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MICROPHOTOMETRIC STUDY OF Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE IN THE OVARIES OF RATS FED ON HIGH FAT DIETS IN RELATION TO 7,12-DIMETHYLBENZ(A)-ANTHRACENE MAMMARY TUMORS

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Female Wistar-Imamichi rats, were given 7,12-dimethylbenz(a)anthracene (DMBA), and then three different high fat (HF) diets containing olive, safflower, and corn oil, respectively, for 120 days. Mammary tumors developed in 8 of 10 rats fed on a corn oil diet (C), 2 of 10 rats fed on an olive oil diet (O), and 2 of 10 rats fed on a safflower oil diet (S). In the tumor-bearing rats fed on O and S diets with DMBA treatment, the levels of the plasma progesterone were higher than those of control rats on the same diets without DMBA treatment. The levels of plasma progesterone of the tumor bearing rats fed on C diet were no difference compared with that of control.

The plasma 17β-estradiol levels in tumor-bearing rats on different diets were similar.

Changes of 3β-hydroxysteroid dehydrogenase (3β-HSD; EC 1.1.1.51) activity in the ovaries of mammary tumor bearing rats fed on HF diets were examined by the microspectrophotometric method. In mammary tumor-bearing rats of HF diets, the activity was higher than that of control rats in the corpus luteum and interstitial gland in the ovaries, but not in the thecal membrane. The activity tended to increase with diet in the order C, S, and O. In particularly, in the ovaries of the tumor-bearing rats fed on O, the secondary corpus luteum (CL 2) and the primary corpus luteum (CL 1) showed high activity. These showed that high content of oleic acid contained in olive oil may induce an increase of progesterone production in their ovaries. In control rats, the activity in CL 2 in the ovaries was lower than that in CL 1.

These results suggest that the increases in plasma progesterone in tumor-bearing rats on O or S may have been caused by prolonged corporus luteum function and the active formation of interstitial glands in the ovaries. This resulted in a low incidence of mammary tumors because continuous stimulation of progesterone caused lobulo-alveolar proliferation, thereby protecting the mammary epithelium from carcinogens.

Using 7,12-dimethylbenz(a)anthracene(DMBA) as carcinogen, Carroll and Khor (3, 4) demonstrated that high fat (HF) diets promote the development of mammary tumors. Chan *et al.* (7, 8) also showed that the tumor promoting effect of HF may be mediated by changes in hormones, such as estrogen and prolactin.

It is currently thought that estrogen regulates prolactin secretion *via* the hypothalamo-hypophyseal portal system. Besides acting directly on the mammary epithelium, it also enhances the sensitivity of the mammary tissue to prolactin stimulation (1, 25).

Progesterone is an antagonist of estrogen activity, because it inhibits estrogen mediated growth of target organs. The relation between progesterone and HF diet has not been studied in rats with DMBA-induced mammary tumors. A few studies (1, 2, 14, 25, 30) have shown that progesterone administration reduces the incidence of DMBA- or nitrosomethylurea (NMU)-induced rat mammary tumors (14, 30). Low plasma progesterone was observed in women with a high breast cancer risk (2).

After DMBA administration, the time of tumor induction was shorter and the number of tumors per rat was higher than in virgin control rats that received DMBA only (17). Progesterone administration, like pregnancy, also enhanced the incidence of mammary tumors (17).

In mammary carcinogenesis in rats, progesterone is not an initiator factor for tumor incidence, but with estrogen it has a promoting action on tumor growth (33, 34). The tumors that develop during continuous progesterone treatment have high estrogen receptor(ER) levels (14); this effect of progesterone is apparent from the high ER levels observed in malignant mammary tumors.

These hormones are chiefly produced in the thecal membrane, the interstitial glands, and the corpus luteum of the ovaries. We suggested that these hormones in the ovaries of rats were increased in animals fed on HF diets (12). In studies on DMBA-induced mammary carcinoma in rats fed on HF diets, it should be determined how each endocrine gland in the ovary is affected physiologically with regard to the production of progesterone and estrogen.

From this precursory basis, we used the following animals and experimental methods; Wistar-Imamichi rats, to define the estrous cycle, which show a regular 4-day cycle in this strain, and a microspectrophotometric method (13) to examine endocrine gland of the ovaries, *in situ*.

