the labeling, at least in part, reflects an actual nuclear localization of the radioactive hormone as inferred from the almost exclusive nuclear distribution of the labeling that occurs in some cells (figure 2,b). This nuclear distribution of the labeling, therefore, is considered to reflect the nuclear uptake of DHT.

The extremely low background in figure 2,a and b, and the absence of the labeling of the cells processed identically but incubated without hormone eliminate the possibility that the labeling of the cells is due to positive chemographical artefacts (figure 2,e).

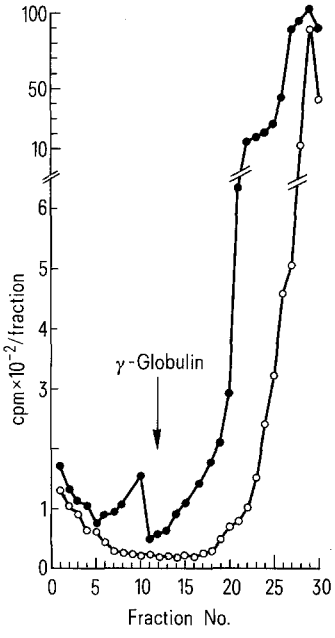

Fig. 1. Sedimentation analysis of ^{3}H-DHT binders by sucrose density gradient. Fibroblast cytosol was incubated with 5 nM ^{3}H-DHT with or without 500-fold unlabeled DHT for 2 h at 0–4 °C and subsequently layered on 5–20% sucrose gradients prepared in TED buffer. The gradients were centrifuged at 178,000 × g for 18 h and fractions were collected by puncturing the bottom of the tubes. ^{14}C-labeled bovine γ-globulin (7.0 S) was used as a sedimentation marker (indicated by arrow). ●——●, fibroblast cytosol incubated with 5 nM ^{3}H-DHT; ○——○, fibroblast cytosol incubated with 5 nM ^{3}H-DHT and 2.5 μM unlabeled DHT. Note the absence of peaks of bound radioactivity in an incubation containing excess unlabeled DHT.

The number of grains on fibroblasts (mean ± SE/cell of determinations in 50 cells)

Experimental group	No. of grains
Control	5.34 ± 0.52
^{3}H-DHT	15.86 ± 1.42
^{3}H-DHT/DHT	7.26 ± 0.80
^{3}H-DHT/DES	13.15 ± 1.35

The grains were counted for fibroblasts incubated without hormone (control), with ^{3}H-DHT (^{3}H-DHT), with ^{3}H-DHT plus excess DHT (^{3}H-DHT/DHT), with ^{3}H-DHT plus excess DES (^{3}H-DHT/DES). P values: $p < 0.001$ = control vs ^{3}H-DHT; control vs ^{3}H-DHT/DES; ^{3}H-DHT vs ^{3}H-DHT/DHT, $p < 0.05$ = control vs ^{3}H-DHT/DHT.

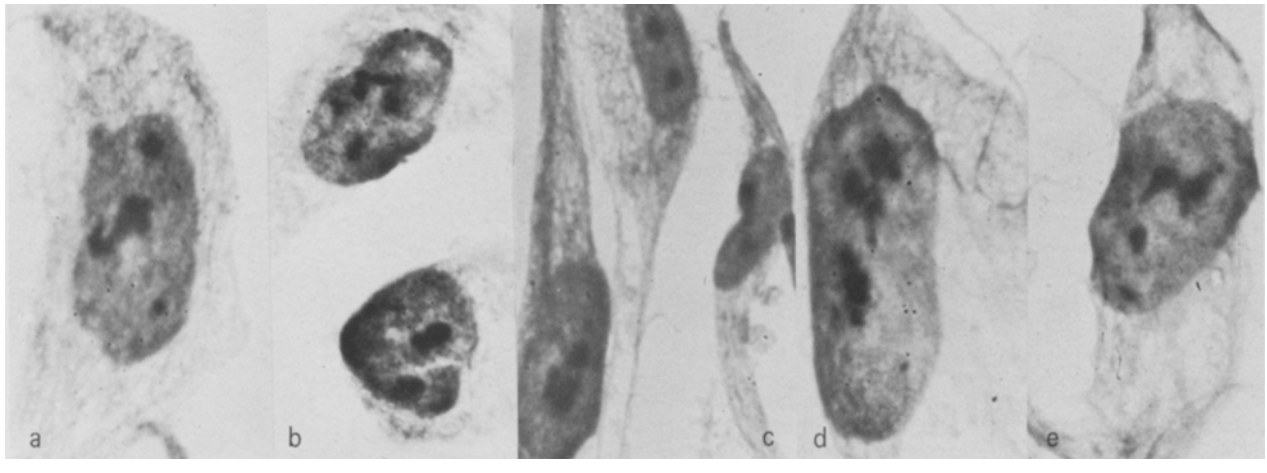

Fig. 2. Autoradiograms of fibroblasts incubated for 3 h at 37 °C with 0.5 nM ^{3}H-DHT alone (a,b) or with a 2000-fold excess of non-radioactive DHT (c) or DES (d). Control incubated in the same experimental conditions but without any radioactive compound (e). Exposure time: 180 days. Stained with hematoxylin-eosin. Magnification × 1400.

In order to assess hormone specificity for the labeling, the same 3-h incubation at 37 °C with 0.5 nM ^{3}H-DHT with the simultaneous presence of a 2000-fold excess of non-radioactive hormone, DHT, or diethylstilbestrol (DES), was carried out. As shown in figure 2,c, no appreciable labeling occurs in an incubation containing excess DHT. DES, on the contrary, elicits no change in the labeling of the cells, even at this excess (figure 2,d). These results indicate the DHT-specificity for the labeling.

