ACTA OBST GYNAEC JPN Vol. 39, No. 5, pp. 842—848, May 1987

ESTABLISHMENT AND MORPHOLOGIC CHARACTERIZATION OF CELL LINE (DMBA-OC-1) FROM 7,12- DIMETHYLBENZ (a) ANTHRACENE-INDUCED RAT OVARIAN CARCINOMA

Akio KATAOKA, Masamichi KOJIRO, Michiaki YAKUSHIJI* and Toshi KATO*

First Department of Pathology, Kurume University School of Medicine, Kurume

**Department of Obstetrics and Gynecology, Kurume University School of Medicine, Kurume*

Synopsis A new cell line (DMBA-OC-1) from 7,12-dimethylbenz (a) anthracene (DMBA) which induced ovarian carcinoma in rat was established and characterized. DMBA-OC-1 cells showed a paving-stone-like growth pattern at around the 10th to 20th passage, but at the point exceeding the 40th passage the cells showed a spindle form, having strong trends both towards flocculation and piling-up. Furthermore, diastase-resistant PAS-positive and hyaluronidase-digested Alcian blue positive substances were observed in cytoplasms. The cells also showed marked phagocytic activity. These findings suggest that DMBA-OC-1 cells are most likely the site of mesothelial cell origin.

Key words: Ovarian carcinoma • DMBA • Cell line • Histogenesis

Introduction

Carcinogenic experiments using chemical carcinogens have a long history with a large number of reports[1)3)~9)13)~16)]. However, ovarian carcinoma has poor experimental systems because of its anatomical and positional difficulty. Ovarian carcinoma induced by the method embedding intraovarially in rats (the clipping method) of 7,12-dimethylbenz (a) anthracene (DMBA) reported by Kato et al.[6)] in 1973, is similar to human ovarian carcinoma morphologically. Although many studies on its histogenesis and biological characteristics in vivo have hitherto been made[3)4)9)13)16)], little investigation has yet been made in vitro[13)~15)].

The studies of in vivo and in vitro of this ovarian carcinoma suggest to provide the useful indices for treatments and histogenesis of human ovarian carcinoma. In order to elucidate the histogenesis and biological characteristics of the DMBA-induced ovarian carcinoma, the author established cell line from DMBA-induced ovarian carcinoma in rat.

Materials and Methods

1. Materials

DMBA-induced rat ovarian carcinoma was subinoculated in the back of female Wistar rats aged within 48 hours after birth. After 10 passages a part of the tumor was subjected to the tissue culture[6)7)].

2. Tissue culture medium

DM 170 (Kyokuto Co., Tokyo, Japan) supplemented with 100μg/ml Penicilin, 10μg/ml Streptomycin (GIBCO, Chagrin Falls, Ohio, USA), 35μM/ml sodium bicarbonate and 20% fetal bovine serum (FBS) (M.A. Bioproducts, Maryland, USA) was used as the primary culture medium (PCM). DM 170 with 10% FBS was used as maintenance medium.

3. Tissue culture

The ovarian carcinoma tissue was washed in PCM and then minced with two surgical blades into 1 to 3-mm small pieces. The small tissue fragments in PCM were filtered through a 20μm pore mesh to remove blood, washed in fresh Ca^{2+} and Mg^{2+}-free phosphate buffer saline (PBS) (Nissui Co., Tokyo, Japan), centrifuged (800 rpm 5 min at 4℃), and the pellet, suspended in 20ml of PBS with collagenase (type IV, 473u/ml, 0.5mg/ml, Sigma, St. Louis, USA) was incubated at 37℃ in 5% carbone dioxide in air. The undigested tissue fragments were again treated with collagenase in PBS for 30 min to 60 min in all. Then, fragments were filtered through a 100μm pore mesh to remove undigested tissue. The cell suspension was centrifuged (800 rpm 5 min at 4℃), and resuspended in PCM and cultured in the same manner. The medium was changed two to three times a week. The cells were frozen and stored in liquid nitrogen in a medium of 10% dimethyl sulfoxide in culture

medium.

4. Growth curve and doubling time

The growth curves were prepared using the cells of the 20th and 40th passages. After washing with PBS the cells were separated from the flask using 0.1% trypsin-EDTA; cell count of 10^5 was disseminated on a 35mm dish; and it was incubated in 2ml of the same cultured solution. Calculation of the cells were measured after adding trypan blue every 12 hours after the logarithmic growth phase. The doubling time of the cell population was estimated in the logarithmic growth phase.