Weisz and Zoller (29) have reviewed a quantitative cyctochemical bioassay for hormones in the ovary. They used Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD; EC 1.1.1.51) as a marker of steroid hormones.

We used their method to investigate the production of steroid hormones in each gland in the ovaries of rats with DMBA-induced mammary carcinoma fed on HF diets, and then measured the progesterone and 17β-estradiol levels in the plasma by radioimmunoassay.

MATERIALS AND METHODS

Animals:

Thirty-nine virgin female Wistar-Imamichi rats 45 days old, were purchased from Hanshoku Kenkyu-sho (Saitama, Japan), and were housed in individual cages at a temperature of $-(24\pm1°C)$, humidity-(50%), and light-(12 h/day) controlled room. The animals were given a commercial diet (Oriental Yeast, Co., Tokyo, Japan) and tap water, *ad lib.* until they were about 50 days old.

Diets:

The composition of diets is shown in Table 1. The diets were stored at $-20°C$

TABLE 1. *Composition of semisynthetic diets* (%)

	Rate
Fat	30
Casein	23
Sucrose	10
Corn Starch	24
Cellulose	6.7
Salt mix.[1)]	5.2
Vitamin mix.[2)]	1.1
Total Energy (kcal/g)	4.7

1), 2) Oriental mixture, Oriental Yeast Co. Ltd., Tokyo.

TABLE 2. *Fatty acid composition of oils* (*wt* %)

Fatty acids*	Shorthand designation	Corn oil	Safflower oil	Olive oil
Lauric	12:0	—	—	0.04
Myristic	14:0	—	0.10	—
Palmitic	16:0	11.0	6.54	11.2
Palmitoleic	16:1	0.03	0.01	1.01
Stearic	18:0	—	—	—
Oleic	18:1	37.93	14.34	75.05
Linoleic	18:2	48.88	77.15	10.52
Linolenic	18:3	0.31	—	0.30
Arachidic	20:0	1.86	1.80	1.88
Arachidinic	20:4	—	0.06	—

*; percent of total fatty acids.

for use in experiments. The corn and olive oil used for experiments were purchased from Nissin Seiyu, Co., Tokyo and Hayashi Junyaku, Ltd., Osaka, Japan, respectively. Safflower oil was donated by Rinol Yushi, Co., Tokyo. The fatty acid composition of these fats, determined by the method of Stoffel *et al.* (27), are shown in Table 2.

DMBA administration:

Five days before DMBA administration, vaginal smears were examined daily to determine the estrous cycle. Induction of mammary tumors was greatest when DMBA was given during the prolactin increasing phase, from 5 : 00–7 : 00 P.M. of the proestrous stage (20). We also administered 10 mg of DMBA (Eastman Kodack, Rochester, N. Y.) dissolved in 1 ml sesame oil through a stomach tube (Natsume KN-384, Japan) at 5 : 00 P.M. of the proestrous stage to about 50-day-old rats. Control animals received 1 ml of sesame oil only. DMBA was purified with methanol-water (5). On day 4 after the first dose of DMBA, the animals were again given the same dose of oil with or without DMBA. Then they were divided

into three groups of 10 animals each and pair-fed on the above experimental diets for 120 days. Three control animals were used for each group. During this experiment, they were weighed weekly and palpated daily for detection of mammary tumors for 120 days after the second administration of DMBA, and then killed. Ten days before sacrifice, vaginal smears were examined daily to determine the estrous stage. Blood samples from all animals were obtained from the *vena cava inferior* under ether anesthesia at 5 : 00 P.M. of the proestrous stage in a cold room. Palpable tumors were carefully examined and their diameters were measured. Then resected tumors were fixed in 10% cold buffered formalin for one week, and embedded in paraffin. Tissue slices of 4 μm thickness were stained with hematoxylin-eosin (HE) and Azan of Heidenhain. The ovaries were removed, weighed and, then promptly frozen in freon gas (−40°C) and cut into 7 μm thick slices on a Bright Cryostat (England). One section was stained with HE to confirm the measurement site, and the other was used for enzyme histochemical study.

Determination of Progesterone and 17 β-estradiol levels:

Radioimmunoassay of plasma progesterone and 17 β-estradiol was performed in Otsuka Assay Laboratories, Tokushima.

