Establishment of a cell line of human endometrial adenocarcinoma in vitro

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A new in vitro cell system, Line human endometrial cancer-one (HEC-1), derived from adenocarcinoma of human endometrium has been established and has successfully proved stable proliferation in continuous tissue culture since May, 1968. The cytologic findings of HEC-1 reveal such anaplastic features as anisonucleosis, nucleolar pleomorphism, and piling-up tendency in cellular arrangement. Distribution of the chromosome number is found at the diploid range, and the apparent marker chromosome has been identified without exception. When transplanted into a hamster cheek pouch, HEC-1 demonstrates a definite tumor formation whose histologic picture reveals papillary adenocarcinoma. The original characteristics of endometrial cancer have been precisely maintained in this in vitro culture system.

Compared with cancer of the uterine cervix, carcinoma of the corpus uteri has its unique features, especially those of a close relationship with sex steroid hormones. The association of hyperestrinism and carcinogenesis of the endometrium has been debated for many years, and the animal experiments on malignant transformation of the endometrium with or without estrogen administration have been extensively reported. However, the results have not been very conclusive. And, since few studies of the human endometrium in this field have been done, the precise results have not yet been achieved. On the other hand, clinical data suggest the favorable carcinostatic effect of progestogen for the treatment of endometrial cancer. The scientific proof of this phenomenon, however, has not been cleared yet, mainly because there has been a lack of suitable materials for the fundamental research work.

In detecting the characteristics of human endometrial cancer, the in vitro culture provides one of the most valuable experimental means on a cellular level. For this reason, we have been trying to culture cancer of the body of the uterus and, among those trials, have successfully obtained a cell line from adenocarcinoma of the endometrium, which has been permanently propagating since May 16, 1968. The establishment of the carcinoma in continuous tissue culture has never been reported, and this new cell system, named human endometrial cancer-one (HEC-1), will be quite instrumental for the further investigation of adenocarcinoma of the corpus uteri.

When cultured in vitro, the cancer cells should be properly identified. For this purpose, we tried to analyze the cytologic findings of the primary cultured cells compared to those of normal endometrium. Chromosome analysis is also quite instrumental in identifying the malignant cells. Furthermore, the transplantability of HEC-1 cells to heter-
ologous animals must also be evaluated, and tissue reconstruction of the established tumor would definitely show the original features of the nonstructured culture cells.

**Material and methods**

The patient was a 71-year-old woman, gravida 4, para 4, last pregnancy at 25 years of age, who noted a small amount of vaginal spotting intermittently for one month's duration. She had had no vaginal bleeding since menopause at 50 years of age. The blood type was B and Rh(D) positive. With the clinical diagnosis of Stage IA endometrial cancer, the patient underwent vaginal simple total hysterectomy and bilateral salpingo-oophorectomy on May 16, 1968. The vaginal approach was preferred to the abdominal one as the method of the lesser surgical intervention for this aged woman. The extirpated uterus, normal in size, showed circumscribed tumor growth in the lower segment of the corpus uteri. The histologic findings of the tumor revealed moderately differentiated papillary adenocarcinoma (Fig. 1). The patient's postoperative course was uneventful.

**Culture studies.** Primarily, an explant plasma clot culture was made for the first 3 generations, and then cultured cells were transferred into the monolayer system which has been precisely maintained up to the present. As for the explant culture, the tissue material was obtained from the central portion of the malignant lesion in the surgically extirpated uterus and was immediately placed in culture. The tumor specimen was minced into fragments 2 to 3 mm. in size, and 3 pieces of these were mounted separately on each glass cover slip, which was coated by the matrix of chicken plasma and embryonal extract. Two cover slips, placed back to back, were cultured in each roller tube containing 2 ml. of culture medium. Twenty tubes were prepared for this experiment and placed in a drum which rotated 12 times an hour. Daily observation of the living cells was done with a microscope. After approximately 2 days of a stationary period, each specimen developed favorable outgrowth. Some of the specimens were fixed and stained for closer observation.

In order to subculture, a cross incision was made in the culture explant with a razor blade, and each piece, one fourth the size, was re-explanted in the same manner.

At the third generation, 2 cover slips with well-grown cells were replaced from a roller tube to Petri dishes and cultivated in 5 per cent carbon dioxide and 95 per cent air for one week. Then the cells were dispersed into a single-cell suspension by a mixed solution of 0.1 per cent trypsin and 0.02 per cent ethylenediaminetetra-acetate (EDTA) in order to transfer them into a monolayer culture. The dispersion was assisted by rather forcible pipetting to detach the cells from the matrix of the plasma clot more easily. The cell suspension was floated to the bottom of the stationary culture bottle. Subculture was made every 7 to 10 days, being diluted to three- to five-fold of the cells at maintenance culture.