5. Histopathological observation

The original tumor was embedded in paraffin after fixation in a 10% formalin and stained with hematoxylin and eosin (H & E), periodic acid and Schiff (PAS) with or without diastase digestion, Alcian blue (pH 2.5) with or without hyarulonidase digestion, and Mayer's mucicarmine. As for routine observations, beside phase-contrast microscopic observations, the cells cultured on a tissue culture chamber slide (Lab-Tek Products, Div Miles Laboratories, IL) were fixed for 1 hour in a 10% formalin and stained with H & E, Giemsa, PAS with or without diastase digestion, and Alcian blue (pH 2.5) with or without hyaluronidase digestion. Then they were further stained with Papanicolau after fixing in 99% ethanol for 12 hours.

For electron-microscopic examination, the original and transplanted tumors were prefixed with Karnovsky's solution, washed three times with cacodylate buffer solution, postfixed with OsO_4, dehydrated with ethanol, and embedded in Epon 812. The monolayer-cultured cells were separated with a 2.5% glutaraldehyde dissolved in a 0.1 M Na-cacodylate buffer solution, postfixed with 1% OsO_4 and embedded in Epon 812. Ultrathin section were observed by a H-500 (Hitachi, Tokyo, Japan) after uranylacetate and lead citrate stains.

6. Heterotrlansplantation and tumorigenecity

Six-week-old female nude mice (BALB/c, nu/nu, CLEA Japan, Tokyo) were used to examine the tumorigenecity of DMBA-OC-1. Five million cells at 30th and 40th passages suspended in 30μl DM 170 with 10% FBS were inoculated intraperitoneally and subcutaneously into the back of four mice. The mice with ascites were observed light and electron microscopically. Paraffin sections were stained with H & E, PAS with or without diastase digestion, and Mayer's mucicarmine.

7. Chromosomal analyses

The cultured cells of the 25th and 50th passages and those of the fifth reculture from the tumorigenesis after transplantation in nude mice were used; 0.02μg/ml of colcemid, the final concentration, was added to the cultured suppernatant of the cells being present at the logarithmic growth phase; the samples were prepared by collecting cells at metaphase and treated by the trypsin G-band method[8]; and 100 cells of the samples were analyzed.

8. Test of phagocytosis

In order to examine the phagocytic activity of the cultured cells, the cells at the 50th passages were added with culture medium and India ink, and incubated at 37℃ in 5% carbon dioxide in air.

9. Analyses of estrogen receptor and progesterone receptor

Studies were made of estrogen receptor and progesterone receptor by the dextran coated charcoal method using 1g of a mass of tumor tissue.

Results

1. Establishment of the cell line (DMBA-OC-1) from the DMBA-induced ovarian carcinoma in rats.

Although fibroblast grew vigorously after starting primary culture, the tumor cells also formed colonies by making fibroblasts as a feeder layer. The colonies consisted of round cells going in paving-stone pattern. It afterwards continued to proliferate and the tumor cells became apparently predominant at the 10th passage. After the 20th passage the tumor cells showing a trend to flocculation were isolated and the colony consisting of nearly entire tumor cells alone was obtained.

2. Morphology of DMBA-OC-1 cell

Light-microscopically, the original tumor had 1 to 2 prominent nuclei and showed an undifferentiated form in which the tumor cells having a large nucler-cytoplasm (n/c) ratio proliferated in a solid fashion (Fig. 1). At around the 10th to 20th passages, cells similar to the original tumor showed a cobble-stone like arrangement and proliferated with a partial invagination (Fig. 2a), but at the point exceeding the 40th passage the cells showed a spindle form, having strong trends both towards flocculation and invagination (Fig. 2b). Furthermore, diastase-resistant PAS-positive and

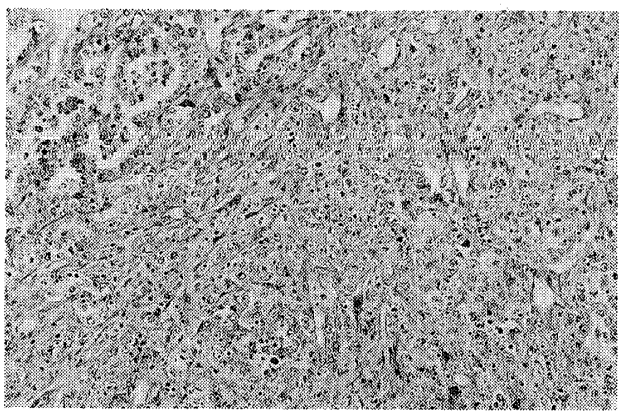

Fig. 1. Histologic finding of DMBA-induced ovarian tumor. Tumor cells proliferate in a solid growth pattern with glandular structure in part. (H & E stain, ×100)