3 β-HSD assay:

The enzyme reaction was carried out by the method described previously (13).

After reactions, the medium was washed off with cold distilled water (4°C) and tissues were fixed in cold buffered formalin containing 10% PEG at 4°C for 30 min. PEG was added to these solutions as a tissue stabilizer. The sections were rewashed with cold distilled water (4°C), rinsed with 1% acetic acid, and then stained with 1% naphthol yellow S(NYS, Wako Chemical Co., Osaka, Japan) by the method of Tas *et al.* (28). After prompt dehydration with tertiary butyl alcohol, the sections were dried and mounted in liquid paraffin (Merk Chemical Co., Darmstadt).

Microspectrophotometry:

For microspectrophotometric assay of 3 β-HSD, we applied the double staining method of Yamada *et al.* (32) showing the relative ratio of absorbance (RA) of diformazan produced by the enzyme reaction using NBT as hydrogen acceptor and NYS stained with protein. The absorption of the tissue was measured with a Zeiss UMSP I micro-spectrophotometer (Carl Zeiss). An objective of 10X was used and the spot size was set at 32 μm. The stage was scanned linearly at 33.3 μm/sec as shown in Fig. 1. Then the stage was moved manually horizontally about 50 μm and the scanning was repeated. All areas of each tissue were scanned. The number of measuring points differed in area in different tissues being narrow in the thecal membrane and wide in the corpus luteum. Therefore, the number of measuring points was 30 in the thecal membrane and the interstitial gland, and 100 in the corpus luteum. RA values on each tissue were caluculated by the formula of Nakae *et al.* (21), using values measured at 450 and 580 nm, respectively.

Statistical analysis:

The significance of differences between values was calculated by Student's t-test.

RESULTS

During the 10 days before sacrifice, the estrous cycle of each rat was regular. As shown in Table 3, there was no difference among the groups in the rate of increase