**Growth medium.** Twenty per cent calf serum and 80 per cent Hank's solution were chosen for the plasma clot culture, and for the monolayer culture 15 to 20 per cent calf serum with TC-199 supplemented by 100 U. per milliliter of penicillin and 100 g per milliliter of streptomycin was utilized. Serum was obtained from a calf approximately 4 months old. We tried to use fetal calf sera, but these were not effective. One of the reasons may possibly be that progesterational agents were contained in the fetal calf sera, although this will remain unsolved until a thorough evaluation can be completed. Culture media were exchanged once every 2 to 3 days.

**Preparation for chromosome analysis.** Approximately $5 \times 10^4$ to $1 \times 10^5$ cells were inoculated into a square tube (40 by 17 by 8 mm. in size, MA-8 Miharu) which contained a cover slip, 32 by 12 mm. in size. Following 3 to 7 days of usual culture, the medium was exchanged with the one containing Colcemid, $5 \times 10^{-7}$ moles per liter, and the cells were incubated for one to 2 hours in order to arrest the metaphase. The slip with HEC-1 was treated by a hypotonic solution, being carefully placed into 0.7 and 0.5 per cent sodium chloride solutions, respectively, for 1 minute and then in 0.2 per cent sodium...
chloride solution for 20 to 40 minutes. Then the cells were fixed by each of 1, 10, and 100 per cent Carnoy solutions (3:1 acetoalcohol), respectively, for one to 2 minutes and then for 15 minutes by the new absolute Carnoy solution. After drying it in the air, the specimen was stained by 10 per cent Giemsa solution (pH 6.8).

For chromosome analysis, 100 metaphase cells were observed in order to calculate the modal ploidy, and 50 of the modal range were counted exactly to evaluate the chromosomal number. Finally, the karyotype of these on the modal number was carefully analyzed in accordance with Denver nomenclature.

Transplantation into the hamster. An immature hamster, approximately 4 weeks old, weighing about 50 grams, was used for the experiment. Sex may not be an important factor, although we customarily used immature female hamsters. The cultured cells were dispersed into single cells by the trypsin-EDTA solution and then were washed with culture media and centrifuged twice. The sediment which contains 1 to 10 x 10⁶ cells was injected into the submucosa of the hamster cheek pouch by a blue syringe with a No. 22 gauge needle. The hamster was anesthetized by 2 mg. of Nembutal* intraperitoneally. In order to enforce immunologic tolerance, cortisone acetate, 2.5 mg. per 50 Gm. of body weight, was given subcutaneously to the hamster on the day when the cells were injected and then once every 4 days.

Results

Morphology and growth characteristics. Most of the outgrowth cells of the primary explant culture were quite obviously of the sheetlike epithelial type which grew in multilayers, and these nuclei were observed to have anisonucleosis, thickening of the nuclear membranes, enlarged nucleoli with pleomorphism, and an occasional presence of more than 2 nucleoli in each nucleus (Fig. 2). Simultaneously, normal endometrium was also cultured as control material, and these findings (Fig. 3) definitely differed from those of cultured cells of endometrial cancer. Some roller tubes showed fibroblastic outgrowth rather than epithelial cells, and these were discarded. One which revealed epithelial outgrowth was selected for subculture. The cell population became a quite pure epithelial cell growth through repeated subculture.

After the transfer at the third generation to a monolayer culture, the cultured cells which presented mainly a firm growth curve appeared morphologically to be exactly polygonal epithelial cells in shape, showing a jigsaw puzzle-like cellular arrangement (Fig. 4). Monolayer-cultured cells were also arranged in a wheeled pattern, especially in the rapidly growing area (Fig. 5). Their nuclei still retain their original appearance as in the primary culture (Fig. 2). A piling-up tendency in the growing process has also been a prominent feature, and, when confluent, the cultured cells have produced not infrequently cauliflower-like agglomerations (Fig. 6).

Chromosome analysis. Chromosome analysis was performed on the cells at the eighth generation. One hundred and three mitotic figures were carefully studied. Distribution of the chromosomal number is shown in Fig. 7. A majority of the metaphase cells were apparently found at the diploid range. An exact count of this modal range was done for further evaluation, showing the modal number to be 47 (50 per cent) (Fig. 7). A karyotype histogram of the stem cell (Fig. 8) revealed the characteristic feature of the apparent marker chromosome, which is the largest in size and has a submetacentric constriction. This marker chromosome was always present. The short arm was very similar in its length to that of the A₂ chromosome. A part of the A₂ pair and one of D chromosomes were absent. From these findings, we postulate that a D chromosome translocated to the missing A₂, thus developing the marker chromosome. However, this will remain unsolved until further investigation is carried out. The number of C group chromosomes was calculated at 17. There is a possibility

Fig. 1. Histology of the original tumor showing papillary adenocarcinoma. (Hematoxylin and eosin. Original magnification x170.)