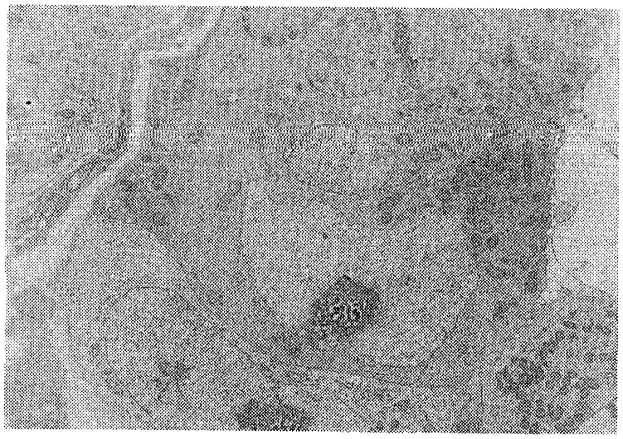

Fig. 3. Electron micrograph of DMBA-induced rat ovarian tumor. Tumor cells show good mutual contact with interdigitations. Cytoplasmic organella are relatively well developed. (×2,500)

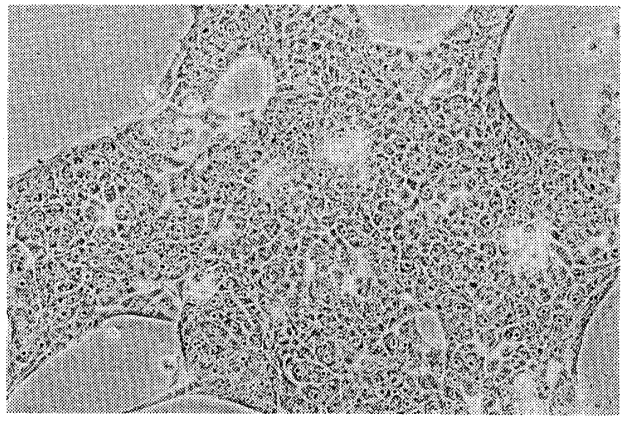

Fig. 2a. Phase contrast micrograph of DMBA-OC-1 cells at the 10th passage. The cells are round to oval in shape and show a paving-stone-like growth pattern. (×100)

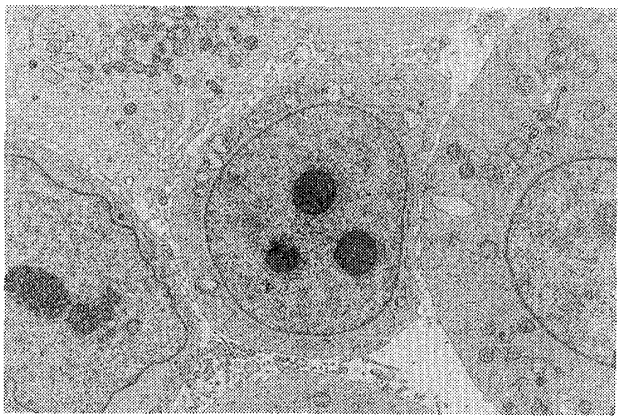

Fig. 4. Electron micrograph of DMBA-OC-1 cells. The cells show poor mutual adherence and microvilli in the cell surface are poorly developed. (×3,000)

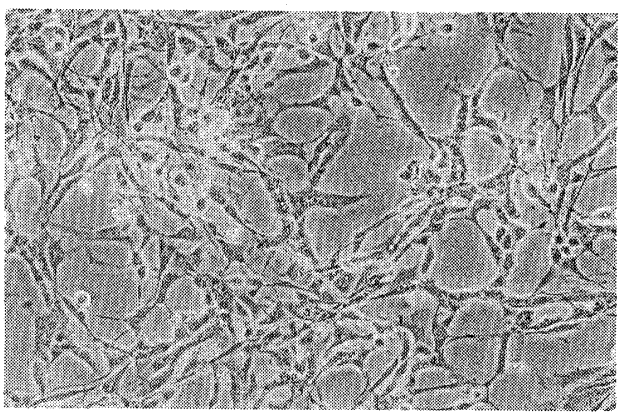

Fig. 2b. Phase contrast micrograph of DMBA-OC-1 cells at the 40th passage. The cells show spindle-shaped and grow in a lacy pattern. (×100)

hyaluronidase-digested Alcian blue positive substances were observed in cytoplasms. In the result, DMBA-OC-1 cells produced hyaluronic acid in cytoplasm.