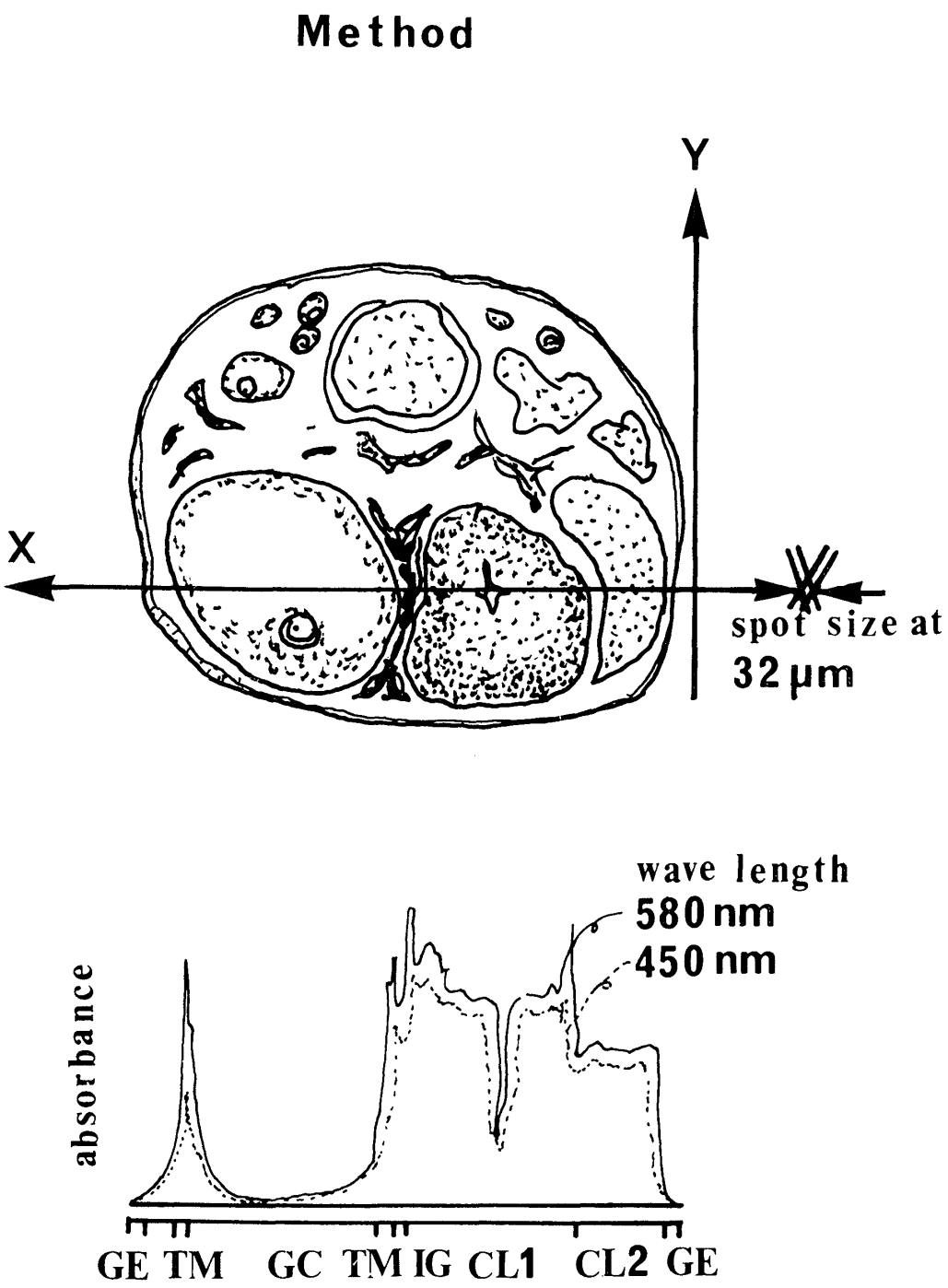

FIG. 1. Diagram illustrating the micro-spectrophotometrical method for 3β-HSD assay. The upper drawing shows that the spectrum of the 32 μm spot at 450 (maximum absorption of NYS) and 580 nm (that of diformazan) is scanned continuously in the direction of X-axis at a scanning speed of 33.3 μm/sec in the ovary. The scanning length was the length from one germical epithelium to the other. Then, the stage was moved manually up the Y-axis at intervals of about 50 μm and the same scanning was repeated. The lower diagram shows the values observed in the thecal membrane (TM), the interstitial gland (IG) and the corpus luteum of primary (CL 1) and secondary (CL 2) in the ovary. No activity was observed in the germinal epithelium (GE). In the granulosa cells (GC), slight activity of 3β-HSD is shown near the thecal membrane, only in the peripheral zone.

