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

Tamotsu Kanzaki,1,2 Hikaru Eto, Akira Umezawa, Tohru Maeda, Hitoo Iwase, and Masatsugu Ito

ABSTRACT

A cell line of a benign human tumor, trichilemmoma, was established in vitro and has been maintained in culture for 1.5 years with more than 30 passages. Plating efficiency was less than 0.1%, and population doubling time was 10 days. Saturation density was $10^6$ cells/sq cm at the time of a monolayer with 98% cell viability. Ultrastructurally, tissue-cultured trichilemmoma cells showed desmosome-tonofilament complexes at cell-to-cell junctions. The tissue-cultured cells synthesized abundant glycogen (50 to 100 $\mu$g/106 cells) as observed in vivo. Gas chromatographic analysis revealed that extracted glycogen was composed of glucose alone. Chromosome analyses with trypsin–Giemsa banding showed an abnormal karyotype with hypodiploid modal numbers of 44 and 45. There were four marker chromosomes observed in 100% of cells in 100 metaphase cells examined. Cells did not grow on fibroblast monolayers or in soft agar in vitro but did induce tumors in athymic nude mice (12 of 15) after the s.c. injection of tissue-cultured cells ($2.5 \times 10^6$ to $4.5 \times 10^7$ cells/mouse). The histological characteristics of the tumors in nude mice were similar to those of the original tumor. This is the first time, to our knowledge, that a benign human tumor cell line has been established in vitro which can induce tumors in nude mice.

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 (Falcon Plastics, Los Angeles, Calif.) using Eagle’s minimal essential medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan), which contained 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 $\mu$g/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,

---

1 This work was supported by research grants from the Japanese Dermatological Association and the Lydia O’Leary Memorial Foundation.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: HE, hematoxylin and eosin; PAS, periodic acid-Schiff.
Benign Human Trichilemmoma Cell Line


Department of Surgery, The Chinese University of Hong Kong

JUNE 1981 2469

About this time, it was found that an extremely large number of cells (10^6 cells/sq cm) were required for subculture to make cells propagate well. By satisfying this requirement, 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. Histochromically, 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 μg of glycogen were present in 10^6 cells.

Chromosome Analyses. One hundred metaphase cells were examined with Giemsa and trypsin-Giemsa stains. Chromosome numbers 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 100 cells examined. 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.
This phenomenon is extremely unusual in cell cultures, because required for the subculture to make cells propagate well. Cell density; more than 10^5 cells/sq cm (a monolayer of cells) finding that the cell propagation greatly depended upon the collagen gel substrates, or 3T3 fibroblast feeder layers is the tumor growth is sometimes 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 immunocompromised 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.

Table 1

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>42</th>
<th>43</th>
<th>44</th>
<th>45</th>
<th>46</th>
<th>47-59</th>
<th>60-69</th>
<th>70-92</th>
<th>&gt;92</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of metaphases</td>
<td>4</td>
<td>12</td>
<td>19</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>21</td>
<td>16</td>
</tr>
</tbody>
</table>

DISCUSSION

Trichilemmoma has been classified under benign pilar epitheliomas of outer root sheath origin (5, 6, 15, 20, 24). Since tumor growth is sometimes 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 immunocompromised 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.

ACKNOWLEDGMENTS

We wish to thank Haruo Takamiya for Mycoplasma cultures and Michiko Ohno, Setsuko Sato, Shichiro Miyazawa, Tomoko Nakagawa, and Yasuo Morohoshi for their excellent technical assistance.

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, × 3, × 160, and × 320, respectively. D and E, PAS stain without (D) and after (E) amylase digestion, × 320 and × 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. × 12,600. Inset, × 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, del(22)(q13), +mar1(M1), +mar2(M2), +mar3(M3) and +mar4(M4). M1, M2, M3, and M4 are marker chromosomes. Trypsin-Giemsa stain, × 8,000.

Fig. 6. In A, tissue-cultured trichilemmoma cells (3 × 10⁷) 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, × 320.
Morphological, Biological, and Biochemical Characteristics of a Benign Human Trichilemmoma Cell Line *in Vivo* and *in Vitro*

Tamotsu Kanzaki, Hikaru Eto, Akira Umezawa, et al.

*Cancer Res* 1981;41:2468-2475.

Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/41/6/2468

**E-mail alerts** Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions** To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/41/6/2468. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.