Morphological, Biological, and Biochemical Characteristics of a Benign Human Trichilemmoma Cell Line in Vivo and in Vitro

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Introduction

Trichilemmoma (5, 6, 15, 20, 24) is a benign human tumor of epidermal appendages, arising from or differentiating toward the trichilemma (outer root sheath of the hair follicle). Although some tumors grow rapidly, it is well differentiated and well organized histologically and is benign biologically (5, 6, 15, 20, 24).

We had a case of trichilemmoma, misdiagnosed as basal cell epithelioma (carcinoma) because of its clinical appearance. We initiated tissue culture of this tumor and have established a benign human tumor cell line in vitro. This report describes the morphological, biological, and biochemical characteristics of this trichilemmoma cell line in vivo and in vitro, including tumorigenicity in nude mice.

Materials and Methods

Clinical Summary. A patient, a 68-year-old Japanese female, showed a large tumor on her scalp in February 1979. In her twenties, the patient had noted an asymptomatic, small (5 x 5 mm in diameter), reddish tumor on the left temporal area, but she had left it alone for over 40 years. The tumor did not change in size during this period. In June 1978, the tumor bled for the first time after a traumatic brushing with a comb and then started to grow aggressively. The tumor was elastic, soft, and 7 x 7 x 3 cm in size (Fig. 1) in February 1979. The surface of the tumor was eroded with telangiectasia. It appeared yellowish and somewhat translucent. The eroded surface was coated with pus. The left cervical lymph nodes were softly swollen and freely movable.

Tissue Culture. The details of the tissue culture methods have been reported previously (14, 17, 18). Briefly, tumor tissue obtained at surgery was minced into 1-cm pieces, and primary culture was initiated in 35-mm plastic dishes containing glucose as a source of monosaccharide, supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.), penicillin (100 units/ml), and streptomycin (100 µg/ml) (Grand Island Biological Co.) in a humidified incubator (New Brunswick Scientific Co., Inc., Edison, N. J.) with 5% CO2 in air at 37°. Tumor cells migrated out from the primary explants on the third day of culture and then propagated slowly but continuously in vitro. The culture became free from fibroblasts after repeated treatments with 0.25% trypsin. Subculture was done with 0.25% trypsin in 0.5% EDTA solution (Toshiba Kagaku Kogyo Co., Tokyo, Japan) every 2 to 4 weeks. Growth factors, 3T3 fibroblast feeder layers, or collagen gel substrates were not used in this culture at any time except when special studies were performed (see below). We did not have HeLa cells in our laboratory. This study was performed by using cells cultured in vitro for 7 to 10 months (6 to 10 passages) after primary culture. Morphological, biological, and biochemical characteristics have not been changed since the study.

Morphological Studies. The light-microscopic observations of the original tumor were made after stains with HE,3 PAS with and without amylase digestion, and Sudan III solutions. For the electron-microscopic studies, the original tumor was fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and processed thereafter for electron microscopy as described previously (14, 17). Thin sections were stained with uranyl acetate and with lead citrate (28) and observed in a Hitachi HU-12A electron microscope at 75 kV. Light-microscopic examination of tissue-cultured cells was made with a Nikon phase-contrast microscope with an attached camera or made after stains with HE or PAS solutions. For electron-microscopic studies, cells were cultured on thin-sectionable plastic coverslips (Microbiological Associates, Inc., Bethesda, Maryland). We did not have HeLa cells in our laboratory. This study was performed by using cells cultured in vitro for 7 to 10 months (6 to 10 passages) after primary culture. Morphological, biological, and biochemical characteristics have not been changed since the study.
Benign Human Trichilemmoma Cell Line

Md.), fixed in situ with 1% glutaraldehyde and processed thereafter as described previously (14, 17).

**Biological Studies.** Population doubling time was estimated by daily count of cells up to 14 days after seeding. Plating efficiency was examined after seeding of $10^5$ to $10^6$ cells onto 35-mm plastic dishes. Saturation density was obtained by counting cells at the time of a monolayer. One hundred to $10^6$ cells/35-mm dish were seeded on fibroblast monolayers (1) or in soft agar (22) to observe cell growth in these conditions.

**Biochemical Studies.** Since tissue-cultured cells synthesized abundant glycogen in vitro, glycogen content in cells was determined by the phenol-sulfuric acid method (9). Extracted glycogen was analyzed by gas chromatography (34) to determine the constituent carbohydrate.