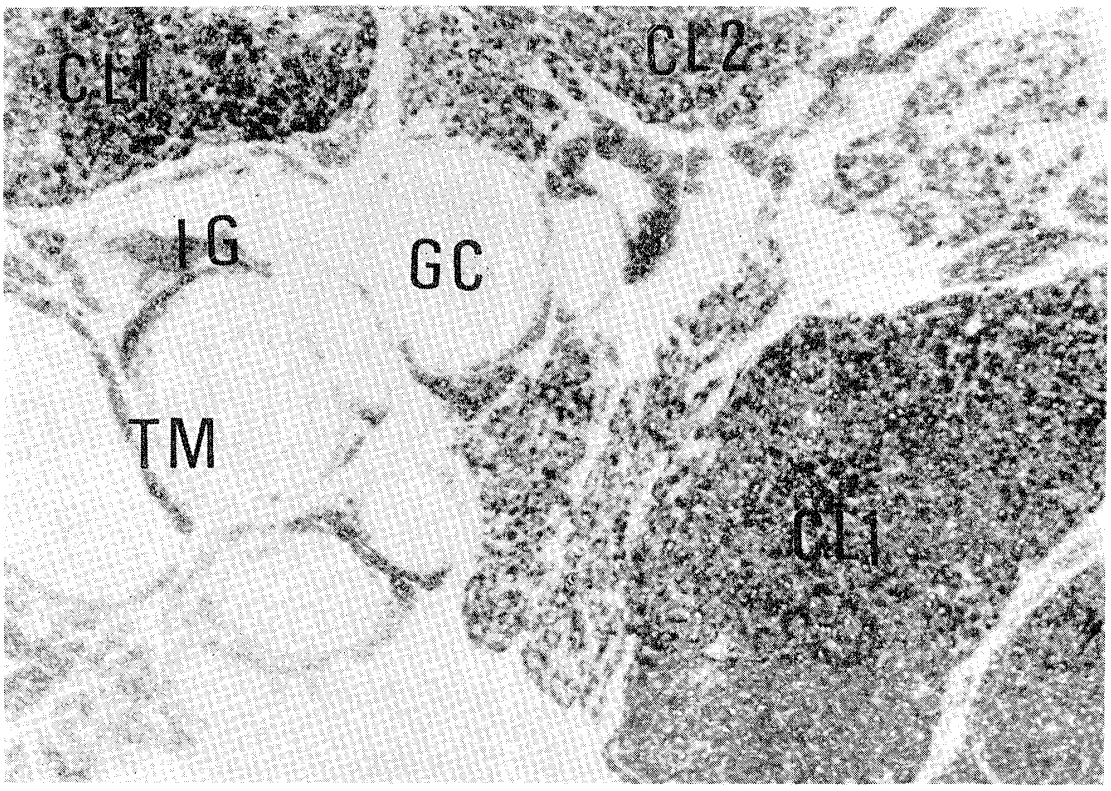

FIG. 2. Photograph of 3β-HSD with DHEA as substrate in the ovary of a tumor bearing rat fed olive oil diet. $\times 72$
Reaction products are observed in the corpora lutea (CL), interstitial gland (IG), and thecal membrane (TM).

TABLE 3. *Mammary tumor incidence following administration of DMBA to rats fed on semisynthetic diets containing different oils*

Diets	Rat body weight (g) initial	Rat body weight (g) final	Minimal days of tumor incidence		No. of rats with tumors (total rats)	Final diameter of tumor (mm)	
	M(SD)	M(SD)	V	M(SD)	V	V	M(SD)
30% corn oil	164(17)	379(38)		83.8(25.0)	8(10)		10.6(9.6)
			68.0			15.5	
30% safflower oil	163(20)	355(23)		91.0	2(10)		11.3
			114.0			7.4	
			109.0			10.0	
30% olive oil	173(12)	361(34)		111.5	2(10)		10.5
			114.0			11.0	

Values (V) are shown as mean (M) $\pm$S.D. in parenthesis.

of body weights. The incidence of palpable mammary tumors in rats fed on HF diets is also shown in Table 3. All of the tumors were adenocarcinomas. The number of tumor-bearing rats was 8 of the 10 rats in the corn oil group, and 2 of the 10 rats in the other groups. Tumor sizes were similar. The latent period of the

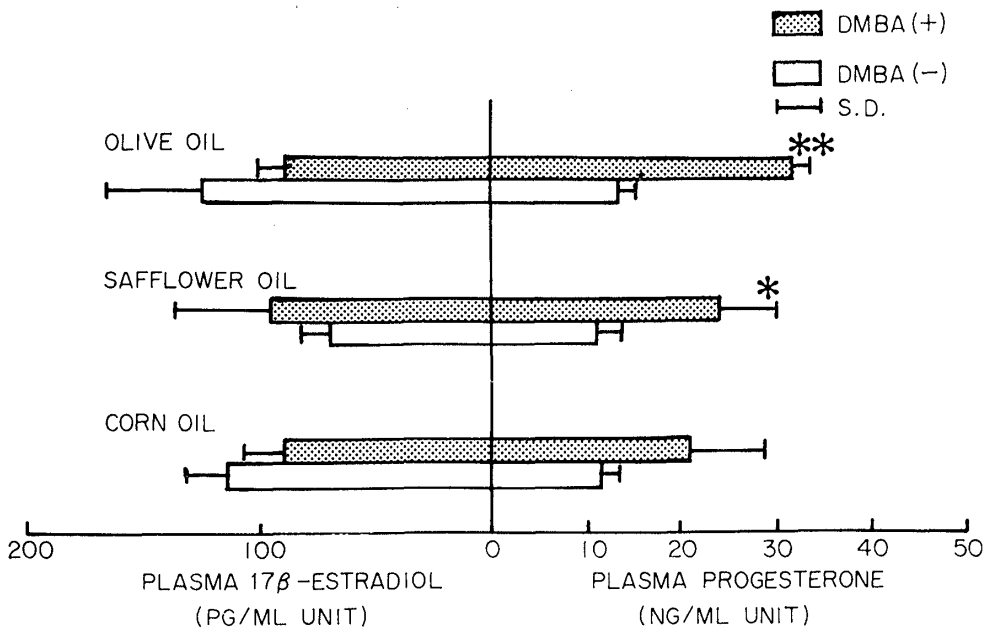

FIG. 3. Plasma progesterone and 17β-estradiol. These measured by radioimmunoassay. Horizontal lines show standard deviation (S.D.). Abbreviations: **, $p \leqq 0.01$; *, $p \leqq 0.05$.