Electron-microscopically, the original tumor cell had a nucleus in the center of the cell. The nuclei were round, but some of them were markedly deformed. Basement membrane was present, and microvilli were noted in the cell surface. In cytoplasm endoplasmic reticulum, mitochondria, lysozome, and ribosome were well-developed in number. Intercellularly, desmosome-like gap junction and interdigitation were observed (Fig. 3). Although the DMBA-OC-1 cells showed nearly the

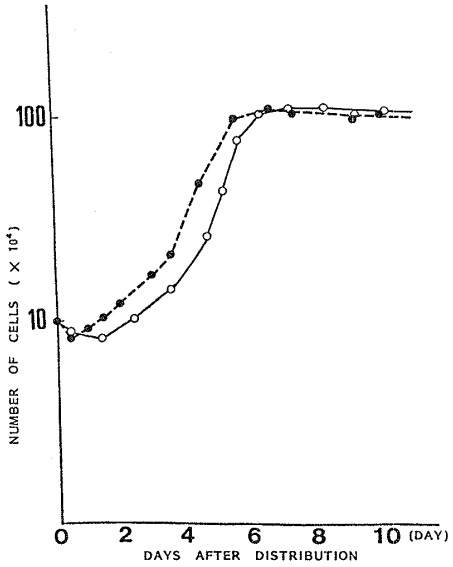

Fig. 5. Growth curve of DMBA-OC-1 cells.
○——○ passage 20, ●·····● passage 40.

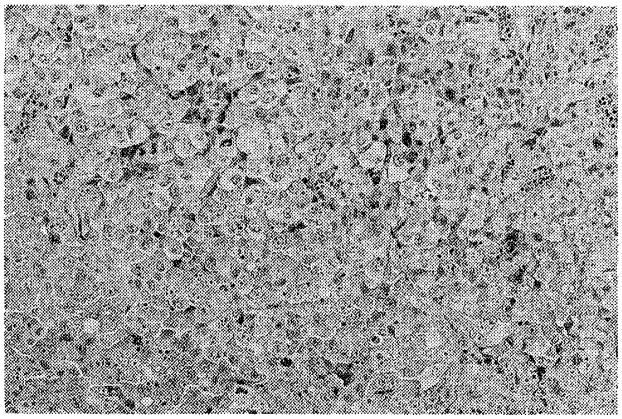

Fig. 6. Histologic finding of heterotransplanted tumor of DMBA-OC-1 cells in nude mouse. The tumor shows a solid growth pattern and resembles the original tumor. (H & E, ×100)

same appearances as the original tumor, they had poor mutual contact with obscure intercellular junctions, and microvilli became poor (Fig. 4).

3. Growth curve and doubling time

The doubling time was approximately 32 hours and saturation density, around $1.4\times10^6/cm^2$ in each passage, but the 40th passage exhibited a shortened lag phase compared with 20th (Fig. 5).

4. Heterotransplantation and tumorigenecity

The cells inoculated subcutaneously in the back of nude mice showed marked tumorigenesis from a week later and leaving the mice as they were led them to death in approximately 6 weeks. The transplanted tumor consisted of the cells growing in a solid pattern very similar to the original tumor (Fig. 6, 7). Intraperitoneal inoculation led to the dark red coloring in the entire abdomen and to death in approximately 4 weeks. Dissection revealed a condition of carcinomatous peritonitis

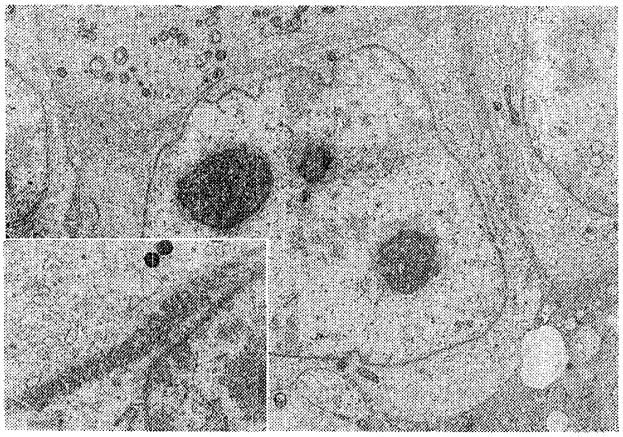

Fig. 7. Electron micrograph of heterotransplanted tumor of DMBA-OC-1 cells in nude mouse. Although the cells resemble the original ones, they lack tight mutual contact. (×2,500)
Inset; well-developed microvilli in the cell surface.

