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Establishment and Characterization of New Cell Line (YMB-1) Derived from Human Breast Carcinoma*)

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ABSTRACT

A continuous cell line (YMB-1), derived from a primary breast cancer lesion has been established and maintained in culture for the past 9 months. Cell colonies consist of polygonal epithelial cells containing junctional complexes and tubular formation. The cell population doubling time is 44 hr and the saturation density at confluency is 2.5×10^5 cells/cm^2. The chromosome modal number is 73 and 3 marker chromosomes are visible. Both cytosol estrogen and androgen receptors are present. However, the cytosol progesterone receptor was not detected by the dextran coated chacoal method. The s. c. injection of 5×10^6 cells from cell culuture into nude mice induced a tumor.

Both the spillage method and cell separation by specific gravity were employed to remove fibroblasts.

INTRODUCTION

The establishment of long-term cultures of human breast cancer cells, especially from primary tumors, is difficult mainly because of fibroblast cells outgrowth[4,11,21,22]. In 1958, Lasfargues & Ozzello[9] succeeded in establishing the first long-term culture of human breast cancer cells. Engel et al.[3] discussed the characteristics of 47 putative human breast carcinoma cell lines. According to that review, 22 of the 47 lines had been derived from human and non-HeLa donors. Of the 22 lines, only 8 had estrogen receptors and all of the lines were derived from patients with pleural effusions or ascites.

Recently, we established a human breast cancer cell line (YMB-1) that has both estrogen and androgen receptors. The methodology of establishment and characterization of the cell line are presented in this report.

MATERIALS AND METHODS

1) *Cell orgin*

YMB-1 cultures were derived from primary carcinoma tissue from the breast of a 55-year-old Japanese female who underwent modified radical mastectomy. The size of the primary tumor was $1.8 \times 1.0 \times 1.0$ in cm. Histologically, the tumor was diagnosed as a well-differentiated ductal carcinoma (Fig. 1). Estrogen receptors were strongly positive as indicated by cytochemical staining[5,12,16].

2) *Preparation of the cell suspension*

The primary tumor was minced in a culture dish containing PBS (−) and cells were teased from the tissue. Released cells were collected in a centrifuge tube to which was added 5 ml of growth medium (RPMI 1640+15% FCS). After allowing the suspension to stand undisturbed for 30 min, the supernatant (Suspension 1) was separated from the pellet of cells at the

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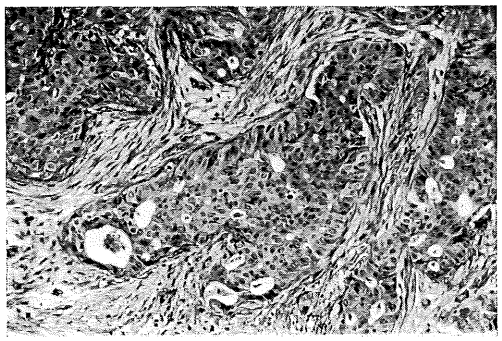

Fig. 1. Primary carcinoma tissue. Histological diagnosis was a well differentiated invasive ductal carcinoma. (×100 H. E.)

bottom of the test tube. The pellet of cells was resuspended in medium and the supernatant (Suspension 2) was removed after the mixture stood for 15 min. This procedure was repeated once more and Suspension 3 was collected after 10 min. Finally, Suspension 4 was obtained by adding growth medium to the remaining pellet.

3) *Cell Culture*

The cell suspensions were transferred either to plastic culture flasks (25 cm^2 growth area, Corning) or culture chambers (Lab-Tek Products) and cultured in a CO_2 regulated incubator (37°C, 5% CO_2, 100% humidity). Growth medium was changed every 2 or 3 days.

4) *Transmission Electron Microscopy*

Cells in monolayer from the culture chamber (Lab-Tek Products) were fixed in 2% gluteraldehyde, post-fixed with 1% osmic acid and embedded in Epon. Thin sections, cut on a Porter-Blum ultramicrotome, were stained with uranyl acetate and lead citrate and examined with a JEOL 100B electron microscope.

5) *Chromosome analysis*

Logarithmically growing cells were treated with Colcemid (10 mM), swollen with a hypotonic solution, and fixed in Carnoy's solution (methanol: acetic acid, 3:1). Following air drying, nuclei were stained with Giemsa for conventional morphological examination.

6) *Biochemical cytoplasmic estrogen, proges-terone, and androgen receptor assay*

After incubation in serum-free medium (HB 101™, Hana Media), cells were harvested with 0.25% trypsin. The dextran-coated charcoal assay was carried out at the Mitsubishi Yuka Labolatory of Medical Science Co., Ltd. The assay was done as published previously[13,14].

