Studies on Canine Oral Papillomatosis

III. Cultivation of Papilloma Cells In Vitro

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Abstract. Canine oral papilloma cells were cultivated in vitro by the trypsinization procedure. The success of primary cell culture depended on the choice of a proper stage of papilloma growth. Dome-shaped papilloma was suitable for cultivation. Cultivated cells were flat, and round or polygonal in shape. They were piled up or overlapped, and keratinized cytologically. From the normal mucosa, fibroblasts and epithelial cells were cultivated only by explant culture, while the epithelial cells were neither keratinized nor piled up.

Infectious oral papillomatosis of dogs has been regarded as a self-limiting neoplastic disease caused by a canine oral papilloma virus (COPV), which is currently classified into the *Papillomavirus* of Papovaviridae. Eye surface, eyelid and skin of young dogs were occasionally susceptible to the virus experimentally [6]. Generally, the cultivation of benign epithelial tumor is difficult because of contamination of bacteria and overwhelming growth of fibroblasts. There has been no report on the cultivation of this tumor in vitro. In the present study the authors attempted cultivation of papilloma cells under several conditions, and compared the cell culture histologically with the papillomatous tissues from which cell culture specimens had

been obtained.

Materials and Methods

Virus and tumor: The strain of COPV used in the present work was originated from a papillomatous tissue removed from a dog in Miyazaki, Japan, in April, 1969. The tumor has been carried to the 12th passage level in 47 dogs up to date. The papillomatous tissues subjected to this study were obtained from young mongrel dogs inoculated with COPV experimentally. The virus and papilloma formation have been described in detail in an earlier publication [4]. The papillomas were removed 2-3 times from the same dogs. To avoid any bacterial contamination, papillomatous tissues on the buccal mucosa were washed with 70% alcohol and rinsed with phosphate-buffered saline (PBS, pH 7.2). This procedure was repeated more than 3 times before operation, and tumors were removed with sterile ophthalmic scissors. After rinsing in 0.5% sodium hypochlorite solution and PBS, the tumors were immersed in PBS until use. Oral mucosal

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samples were collected from the inner side of the lower lips of apparently healthy young dogs, treated similarly and used as controls.

Cell culture: Trypsinized cell culture method and tissue explant culture method were applied.

1) Trypsinized cell culture: The tumor masses were minced with sharp scissors, rinsed in PBS, and digested 3–6 times with 0.5% trypsin for 30 minutes each. Dispersed cells were sedimented by low-speed centrifugation. The packed cells were resuspended in Eagle's medium supplemented with 10 or 20% bovine serum, bacto-tryptose phosphate broth, 100 units/ml of penicillin, 100 μ g/ml of streptomycin and 80 units/ml of nystatin. Aliquots of the cell suspension were placed in test tubes or TD40 flasks containing small coverslips, and incubated at 37°C. The medium was changed every 3–4 days.

2) Tissue explant culture: The pieces of papilloma or normal oral mucosa, approximately 1 to 2 mm in diameter and 1 mm thick, were grown either on the bottom of a petri dish or on a coverslip. They adhered to the underlying surface by drying in a CO₂ incubator during incubation at 37°C for 30 minutes and were covered with growth medium. Some explants were held in a thin chicken plasma-clot, cellulose or collagen.

The cultures were observed daily. Cells grown on a cover slip were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin. Some other cells were fixed in methanol and stained with Giemsa or May-Grünwald Giemsa solution. Gram's and van Gieson's staining were used to find keratinized cultured cells, and acridine orange staining was inclusion bodies. Nigrosin and trypan blue staining were also done for viable cell count.

Histological examination was performed on tumors at stages 2, 3 and 5. The tissues adjacent to the part having served for tissue culture were fixed in 10% neutral buffered formalin. After routine processing and paraffin embedding, sections were cut and stained with hematoxylin and eosin.

The media after cell growth were harvested and inoculated to the oral mucosa of young dogs to determine if the virus was contained.

Results

1. Process of tumor growth and result of cultivation

The process of papilloma growth was divided into 5 stages morphologically: (1) white spot, (2) white spot/dome-shaped, (3) dome-shaped, (4) dome-shaped/cauliflower-like, and (5) cauliflower-like stages. Each

stage occupied 7–10 days and shifted to the next stage continuously.

The whole process of tumor growth and the result of cultivation are summarized in Table 1.

Stage 1: The lesion at this stage was a small white protrusion which appeared at the site of inoculation 20 to 40 days after inoculation. It was too small to apply a trypsinization method. The cell growth and fall of pH in culture fluid were found by the plasma clot method.

Stage 2: At this stage, histological observation revealed papillary growth of the oral epithelium, showing hyperkeratosis, parakeratosis and acantosis. The thickened prickle cell layer was projecting into the corium in a form of branch composed of many large swollen and vacuolated squamous cells which had lost their intercellular bridges. Cells lining the parakeratotic corneum contained many keratohyaline granules. Cornification was observed in the deeper area of the prickle cell layer. Branching cords of the epithelium were lined with stratified basal cell layers. Many of the basal cells took a cubic shape and showed hyperchromatosis. Mitotic figures were readily found in these cells. Nodular proliferation of basal cells was occasionally observed (Fig. 1). The underlying corium presented little changes.