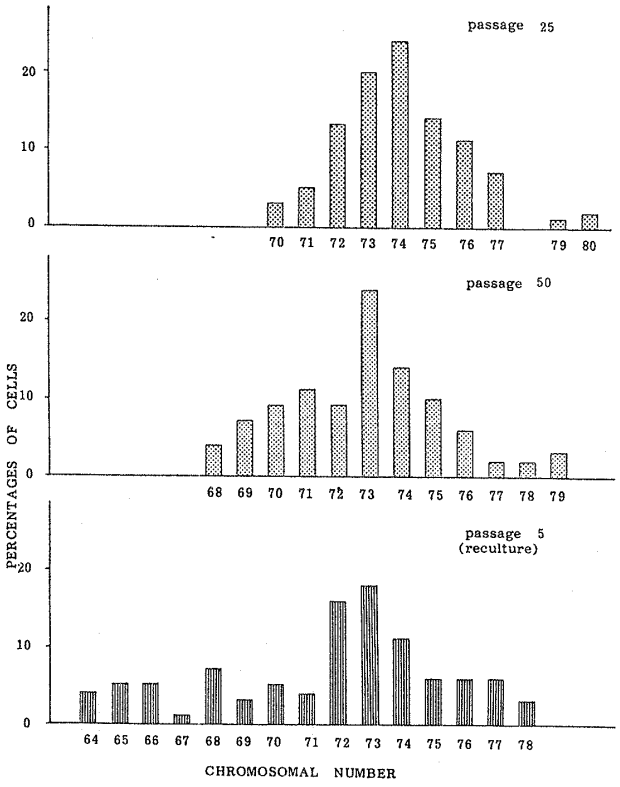

Fig. 8. Chromosome analysis of DMBA-OC-1 cells.

with marked hemoascites and metastases to the liver and spleen. Cells similar to the cultured cells were noted from the ascites.

5. Chromosomal analysis

The mode was present at 74 at the 25th passage and at 73 at the 50th passage and the 5th reincubation. However, abnormal structures such as $1q^+$, $2q^-$ and $4q^-$ were observed in common (Fig. 8).

6. Phagocytosis

Some of DMBA-OC-1 cells showing a spindle form at the point exceeding the 40th passage cultured with India ink, were observed with phagocytosis phase-contrast microscopically and small granules in cytoplasm light-microscopically. Furthermore, phagosome with India ink were also present in cytoplasm electron-microscopically (Fig. 9, 10).

7. Estrogen receptor and progesterone receptor

Any of the original tumor and transplanted tumor in nude mice was negative to both estrogen receptor and progesterone receptor.

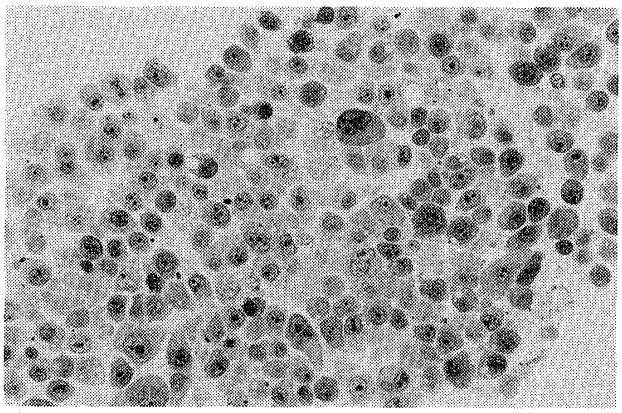

Fig. 9. DMBA-OC-1 cells exhibiting phagocytosis. Fine granules of India ink are observed in cytoplasms of some cells. (1μm Epon section, Giemsa stain, ×400)