**Chromosome Analyses.** Chromosome analyses of 100 tissue-cultured cells were done with conventional Giemsa staining (12) and with trypsin-Giemsa banding by the method of Seabright (30).

**Tumorigenicity.** Fifteen athymic nude mice (NIH strain, Nihon Clea, Tokyo, Japan) were inoculated s.c. with 2.5 X $10^6$ to 4.5 X $10^7$ tissue-cultured cells. Tumorigenicity was observed for 6 to 9 months after inoculation.

**Preservation of Cells by Freezing.** One to 2 million cells were suspended in 1 ml of normal culture medium containing 5% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) and frozen at $-80^\circ$. More than 90% of cells were viable at thaw, but all of them died with swelling in 24 hr when cultured in normal culture medium. However, 30 to 50% of cells were viable and propagated when cells were cultured in Ca$^{2+}$ (2.8 mM)-added medium.

**Mycoplasma Culture.** Tissue-cultured medium containing 1 X $10^6$ cells was spread onto Mycoplasma agar and fluid broths (BRL Division of Becton, Dickinson, and Co., Cockeysville, Md.) and allowed to incubate at $37^\circ$ for 4 weeks.

**RESULTS**

**Light- and Electron-microscopic Studies of the Original Tumor.** The tumor was solid and lobulated (Fig. 2, A and B). Tumor cells were well organized and well differentiated without atypia (Fig. 2, B to E). Cells at the periphery of lobules were cylindrical and palisading on basal membrane (Fig. 2, B to E). The cell shape gradually became cuboidal toward the center of lobules (Fig. 2, B to E) where microabscesses were present (Fig. 2, B, D, and E). There was little evidence of cellular keratinization or cyst formation. The cytoplasm of the tumor cells was clear with HE stain (Fig. 2, B and C). The cytoplasm contained abundant PAS-positive granules which were digested by amylase (Fig. 2, D and E). No cytoplasmic lipid droplets were detected with Sudan III stain. Thus, histological appearances of the original tumor resembled those of trichilemmal cells (outer root sheath of the hair follicle). Fibroblast proliferation, neutrophil infiltration, and hyalin substance were observed in the stroma (Fig. 2, B to E). Electron microscopically, tonofilaments and glycogen granules of dispersed (6) type were observed in cytoplasm, and desmosome-tonofilament complexes were observed at cell-to-cell junction sites (Fig. 3). Particle-like inclusions (45 nm in diameter) were observed in nuclei (Fig. 3).

**Tissue Culture and Cell Kinetics.** Many epithelial cells migrated out from the primary explants on the third day of culture and made large sheaths (7 mm in diameter) on the fifth day. However, cell propagation was very slow, and it took 10 weeks to make a 20-mm-diameter sheath. The first passage was successfully done at the tenth week. Subculture was repeated every 4 weeks until the sixth month in culture (fourth passage). About this time, it was found that an extremely large number of cells ($10^6$ cells/sq cm) was required for subculture to make cells propagate well. By satisfying this requirement, cells propagated steadily, and subculture was done every 2 weeks. The plating efficiency was very poor, less than 0.1%. When $10^4$ cells were seeded in 35-mm dishes, only a few (3 to 5) colonies consisting of more than 50 cells were made in 4 weeks. Cell propagation greatly depended upon cell density. When $10^5$ cells/sq cm were seeded, cell number became double on the tenth day (the population doubling time was 10 days). The cell viability was 98%. When $5 \times 10^6$ cells/sq cm were seeded, it took 4 to 8 weeks to reach a monolayer. The saturation density at a monolayer was $10^6$ cells/sq cm. When $10^5$ cells/sq cm were seeded, many cells died slowly with cytoplasmic vacuolization, and the propagation to death ratio of cells was one. Thus, these cells did not reach a monolayer in 4 months of culture. When culture was continued after a monolayer, cells propagated continuously, making double to triple layers in 10 to 14 days from a monolayer. Cells started to pile up after reaching triple layers. In the older culture, more than 4 weeks in culture, some cells of the uppermost layer became flat and detached from the underlying cells. These cells looked like stratum corneum cells in vivo.

**Morphological Studies of Tissue-cultured Cells.** Light microscopically, cells were flat and epithelioid (Fig. 4, A and B). Electron microscopically, tonofilaments and desmosome-tonofilament complexes were observed in cytoplasm and cell-to-cell junction sites, respectively (Fig. 4C). Half desmosomes were observed at the cell membrane where it was lying against plastic substrate. Glycogen granules of the dispersed type were observed in cytoplasm (Fig. 4C). Intranuclear inclusions observed in the original tumor were not observed in tissue-cultured cells. In older cultures, the cell membrane of flattened cells was highly invaginated. The inner cell membranes in these cells were thickened; i.e., marginal bands (13) were formed, thus showing evidence of cellular keratinization in vitro.