7) *Cytochemical staining for the estrogen receptor assay*

The detailed methodology for identification of cell estrogen receptor has been published previously[5,12,16]. Briefly, after fixation with 95% alcohol, the cells on the Lab-Tek culture slides were overlaid with 0.1 ml of fluorescent estradiol conjugate (17-estradiol-6-carboxy-methyl-oxime-bovine serumalbumin fluorescent, Zeus Scentific, Inc USA). The cells were examined using an Optiphoto fluorescent microscope (Nikkon).

8) *Cell-doubling time*

Cellular proliferation of cells incubated was measured by counting the number of cells from freshly trypsinized cultures. Counts were made every 24 to 48 hr for 10 days.

RESULTS

1) *Establishment of cultures*

Fibroblast outgrowth was uncontrollable in cells cultured from Suspension 1, 2, and 3. Only one subculture was possible from Sus-

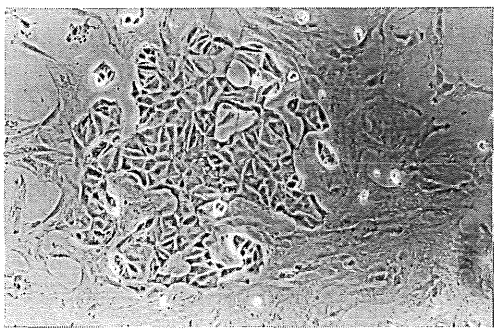

Fig. 2. Phase microscopy from Suspension 3. Epitherial colony is surrounded with many fibroblasts. 11 days after culture (×100)

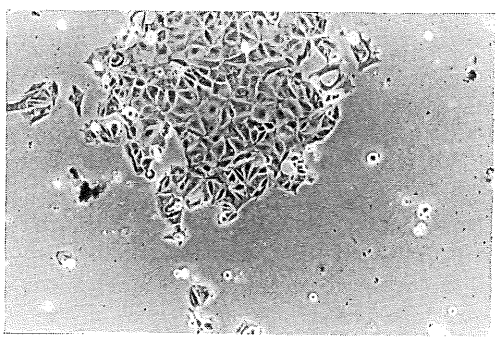

Fig. 3. Phase microscopy from Supension 4. Note a pure epithelial colony. No fibroblast outgrowth is seen. 11 days after culture (×100)

pension 3 (Fig. 2). Suspension 4 had pure epithelial colonies and no fibroblast contaminations (Fig. 3). After two subcultures, cellular proliferation gradually diminished. By 3 monhs after the initiation of the culuture most colonies had disappeared. However, after 4 months, the few remaining epithelial cells began to form colonies. After 5 months, one colony that had detached from the flask was trypsinized and transferred to a new flask. This colony (YMB-1, subline C) is now in its 20th passage and the cells continue to proliferate.

2) *Morphological features*

Colonies consisted of epithelial and polygonal cells. Extensive piling up of cells was ubiquitous. The cells had grown as tightly packed colonies but the colonies seldom merged to create a confluent monolayer. Culture cells partially formed tubular like structure resembling the orginal tissue (Fig. 4).

With electron microscopy, cell nuclei were seen to be ellipitical and their nucleoli were prominant. A few profiles of the rough endoplasmic reticulum and microvilli were evident (Fig. 5A). Fig. 5B shows tubular formations with many microvilli on the luminal surface and junctional complexes consisting of desmosomes and tight junctions between the lateral surfaces of cells near the lumen.

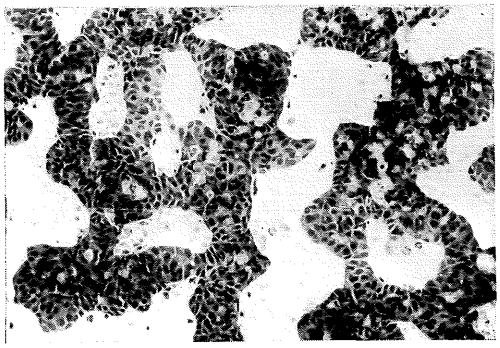

Fig. 4. Established cells on the culture chamber. Colonies consisted of epithelial and polygonal cells. Culture cells partially form tubular like structure. (×100 H. E.)

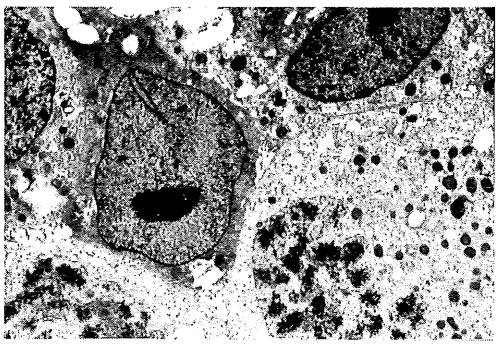

Fig. 5A. Electron microscopy (Lower magnification). Nucleoli are prominant and nuclei are ellipitical.