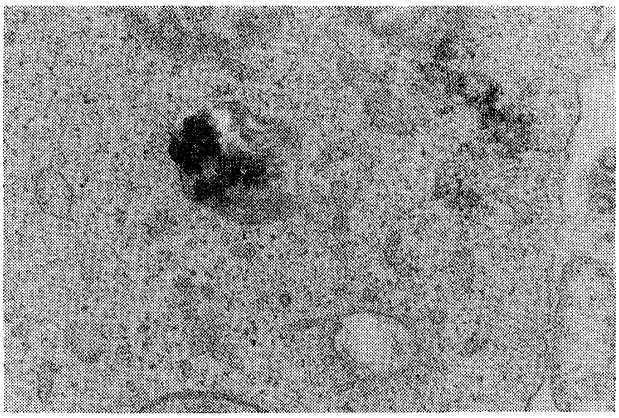

Fig. 10. Electron micrograph of DMBA-OC-1 cell with phagosome containing India ink. (×20,000)

Discussion

The experimental ovarian adenocarcinoma was first reported by Kato et al.[5)~7)] by means of the intraovarian embedment of DMBA. According to the fact that the tumor developed exhibited consecutiveness to germinal inclusion cyst, it was assumed to be of ovarian-superficial epithelium origin[16)]. Sekiya et al.[13)~15)] reported that adenocarcinoma was seen in 39% of the developed ovarian tumors using the clipping method and considered that adenocarcinoma arose from adenoma in the hilus ovarii based on the observation in vitro. By contrast, Nishida et al.[9)] mentioned that direct carcinogenesis to the ovarian superficial epithelium of DMBA silk pierced blindly was liable to be more assumable rather than that silk passed the hilus ovarii in the ovarian carcinoma prepared by the same method. Furthermore, Adams et al.[1)] have succeeded in isolation culture of ovarian superficial epithelium in rats (ROSE cell), infected it with murine sarcoma virus, and reported that the tumor formed in immunosuppressed rats resembled morphologically ovarian endometrioid stromal sarcoma. Concurrently they described that the ROSE cell was useful in the study of histogenesis of common epithelial tumor and pointed out that the method of Kato et al.[6)] would have a difficulty in identifying which cell exhibits tumorigenesis.

DMBA-OC-1 cells showed tumorigenecity in nude mice, and led them finally to death. Its histological features are very similar to the original tumor, and indicate that it is a cultured-cell line originated from malignant tumor. Additionally the cells showed a paving-stone arrangement at the beginning of the culture, and exhibits trends toward piling-up and flocculation after the 40th passage; the cytoplasms became more spindly and showed non-epithelial cell-like proliferation. These changes are not found in other reports. These cells showed marked phagocytosis and contained hyaluronic acid in cytoplasms but there is no report of the ovarian carcinoma cell lines with phagocytosis. The characteristics, such as phagocytosis, hyaluronic acid activity, morphological change of spindley cytoplasms and non-epithelial cell-like

proliferation in the culture cells suggest mesodermal cell origin rather than epithelial cell origin[2)11)]. Parmley et al.[10)] considered that the ovarian superficial epithelium is mesodermal origin, and mesothelial proliferations in and around the ovary are common reactions to a variety of focal irritants, infections and neoplasm. To our knowledge, there are two hypotheses in the histogenesis of DMBA-induced ovarian carcinomas. One is "ovarian superficial epithelium" origin[9)] and another is "adenoma of the hilus ovarii" origin[13)]. The evidence that the DMBA-OC-1 cells are similar to the mesothelium on the morphological and biological characteristics in vivo and in vitro may suggest that the DMBA-induced ovarian carcinoma is ovarian superficial epithelium origin.

Although both estrogen and progesterone receptors were not found in the original tumor and transplanted tumor in nude mice, Katabuchi[4)] noted the estrogen receptor in 8 of 10 DMBA-induced cases. Kamura[3)] noted the progesterone receptor in 2 of 7 DMBA-induced cases. They reported accelerative effects on proliferation in vivo and in vitro by means of estrogen load and progesterone load, respectively. However, they further reported that since the same accelerative effect was noted in receptor-negative cases when loaded with progesterone, the method of measuring the progesterone receptor may possibly induce a false negative results in comparison with the estrogen receptor. Therefore, its nonspecific and accelerative effect requires probably further studies.

Acknowledgments

The author wishes to thank Mr. K. Takashima for photomicrographs, and Miss Y. Konomi for technical assistance.