**Biochemical Studies (Glycogen Analysis).** Virtually 100% of the cells synthesized abundant glycogen in vitro. Histochemically, cytoplasm stained strongly with PAS stain (Fig. 4A) but did not stain after amylase digestion (Fig. 4B). Electron microscopically, glycogen granules were observed in cytoplasm (Fig. 4C). Qualitatively, the glycogen fraction extracted from these cells showed only one peak at the glucose position by gas chromatography, meaning that the glycogen fraction was composed of glucose alone without other monosaccharides such as fucose, mannose, or galactose. Quantitatively, 50 to 100 $\mu$g of glycogen were present in $10^6$ cells.

**Chromosome Analyses.** One hundred metaphase cells were examined with Giemsa and trypsin-Giemsa stains. Chromosome number varied from 42 to more than 150 with hypodiploid modal numbers of 44 and 45 (Table 1). Four marker chromosomes were identified by trypsin-Giemsa banding (Fig. 5). All of these 4 marker chromosomes were present in 100% of cells. A typical karyotype was 45,XX,1p+, del(1)(p13), del(3)(p13), 11q+, -15, -18, -19, -21, -21, del(22)(q13), +mar1, +mar2, +mar3, +mar4 as shown in Fig. 5.
Tumorigenicity. Tissue-cultured cells (2.5 \times 10^6 to 4.5 \times 10^7) were injected s.c. into 15 nude mice. When less than 10^7 cells (2.5, 5.0, and 7.5 \times 10^6 cells each) were injected into 3 mice, no mouse developed a tumor. However, when more than 1.5 \times 10^7 cells (1.5, 3.0, and 4.5 \times 10^7 cells, each in 4 mice) were injected, tumors appeared in all mice (12 of 12). Small tumors first appeared in 2 to 3 weeks, and these tumors then grew continuously. Tumors reached 1 to 2 cm in diameter in 2 to 3 months (Fig. 6A). Tumor cells grew well when placed back in the culture. These tumors have been serially passed through 6 generations of nude mice and have maintained histological patterns of growth which resembled those of the original tumor (Fig. 6B).

No Mycoplasma was detected in repeated cultures.

DISCUSSION

Trichilemmoma has been classified under benign pilar epitheliomas of outer root sheath origin (5, 6, 15, 20, 24). Since tumor growth is very rapid and since tumor cells may be clear with HE stain, at one time, trichilemmoma was confused with clear cell carcinoma (21), sebaceous carcinoma (4, 31), or sweat gland carcinoma (33). A similar tumor, named proliferating trichilemmal cyst (35) or pilar tumor of the scalp (20), is also considered to originate from or differentiate toward the outer root sheath of the hair follicle, but this tumor shows extensive keratinization and cyst formation (7, 20, 27, 35). Similar tumors and tumors seemingly identical to trichilemmoma or proliferating trichilemmal cyst have been reported as trichochlamydocarcinoma (16). One of 7 cases was reported to have metastasized to a regional lymph node (16). Although our clinical impression was that the tumor was malignant, it was benign histologically. No lymph node metastasis was present despite such a long period of neglect. The patient was healthy and showed no local recurrence or distant metastasis 1.5 years after the surgical excision.

Histogenesis of trichilemmoma is unknown, but it has been suggested that the cause is a papilloma virus infection (2, 11). If so, the intranuclear inclusions observed in this tumor may be viral inclusions, although these particles (45 nm) were slightly smaller than those of typical papilloma viruses (53 nm). Further studies are certainly needed to elucidate the onogenesis of pilar epithelioma.

The reason we have successfully established a cell line of a benign human tumor without using epidermal growth factors, collagen gel substrates, or 3T3 fibroblast feeder layers is the finding that the cell propagation greatly depended upon the cell density; more than 10^6 cells/sq cm (a monolayer of cells) were required for the subculture to make cells propagate well. This phenomenon is extremely unusual in cell cultures, because the cell propagation is well known to cease or decelerate in the most benign or malignant cells after reaching a monolayer. It is not known at present why the cell propagation accelerates rather than declines after reaching a monolayer in this cell line.