Fig. 2. Magnified view of the primary cultured cells revealing such anaplastic features as anisokaryosis, thickening of nuclear membrane and nucleolar pleomorphism. (Giemsa. Original magnification x400.)

Fig. 3. Primary cultured cells of the normal endometrium. Compare with Fig. 2. (Giemsa. Original magnification x400.)
Fig. 4. Phase-contrast microscopic findings of the monolayer-cultured cells revealing the sheet of polygonal cells with jig-saw puzzle-like arrangement. Eleventh generation.

Fig. 5. Monolayer-cultured cells showing a wheeled pattern on the right upper portion. Thirty-third generation. (Giemsa. Original magnification ×100.)

Fig. 6. Piling-up tendency of the cells producing cauliflower-like aggregations. Phase-contrast. Eleventh generation.
that a pair of X chromosomes may be included in this group. However, we could not confirm the apparent sex chromatin body.

**Heterotransplantation.** Submucosal transplantation of the cultured cells was carried out on the cheek pouches of hamsters where definite tumor formation took place. A histologic picture of this resultant tumor (Fig. 9) demonstrated the typical papillary and adenomatous patterns and, under high magnification, revealed the malignant features of the tumor, which were precisely the same as that of adenocarcinoma. Therefore, these findings reveal that these in vitro cells still maintain the potential to disclose their original characteristics, if they are in the appropriate condition.

**Comment**

Among human cancers in the gynecologic field, in vitro cell lines, such as HeLa cells\(^4\) of epidermoid cancer of the cervix and the BeWo line\(^5\) of choriocarcinoma, have been used in experimental research. A similar and appropriate experimental system with an authentic cell line of human adenocarcinoma of the endometrium has now been developed and presented.

Certain key points should be kept in mind when culturing cancer material. First, it must be certain that cancer cells and not normal cells are put into culture. For this, the method of primary cloning\(^6\) has often been used, but it is not successful in human cancer. The classical plasma clot culture has been a convenient method for a primary culture and has a favorable success rate. The famous cell lines, including HeLa cells\(^4\) were reportedly obtained by this method and then transferred to the monolayer system which enabled a more detailed analysis in many respects. We tried various methods and finally adopted a very simple one, which happened to be the old formula of culture. In addition, we subcultured 3 times by the plasma clot method, selecting the tube in which the cultured cells showed the epithelial outgrowth. These segregated subcultures apparently imitated a sort of cloning technique. Our multiple studies indicate that cancer of the corpus uteri has been successfully cultured in vitro.

Once placed in vitro, the second point is how stable the characteristics of these cells will remain after a long-term cultivation. We have been working with the HEC-1 line with various approaches ever since the primary culture, and the cell characteristics have remained fairly stable. A chronologic study of this new cell line will be reported elsewhere.

Wakonig-Vaartaja\(^7\) and Baker\(^8\) reported that certain types of cases of endometrial cancer had their own specific marker chromosome. We found that the HEC-1 had a distinct marker chromosome which was quite stable all through the long period of culture. We postulate that the existence of
the marker chromosome is closely related to type of differentiation in each cancer. Relationship between the marker and the character of endometrial cancer, however, still remains unproved.

We have not encountered any previous report, other than that of HEC-1, which investigated the formulation of a glandular or papillary structure when an in vitro cell line was transplanted to a heterologous animal. Easy transplantability and reproducibility of the original in vivo histologic findings make this cell line a convenient experimental system for the study of cancer morphogenesis in papillary adenocarcinoma of the endometrium. In addition, the secreting activity of a functional endocrine tumor serves as the specific marker for the identification of its tumor cell. Patrillo and Gey used the identifiable chorionic gonadotropin, produced by chorioniccarcinoma, to select the functional cell types for culture and observed no alteration of the marker during the long time of cultivation. Likewise, the in vivo gland-forming ability of HEC-1 is also a convenient marker which accomplished the identification of the adenocarcinoma cells, and, through periodic confirmation, it will provide the solid evidence that the cells still keep the stable genetic character of adenocarcinoma.

Since the report of Kelley and Baker, progestogen therapy has been considered to produce a favorable effect, to a great extent, for patients with endometrial cancer. Nordqvist and Kohorn and Tchao independently reported degenerative necrosis of endometrial adenocarcinoma in organ culture when a progestational agent was added to the culture media. The former also stated that deoxyribonucleic acid synthesis of the cultured tissue was suppressed by progestogen. We have data similar to theirs with the use of the HEC-1 (to be published). These reports give support to the clinical application of progestogen for patients with cancer of the corpus uteri. Furthermore, this new cell system may aid in clarifying the precise mechanism of progestogen therapy from both the quantitative and qualitative aspects against the endometrial cancer.

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