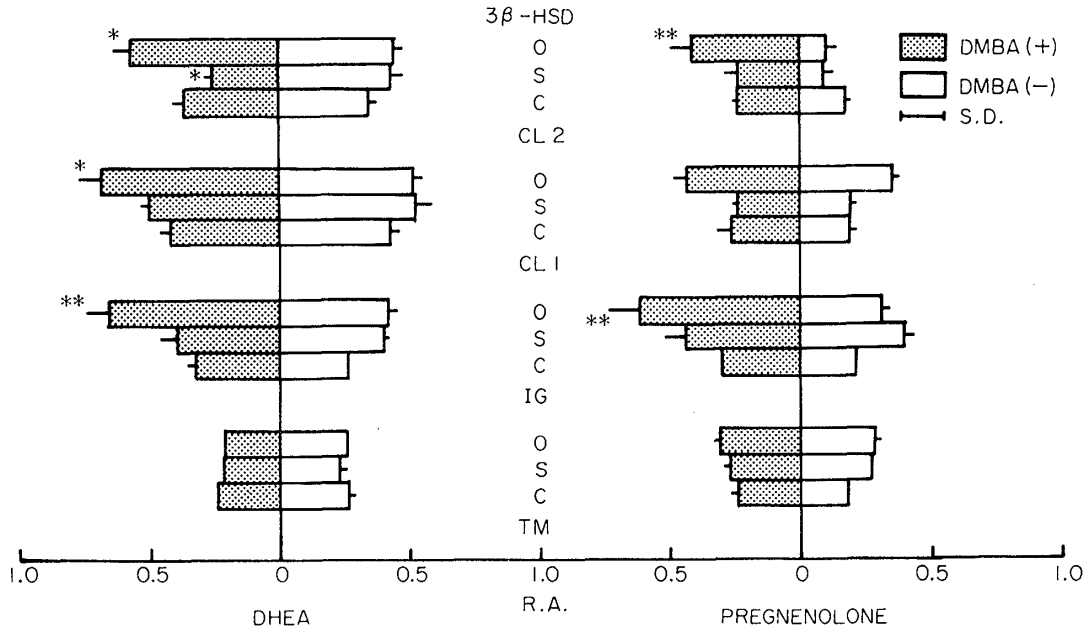

FIG. 4. Changes of RA of 3β-HSD in the ovaries. The experimental groups were fed on olive (O), safflower (S), and corn (C) oil diets, respectively. The ovaries used this study were removed from pairs of animals with histologically similar mammary tumors. 3β-HSD activity was measured as the absorbance at 580 nm relative to that at 450 nm. The number of measuring points were 30 in the thecal membrane and the interstitial gland, and 100 in the corpus luteum. The enzyme reactions of 3β-HSD used DHEA or pregnenolone as a substrate. Horizontal lines show S.D. Abbreviations: TM, thecal membrane; IG, interstitial gland; CL 1 and CL 2, primary or secondary corpus luteum; **, $p \leqq 0.01$; *, $p \leqq 0.05$.

tumors tended to increase in order in the corn oil group, safflower oil group, and olive oil group.

Plasma progesterone and 17 β-estradiol:

The plasma progesterone and 17 β-estradiol levels are shown in Fig. 3. In animals on HF diets, the plasma progesterone level of tumor bearing rats was higher than that of DMBA-untreated rats. The incidence was marked in the groups fed on olive and safflower oil diets. In DMBA untreated rats, the difference in the kind of fat had no effect on the plasma progesterone. The plasma progesterone level of tumor bearing rats in the group fed on olive oil diets was higher than that in the other groups.

There were no significant differences in the plasma 17 β-estradiol levels of the groups.

Histochemical measurement of 3 β-HSD in the ovary:

3 β-HSD activity is shown in Fig. 4. The activity of the thecal membrane, which may have secreted estrogen, did not change in this study.