Very poor plating efficiency, slow propagation, and no growth on fibroblast monolayers (1) or in soft agar (22) in vitro suggest that these cells are benign. A pile-up phenomenon which was observed in this cell line does not necessarily indicate cancer, because benign epidermal keratinocytes pile up in vitro (8, 23, 29). It was therefore a surprise to observe a successful growth of tumors in nude mice after the injection of these benign tumor cells. In the literature, however, benign human tumors have been reported to grow or produce tumors in athymic nude mice; a benign teratoma and 3 other benign tumors have been reported to have grown well in immunosuppressed mice (3). A fibrocytic disease with duct papillomatosis showed abundant growth in a nude mouse (26), and a papillary adenoma of the large bowel grew appreciably in a nude mouse (36). Likewise, 3 cases of benign mixed tumors of the salivary gland grew in nude mice (32), and the RPMI 4098 cell line derived from normal blood lymphocytes grew in nude mice (10). Therefore, our benign tumor is apparently not the first one to show tumorigenicity in nude mice. However, all of the above-described benign solid tumors were made by the direct transplantation of tumor tissues and not by the injection of tissue-cultured cells. In other words, none of these tumor cells was developed into a continuous line like ours. Taking advantage of the tissue culture system, our original tumor and its cell line could be extensively investigated and thoroughly documented clinically, morphologically, biologically, biochemically, and karyologically.

Trichilemmoma cells in culture and in nude mice have synthesized abundant glycogen, as do normal hair follicle cells and trichilemmoma cells in vivo. Glycogen synthesis in vitro appears to be unique to trichilemmal cells, because epidermal keratinocytes and squamous cell carcinoma cells [COLO 16 (25)] do not synthesize glycogen appreciably in vitro. 4 The glycogen metabolism in these cells in vitro has been reported elsewhere (19).

Chromosome analysis revealed the presence of 4 marker chromosomes in 100% of the metaphases analyzed. One marker (M1) appears to be a metacentric. Banding pattern indicated that the M1 marker was an 18p+. Other markers (M2, M3, and M4) were too small to identify their origins. The most interesting and significant finding is the presence of 4 marker chromosomes in all cells examined. This result strongly suggests that the large tumor observed clinically was monoclonal in origin.

In summary, we have reported the establishment of a cell line of a benign human tumor, trichilemmoma, in vitro. To the best of our knowledge, this is the first time a long-term cell line has been established from a benign tumor of the human skin.

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REFERENCES

Fig. 1. The original tumor (trichilemmoma) of the head.

Fig. 2. Histological sections of the original tumor. The tumor is solid and lobulated (A and B). Tumor cells are well organized and well differentiated (B to E). Cells at the periphery of lobules are cylindrical and palisading on basal membrane (B to E). Tumor cells are clear with HE stain (B and C). The cytoplasm stained with PAS (D) but did not stain after amylase digestion (E). Microabscess is present in the center of tumor lobules (B, D, and E). Neutrophil infiltration, fibroblast proliferation, and hyalin substance are present in the stroma (B to E). A to C, HE stain, x 3, x 160, and x 320, respectively. D and E, PAS stain without (D) and after (E) amylase digestion, x 320 and x 320, respectively.
Fig. 3. An electron micrograph of the original tumor. Tonofilaments (T), desmosome-tonofilament complexes (D-T), and glycogen granules (G) are seen. Intranuclear inclusions (Ic) which are magnified in the inset are observed. $\times$ 12,600. Inset, $\times$ 67,000.
Fig. 4. Tissue-cultured trichilemmoma cells. A and B, PAS stain without (A) and after amylase digestion (B), × 320 and × 320, respectively. C, an electron-microscopic photograph showing tonofilaments (T), desmosome-tonofilament complexes (D-T), and glycogen (G). × 40,000.
Fig. 5. Metaphase of a tissue-cultured trichilemmoma cell with 45 chromosomes. XX, 1p+, del(1)(p13), del(3)(p13), 11q+, -15, -18, -19, -21, -21, del(22)(q13), +mar1(M1), +mar2(M2), +mar3(M3) and +mar4(M4). M1, M2, M3, and M4 are marker chromosomes. Trypsin-Giemsa stain, x 8,000.

Fig. 6. In A, tissue-cultured trichilemmoma cells (3 x 10^7) were inoculated into a nude mouse. Ten weeks after the injection, a large tumor developed at the site of inoculation. B, the histological appearance of the tumor in a nude mouse. Note the remarkable similarity of the tumor histology in the nude mouse to that of the original tumor in Fig. 2. HE stain, x 320.
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