Such scanty cell growth and drop of pH of culture fluid as found in stage 1 were also observed by the plasma-clot method. Growth of epithelial-like cells was noticed occasionally in the culture of trypsinized cells, but did not attain to a full sheet formation.

Stage 3: Dome-shaped papilloma persisted for 15–22 days after the appearance. It was a white solid tumor with a smooth surface (Fig. 2). Its growth was more vigorous in this stage than in any other stage.

Table 1. Growth stage of typical papilloma and cell culture in vitro

Papilloma		Cell culture		
Stage of papilloma	Duration (days)	Proportion of cells*		Time for full sheet formation (days)
		Epithelial-like cell	Fibroblast	
1. White nodule	1-7	±	—	
2. White nodule/dome shape	8-14	++	—	
3. Dome shape	15-22	+++	±	2-3
4. Dome shape/cauliflower	23-30	++	++	7
5. Cauliflower	30<	—	+	14<

Remarks.

* +++: Many cells grew rapidly.

++: Cells grew.

+: A few cells grew.

±: Very few cells grew.

—: No cells grew.

The neoplastic proliferation of basal cells and the degeneration of squamous cells were the most prominent histological features of this stage. The prickle cell layer became thicker with swollen and vacuolated squamous cells. These cells had pyknotic nuclei. Some of them had eosinophilic inclusions in the cytoplasm. Some basal cells were stratified and invaded the prickle cell layer in mass. They were small in size and had a hyperchromatic nucleus. Many mitotic figures were found in that layer (Fig. 3).

When the papilloma tissue was cultured by the trypsinization method, newly formed epithelial-like cells appeared 2-3 days after seeding. The number of these cells was twice as large as the initial number of epithelial cells. The cell metabolism was very active. Very few fibroblastic cells could be seen at the rim of the sheet. For the first 3 days, epithelial-like cells formed a full sheet (Figs. 4, 5).

Stage 4: In this stage papilloma had a typical wart appearance. Epithelial-like cells could be cultured. The population of fibroblastic cells increased. A full sheet

was formed by epithelial-like cells and fibroblastic cells about 1 week after seeding. Both epithelial-like and fibroblastic cells formed a clear boundary line and multiplied.

Stage 5: The papillomatous growth was more prominent in this stage than in any other stage. Thus, many tumor masses were easily biopsied. The tumor growth stopped after around 30 days and was followed by spontaneous regression 1-2 weeks later. Epithelial tissue became thinner in this stage. The prickle cell layer was occupied by large vacuolated cells of ovoid shape. Many of the basal cells also showed vacuolation and destructive changes (Fig. 6).

An attempt to culture cells from the tumor of this stage yielded almost negative results; that is, only a few fibroblastic cells were cultured, and it took 2 weeks or more for a cell sheet to be formed.

2. Characters of epithelial-like and fibroblastic cells

The cell suspension prepared from papillomatous tissue contained large and small round cells, red blood cells, and fragments.

Large round cells were attached to the glass surface and took the same shape as the epithelial-like cell, while small round cells took the fibroblastic shape. The cell suspension prepared from dome-shaped papilloma contained many large round cells in contrast to the cell suspension from the cauliflower-like stage which contained many small cells.

The epithelial-like cells were flat, and round or polygonal in shape. They contained a nucleus round or oval in shape situated at the center of the cell. They showed so strong lateral cohesion that their boundary was not clear. There were one or two nucleoli and/or pseudonucleoli of irregular shape in the nucleus. The diameter of each cell varied according to the density of cell growth. It was short at a high density and long at a low density. The greater part of the cell sheet was bi-layered. Partially, cells were piled up upon one another. The upper cells of bi- or multi-layered cells were eosinophilic and Gram-positive. They had essentially the same morphological features as keratinized cells of oral papilloma (Fig. 7). The lower cells were also attached to the glass surface, but were not keratinized. Significant changes were observed on the 4th day, when some epithelial-like cells were detached from the glass surface like the case of CPE, and floating necrotic cells increased. Occasionally, in place of the epithelial-like cells detached, fibroblastic cells multiplied (Fig. 8). The CPE-like change of cultured cells was neither stopped nor accelerated by medium change.

An interesting change was observed on the rates of viable cells and necrotic epithelial-like cells in culture. The rate on necrotic cells was less than 5% of the cells present at the beginning of culture. It increased to 20–30% in 2–3 days. On the 4th

day it was 50%. Cells attached to the glass surface were almost all viable, but did not increase in number. On the contrary, overlapping cells were necrotic and increased in number as days went by. In these conditions they survived for several weeks. More than 95% of the fibroblastic cells continued to be in a viable state.

When the primary cell culture was subcultured by the conventional trypsin treatment, no epithelial-like cells were attached to the glass surface or grew.