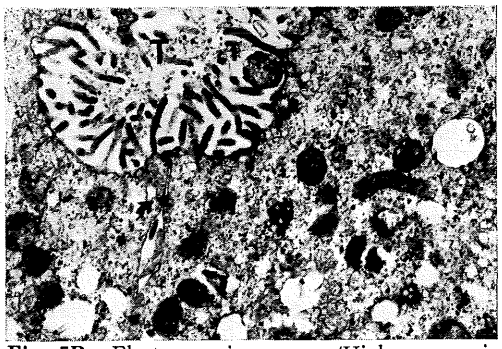

Fig. 5B. Electron microscopy (Higher magnification) Note desmosomes (→) and tubular formation (T) with many microvilli.

3) *Proliferative ability*

Growth curves of cells incubated in MEM+10% FCS showed that the population doubling time was 44 hr. The cell saturation density was 2.5×10^5 cells/cm^2 (Fig. 6).

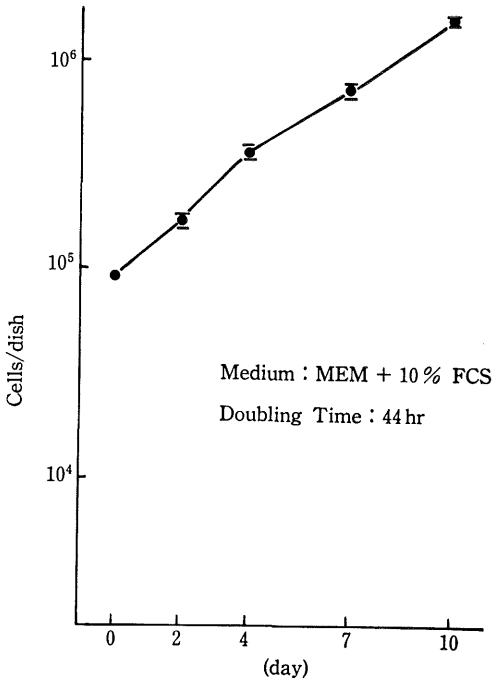

Fig. 6. Growth curve in MEM+10%FCS Doubling time is 44 hr and saturation density is 2.5×10^5 cells/cm^2.

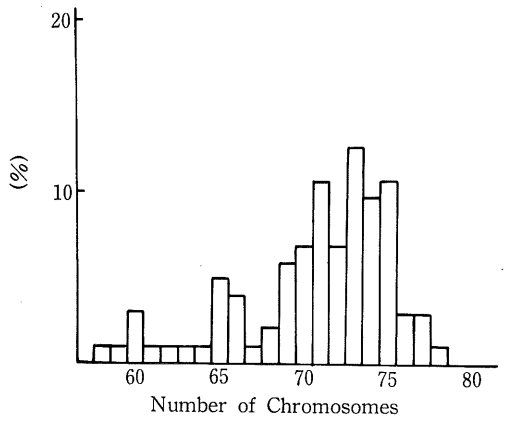

Fig. 7. Chromosome analysis at 8th passage Modal number is 73 in 103 observed cells

4) *Chromosome analysis*

The chromosome modal number at 8th passage was 73 (Fig. 7). Three abnormally large chromosomes (two acrocentric and one submetacentric type) are distinguishable with conventional staining (Fig. 8, M).

5) *Estrogen, Progesterone, Androgen receptor assay*

By the dextran coated charcoal method, estrogen receptors (binding sites: 38.0 fmol/mg cytosol protein, Kd: 31.8×10^{-10}M, inhibition rate: 93.2%) and Androgen receptors (binding sites 50.9 fmol/mg cytosol protein, Kd: 7.1×10^{-10}M, inhibition rate: 80.4%) are seen. However, the progesterone receptor was not detected.

Cytochemical staning of estrogen receptors shows many granular fluorescence in the cytoplasm (Fig. 9).