References

1. *Adams, T. and Auersperg, N.:* Transforming of cultured rat ovarian surface epithelial cells by Kirsten Murine sarcoma virus. Cancer Res., 41: 2063, 1981.
2. *Ebihara, Z. and Fukushima, N.:* Histological reaction of serosa. Tr. Soc. Path. Jpn., 70: 163, 1981.
3. *Kamura, N.:* Hormonal impacts on DMBA-induced rat ovarian cancer, detection of hormone-receptor, and resultant morphologic changes after progesterone application. J. Jpn. Soc. Cancer Ther., 18: 91, 1983.
4. *Katabuchi, H.:* Hormonal inpacts on DMBA-induced rat ovarian cancer detection of hormone-receptor, and resultant morphological changes after estrogen application. Acta Obst. Gynec. Jpn., 35: 1634, 1983.
5. *Kato, T., Yakushiji, M., Tsunawaki, A. and Ide, K.:* A study of experimental ovarian tumors, produced in rats by the chemical carcinogen, 20-Methylcholanthrene. Kurume Med. J., 20: 159, 1973.
6. *Kato, T., Yakushiji, M., Tsunawaki, A. and Ide, K.:* Ovarian tumors developed in rats recieving chemical carcinogen 9,10-dimethyl-1,2-benzanthrecene. Kurume Med. J., 21: 11, 1974.
7. *Kato, T., Yakushiji, M., Tsunawaki, A., Ide, K., Hirose, N., Araki, S. and Abe, M.:* Studies on experimental formation of ovarian tumors especially, the discussion of the developing process of ovarian tumors following an application of DMBA. Kurume Med. J., 22: 169, 1975.
8. *Levan, G.:* Nomenclature for G-bands in rat chromosomes. Hereditas, 77: 37, 1974.
9. *Nishida, T., Oda, T., Sugiyama, T., Nishida, T., Nishimura, H., Tsunawaki, A. and Yakushiji, M.:* Squamous cell carcinoma arising from an epidermoid cyst in the ovary of a rat treated with 7,12-dimethylbenz (a) anthracene. Jpn. J. Cancer Res. (Gann), 73: 153, 1982.
10. *Parmley, T. and Woodruff, J.D.:* The ovarian mesothelioma. Am. J. Obstet. Gynecol., 120: 234, 1974.
11. *Rhodin, J.A.G.:* An atlas of ultrastructure. Sunders, Co., London, 68. 1963.
12. *Scully, R.E.:* Ovarian tumors. Am. J. Pathol., 686. 1977.
13. *Sekiya, S., Endoh, N., Kikuchi, Y., Katoh, T., Matsuura, A., Iwasawa, H., Takeda, B. and Takamizawa, H.:* In vivo and in vitro studies of experimental ovarian adenocarcinoma in rats. Cancer Res., 39: 1108, 1979.
14. *Sekiya, S., Iwasawa, H., Yamauchi, K., Kubota, K. and Takamizawa, H.:* Effect of cis-Dichloridiammineplatinum (II) on cloned ovarian adenocarcinoma cells of the rat in vitro. Gynecol. Oncology, 12: 14, 1981.
15. *Sekiya, S., Iwasawa, H. and Takamizawa, H.:* In vivo studies on the antitumor effect of cis-dichloradiamineplatinum (II) against transplantable ovarian adenocarcinoma cells of the rat. Gynecol. Oncology, 16: 85, 1983.
16. *Tsunawaki, A.:* A study of experimental ovarian tumor. Acta Medica, 47: 48, 1977.

(Accepted: No. 6082, Novenber 14, 1986)

概要 7,12-dimethylbenz (a) anthracene (DMBA) を卵巣に直接局所的に作用されることで発生するラット卵巣癌の培養細胞株 (DMBA-OC-1) を樹立し，その生物学的特性の観察を試みた．

1) DMBA-OC-1 細胞は，10代から20代までは敷石状の増殖様式を示した．しかし，40代を越えた頃より，紡錘形を呈し，浮游性や重積性が強い増殖様式となつた．

2) 胞体内には，ジアスターゼ非消化性の PAS 染色陽性顆粒で，ヒアルロニダーゼ消化性の Alcian-blue 染色陽性顆粒が認められた．

3) DMBA-OC-1 細胞は著明な貪食能が認められた．

これらの所見は，DMBA-OC-1 細胞が，中皮細胞由来とする考えを支持するものと考えられる．