When DHEA was used as the substrate in the 3 β-HSD reaction, the activity in the interstitial gland and corpus luteum in the tumor bearing rats did not differ from that of the DMBA untreated-rats in the corn oil group. The activity of the DMBA-untreated rats in the corn oil group was somewhat lower than that in the other oil groups, although there was no difference between the activities in the safflower and olive oil groups. Differences between the tumor bearing rats and the DMBA-untreated rats were observed in the olive oil and safflower oil groups. The activity of the tumor-bearing rats fed on the olive oil diet was consistently higher in individual tissues than that of DMBA-untreated rats, while the activity of the safflower oil group was only lower in the secondary corpus luteum (CL 2). The activity in the tumor-bearing rats was the highest in the olive oil group. In general, the activity of these glands tended to increase, in order in the corn, safflower, and olive oil groups, respectively.

We also investigated reactions of 3 β-HSD with pregnenolone as substrate. The activity was generally higher in tumor-bearing rats than that in DMBA-untreated rats, and was particularly marked in the corpus luteum of the olive oil group. These results were similar to those on 3 β-HSD with DHEA as substrate. The activities in CL 2 in the tumor bearing rats fed on olive and safflower oil diets were similar to those in the primary corpus luteum (CL 1).

In DMBA-untreated rats, the activity in the CL 2 in the safflower oil group was about one-half that in the CL 1, and that in the CL 2 of the olive oil group was about one-third of that in the CL 1. There were no changes in the activity in the corn oil group.

Morphological observation:

The size, shape, and weight of the ovaries were similar in DMBA-treated and untreated rats fed on HF. The atretic follicle and interstitial gland tended to increase slightly in the tumor bearing rats. The corpora luteal cells had compact acidophilic cytoplasm which contained Azan positive granules. These were remarkable in the olive oil group.

DISCUSSION

The dietary factors that influence cancer development in rodents have been

reviewed by Reddy *et al.* (23). HF containing poly-unsaturated fatty acids are more effective than those containing poly-saturated fatty acids for promotion of mammary tumors induced by carcinogens. The effects of oleic and linoleic acid are particularly marked.

In this study, we used HF diets containing olive, safflower and corn oil containing a high content of oleic acid, linoleic acid, and their mixtures, respectively. The incidence of mammary tumors was high in rats fed on corn oil diet, and the latent periods decreased. Many researchers showed marked increases in incidence of mammary tumors in animals on linoleic and oleic acid diets, as well as corn oil diet. However, the incidence of tumors in this study was low on both olive oil and safflower oil diets.

A 3% addition of linoleic acid to tallow or coconut oil diets, on the whole, resulted in a high incidence of mammary tumors (16), where addition of less than 2% linoleic did not effect the incidence of mammary tumors (19, 24). If the incidence of tumors depends only on the concentration of linoleic acid, the group fed on the safflower oil diet, containing a high concentration of linoleic acid, should always show a high incidence of mammary tumors. But our data did not show a high incidence of mammary tumors in the group fed on safflower oil diets, although the content of linoleic acid in this oil was more than 70%, as shown in Table 1. The present data, therefore, do not support the notion of Hopkins and Carroll (16), that it is the concentration, rather than the type, of fat in diet that is critical in enhancing mammary carcinogenesis. The balance of fatty acids in the oil may be more important than their concentration.

The Wistar-Imamichi rats used in this study are good models for investigating endocrine function, because of the stability of the estrous cycle; a regular 4-day cycle (22). Sprague-Dawley (SD) rats have generally been used for the experiments on DMBA-induced mammary tumors. Consequently, it is thought that the difference in the strain may have affected these results. Chan *et al.* (9), however, reported that similar effects were induced by both DMBA administration and on HF diet in SD and F344 rats. This finding suggests that the difference of the strain had little effect in this study.

There are few reports about the levels of plasma progesterone in rats with DMBA-induced mammary tumors on HF diets, although there are many reports on their plasma estrogen and prolactin levels (7, 8, 9). In the present study, we observed that the level of the plasma progesterone was higher in tumor-bearing rats than in DMBA-untreated rats. The increase was remarkable in the groups on olive and safflower oil diets, although there was no significant difference between the levels of plasma 17 β-estradiol in these animals.