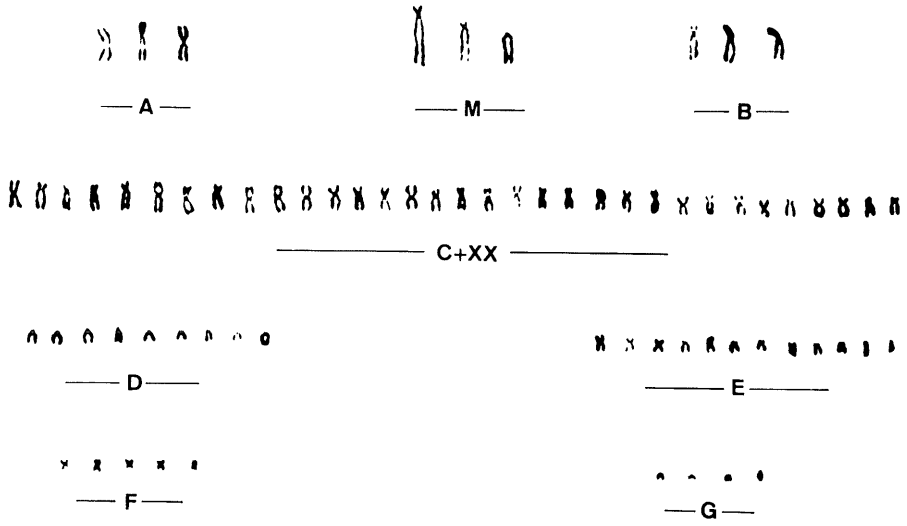

Fig. 8. Karyotype at 8th passage Three abnormal large chromosomes (M) are ditinguishable

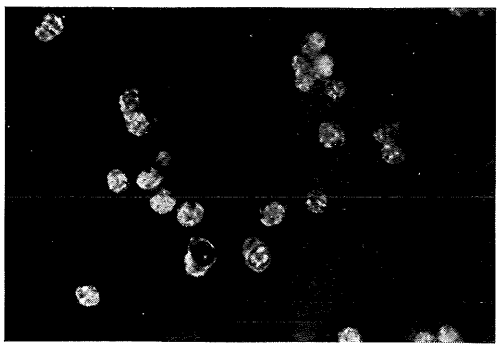

Fig. 9. Cytochemical staining of estrogen receptors. Note many granular fluorescence in the cytoplasm.

6) *Tumorigenicity*

Four nude mice were injected subcutaneously with 5×10^6 cells. One mouse produced a tumor (7 mm diameter) after 3 weeks. In soft agar culture, an average of 25 colonies (over 2^5 cells) were seen in three dishes seeded 5×10^4 cells (plating efficiency 0.05%).

DISCUSSION

The greatest difficulty in establishing long-term culture of human breast carcinoma cells is in controlling fibroblast outgrowth[4,11,21,22]. Several methods to prevent fibloblast outgrowth have been described, ie, incorporation of D-

valine instead of L-valine in the medium, hormonal supplementation and/or growth factors to serum-free medium[6,11], enzymatic technics[10,18] and the use of anti-fibroblast monoclonal antibodies[17]. However, these methods have not been universally successful in completely eliminating fibroblasts.

Separation of fibroblasts from epithelial cells at the initial period of primary culturing appears to be most effective for the establishment of long-term epithelial cell cultures. Although it is described in detail, the selection of epithelial cells by means of a Ficoll density gradients[7,20] is not technically simple and requires relatively large tissue samples because of the low recovery rate of cells after separation. Recently, Hiratsuka et al.[8] described a simple method for removing fibroblasts at the initial stages establishing cell cultures. Following cell dispersion with collagenase, epithelial and fibroblast cells were separated by allowing them to slowly settle in a centrifuge tube contaning growth medium. The differential rate of settling between the two cell types yielded enriched epithelial cell cultures. However, in our experiment, fibroblast outgrowth was still uncontrollable by the above procedure. To reduce the number of fibroblast cells, the spillage method[9] which produces fewer fibroblasts than the collagenase digestion method, was used with the settling technique of Hiratsuka et al.[8] YMB-1 cells were established with these methods and free of fibroblast contamination.

The cell population doubling time is 44 hrs and saturation density is 2.5×10^5 cells/cm^2. The chromosome modal number is 73 and 3 marker chromosomes are visible. Both cytosol estrogen and androgen receptor are present. YMB-1 cells microscopically formed epithelial colonies and tubular-like structures resembling the original tissue. With electron microscopy, desmosomes, tonofibrills and tubular formations were observed.

Electron microscopical findings and precence of estrogen and androgen receptors strongly support for mammary origin[2,15] and potential usefulness for in vitro model system of hormonal responsiveness.

According to the review by Engel & Young[3] all of 7 cell lines possessing both estrogen and androgen receptors were derived not from primary lesions but pleural effusion or ascites. Conversely, YMB-1 was derived from a primary lesion. On the stand point of cell origins, YMB-1 is thought to be an unique and interesting cell line.

It may be the interesting problem whether any biological differences exist between cell lines derived from primary lesions and metastatic lesions. Experiments to characterize the responsiveness to variable hormones, production of α-lactoalbumine[19] and casein[1] are being conducted.

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