The labeling of fibroblasts from each incubation was quantitated by counting the grains (table). The background as assessed from the labeling present on fibroblasts incubated without hormone was 5.34 ± 0.52 grains/cell (mean $\pm$ SE of determinations in 50 cells). Fibroblasts from an incubation containing ^{3}H-DHT had 15.86 ± 1.42 grains/cell (mean $\pm$ SE of determinations in 50 cells). Incubation of cells with excess DHT besides labeled hormone reduced the labeling to 7.26 ± 0.80 grains/cell (mean $\pm$ SE of determinations in 50 cells), a decrease by 81.7% from the value for an incubation containing labeled hormone alone when corrected for the background. On the contrary, the simultaneous presence of excess DES in an incubation gave the value of 13.15 ± 1.35 grains/cell (mean $\pm$ SE of determinations in 50 cells), showing no change in the labeling despite a 25.8% decrease in terms of the values corrected for the background. These data add further verification to the findings obtained from photographs.

This study provides direct evidence for a cytoplasmic and nuclear localization of DHT binding sites in cultured human fibroblasts, in the cytosol of which the existence of DHT receptors was also confirmed by sucrose gradient analysis carried out in parallel.

1 Acknowledgments. Gratitude is extended to Dr K. Yasuhira and Mr T. Matsushita for pertinent advice and technical assistance, respectively, and to M. Ohara for criticism of the manuscript.

2 Reprints request should be addressed to T. Takeda, Department of Pathology, Chest Disease Research Institute, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

3 B.S. Keenan, W.J. Meyer, III, A.J. Hadjian, H.W. Jones and C.J. Migeon, J. clin. Endocr. Metab. *38*, 1143 (1974).
4 S. Weiller, C. Le Goascogne and E.E. Baulieu, Exp. Cell Res. *102*, 43 (1976).
5 J.E. Griffin, K. Punyashthiti and J.D. Wilson, J. clin. Invest. *57*, 1342 (1976).
6 G.A. Bray, Analyt. Biochem. *1*, 279 (1960).
7 R.G. Martin and B.N. Ames, J. biol. Chem. *236*, 1372 (1961).

A histochemical study of leucine amino peptidase activity in the testes of immature and mature guinea-pigs

S. Chanda*, P.B. Patra, Ratna Chanda and C. Deb

Department of Physiology, University College of Science and Technology, 92, A.P.C. Road, Calcutta-700009 (India), 27 December 1979

Summary. The distribution of the enzyme leucine amino peptidase (LAP) has been studied histochemically in immature and mature guinea-pig testes. Immature guinea-pig testis showed a very feeble LAP activity, while in mature ones strong LAP activity was noted. The present communication indicates a direct relationship between the activity of the enzyme LAP and sexual maturation.

Leucine amino peptidase (LAP) activity has been demonstrated in several endocrine glands[1-3] including the testis of different vertebrates[4]. Further it has been reported that the activity of the enzyme LAP is enhanced during HCG-induced hyperactivity of Leydig cells, while estrogen-induced blockage of pituitary gonadotropins diminished the activity of this enzyme in guinea-pig testis[5]. The present investigation has been undertaken to correlate histochemically the activity of the enzyme LAP with testicular maturity in guinea-pig.

Materials and methods. 8 mature and 8 immature male guinea-pigs were taken. The animals were killed and the testes were dissected out. Pieces of testes were fixed in Bouin's fluid for histological studies.

Testicular pieces were also fixed in chilled buffered formol (10%) for 3-4 h for histochemical demonstration of the LAP activity. Frozen sections of prefixed tissues were cut at 20 µm in a cryostat at -20 °C, collected over pre-cleaned coverslips, and immersed in an appropriate medium for LAP activity according to the improved method of Nachlas et al.[6], using leucyl-4-methoxy-napthylamide as substrate. Parallel sections, incubated in substrate free medium, served as controls. Paraffin sections were cut at 6 µm and stained with PAS-haematoxylene for histological studies.

Results. PAS haematoxylene stained sections of testes showed the presence of numerous spermatozoa and prominent Leydig cells in the mature ones and their absence in the immature ones, which confirmed the maturity and immaturity of the respective testes. Histochemical studies revealed the presence of LAP activity in Leydig cells of both mature (figure 1) and immature (figure 2) guinea-pig testes, but the intensity of reaction of this enzyme was significantly higher in mature ones than in the immature ones.

Discussion. In the present investigation a marked rise in the LAP activity has been observed histochemically in Leydig

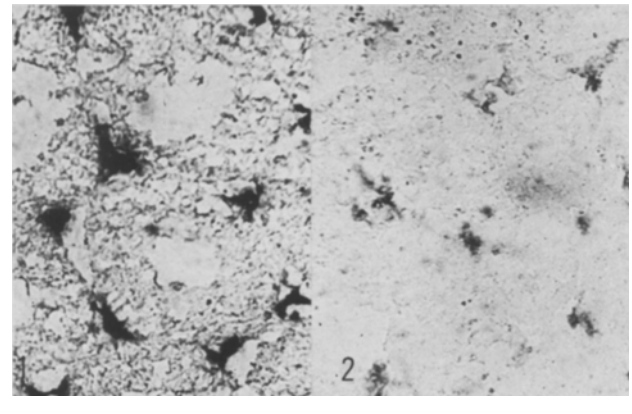

Fig. 1. Leucine amino peptidase in testis of mature guinea-pig. ×69.
Fig. 2. Leucine amino peptidase in testis of immature guinea-pig. ×69.