It was difficult to investigate the original function of the cells which secreted steroid hormones in the ovaries because these cells could hardly be separated. By using the microspectrophotometric technique, we could easily study the productive ability of steroid hormones in the thecal membrane, interstitial gland, and corpus luteum, *in situ*. In general, it is thought that the thecal membrane secretes estrogen, and the corpus luteum produces progesterone. The interstitial gland may produce both these hormones (15).

3 β-HSD promotes four catalytic reactions. In this study, we investigated two representative reactions: the conversions of pregnenolone to progesterone and

DHEA to androstendione.

In the tumor-bearing rats fed on HF diets, 3 β-HSD activity increased in both the interstitial gland and the corpus luteum. Also, 3 β-HSD activity increased in the order of corn oil group, safflower oil group, and olive oil group. The activity in CL 2 was slightly less than with that in CL 1 in the safflower and loive oil groups. In the DMBA-untreated rats, the activity in CL 2 was about one-half or one-third of that in CL 1. These results suggest that the production of progesterone in corpus luteum has been maintained for a long-term in rats with DMBA-induced mammary tumor fed on HF diets. The activity in the corpora lutea was particularly marked in the olive oil group. Also, the activity in the interstitial gland increased in their group.

There was no difference between the 3 β-HSD activities in the thecal membrane of tumor-bearing rats and DMBA-untreated rats. These findings are consistent with results on the plasma of levels 17 β-estradiol in these animals.

An effect of HF in the ovaries of tumor bearing rats was observed only at sites of progesterone production; *i.e.*, in the corpus luteum and the interstitial gland. Therefore, these results suggest that the increase of plasma progesterone in tumor-bearing rats fed on HF diets may be caused by prolongation of corpora luteal function and active formation of the interstitial gland.

Progesterone is known to antagonize the action of estrogen. Sherman and Kreman (26) suggested that inadequate corpora luteal function is a risk factor of human breast cancer because it permits excessive estrogen activity due to a lack of the normal modulating action of progesterone. Low levels of plamsa progesterone are observed more frequently in breast cancer patients than in normal women (2). Progesterone administration reduces both the incidence (30) and the multiplicity (14) of mammary tumor rats induced by nitrosomethylurea (NMU).

Huggins *et al.* (17) induced pregnancy in rats after DMBA-administration and found the time for tumor induction was less and the number of tumors per rat was more than control virgin rats treated with DMBA. In addition, they showed that, like pregnancy, administration of progesterone enhanced induction of mammary tumors by DMBA in rats. Jabara (18) also showed that progesterone administration increased the incidence and number of tumors, but reduced the time of tumor induction.

Yoshida *et al.* (33, 34) reported that in the presence of estrogen only, many papilloma were observed in the mammary glands but in the presence of estrogen and progesterone, most tumors were adenocarcinomas. They proposed that plasma progesterone was a promoter of mammary tumors, and plasma 17 β-estradiol was an initiater. The tumors that developed during continuous progesterone treatment generally have high levels of estrogen receptors (ER) (14). These results suggest that progesterone is possibly a malignant factor of mammary tumors.

High contents of oleic and linoleic acid may cause growth of epithelial cells of the tumor and mammary glands (31). When progesterone was administered before the first dose of carcinogen, the incidence of mammary tumors was reduced (14). It is thought that this reduction is to progesterone administration, which may produce sufficient lobulo-alveolar proliferation to protect the mammary epithelium from carcinogens (31). The low incidence of mammary tumors in rats on olive and safflower oil diets in this study might be caused by an elevation of progesterone

production in the ovaries. The corn oil diet group, which showed a high incidence of mammary tumors, did not show a significant increase of estrogen. Chan and Cohen (6) gave the anti-estrogen drug U11, 100A to DMBA pretreated rats fed on HF diets and then measured the incidence of breast tumors. They showed that the tumor enhancing effect of HF may not be mediated *via* the estrogen pathway because the incidence of tumors did not decrease. Our data also support this notice because a high incidence of mammary tumors, induced by HF diets, did not parallel the elevation of estrogen.

For further study of the feeding of HF diets in rats with mammary tumor induced by DMBA, our data suggest that it may be necessary to investigate the relation between ovarian hormones and two different effects, the quality and the balance of the composition of fatty acids.

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