Fibroblastic cells increased in population when papilloma tissues of later stages were used. They grew later than epithelial-like cells (Fig. 8). They did not form a completely adherent cell sheet. They were generally spindle-shaped, but were sometimes extremely changeable to be triangular, rectangular, and polygonal in shape. Their nuclei were oval in shape and had some nucleoli. Mitotic cells were seen frequently. Fibroblastic cells were subjected successfully to at least 6 passages. At the 5th or 6th passages all of them still revealed the character of the fibroblast, except a few which became randomly oriented criss-crossed cells showing a dense growth.

3. Cultivation of normal oral mucosa

Normal oral mucosal cells could not be cultivated by the trypsin dispersing method, whereas epithelial-like cells, as well as fibroblastic cells, could be cultivated by explant culture. Outgrowth of epithelial-like cells was generally detectable at 3–7 days of cultivation. The growing epithelial-like cells spread radially for 2–3 weeks. They grew into a sheet of tightly packed but nonpiled-up cells. These cells were large, flat, and polygonal in shape. Their nuclei were large in size and contained several nucleoli. Keratinization was not clear.

Discussion

Cultivation of tumor cells in vitro has been attempted by many investigators for various purposes since HeLa cells were first established from human cervical carcinoma in 1952. The cultivation of viral papilloma, however, has not been successful for many years. Accordingly, cytological and immunological studies on papilloma cells were restricted. In the present study, papillomatous tissues were successfully cultured in vitro when collected from a proper stage of papilloma growth. Namely, dome-shaped papilloma collected during a limited period of 7–10 days was found to be suitable for cultivation in vitro. The growth period of benign tumor is generally short, while the duration of its stationary state is long. Therefore, it is presumed that the results of cultivation of other papillomas [2, 3, 5] may vary not with the culture method used but with the stage in which tissue material is obtained.

Histopathologically, basal cells at stages 2 and 3 showed an active and neoplastic proliferation which resulted in the formation of a stratified layer or nodular mass. Many mitotic figures were found among these cells. On the other hand, in stage 5, basal cells underwent degenerative changes with few mitotic figures. Squamous cells exhibited degenerative changes with a slight fluctuation in severity principally in stages 2, 3 and 5. They were estimated to be low in proliferative vitality. The cell culture in vitro was successful with tissues obtained in stages 2 and 3, but not with those in stage 5. Thus, the cells proliferating actively in vitro were considered to be neoplastic basal cells.

The spindle-shaped cells were assumed to be fibroblasts derived from the connective tissue of the tumor according to their

growth pattern and morphological characteristics in vitro. Occasionally, they became polygonal or round in shape. The polygonal cells were morphologically similar to epithelial cells. Nevertheless, they were distinguishable from these cells by their growth pattern and the shape of their nuclei.

Cheville et al. [1] described two main morphological features of canine oral papilloma: (1) growth of basal cells followed by keratinization and (2) appearance of large swollen cells with intranuclear inclusion bodies. In the present study, such growth of epithelial cells in vitro and keratinization as noticed in the case of tumor in vivo were observed. The detachment of clustering cells from the glass surface appeared to resemble viral CPE, suggesting the existence of virus-producing cells, although neither large swollen cells nor inclusion bodies were observed in vitro. Cultured cells and medium inoculated into the oral mucosa of young dogs were negative of papilloma formation. It was supposed that the amount of infective virus inoculated might not be enough to produce papilloma in the oral mucosa of the dog [4].

Canine oral papilloma virus has been known to lack pathogenicity for mouse, hamster and guinea pig. It has not been cultivated in dog kidney cells, HeLa cells, Vero cells, BHK cells or any other dog tissue cell. It is also well known that no cell line from rabbit papilloma produces viral particles, and that few papilloma viruses can be cultivated in vitro. It is probably needed to develop any procedure for cultivation of epithelial cells which will produce papilloma virus in vitro.

References

- [1] Cheville, N. F., and Olson, C. (1964). Cytology of the canine oral papilloma. *Amer. J. Path.*,

- 45, 849-872.
- [2] Hellström, I., Evans, C. A., and Hellström, K. E. (1969). Cellular immunity and its serum-mediated inhibition in Shope-virus-induced rabbit papillomas. *Int. J. Cancer*, 4, 601-607.
 - [3] Ishimoto, A., Oota, S., Kimura, I., Miyake, T., and Ito, Y. (1970). In vitro cultivation and antigenicity of cottontail rabbit papilloma cells induced by the Shope papilloma virus. *Cancer Research*, 30, 2598-2605.
 - [4] Konishi, S., Tokita, H., and Ogata, M. (1972). Studies on canine oral papillomatosis. I. Transmission and characterization of the virus. *Jap. J. vet. sci.*, 34, 263-268 (in Japanese with English summary).
 - [5] Morgan, H. R., and Balduzzi, P. C. (1964). Propagation of an intranuclear inclusion-forming agent from human condyloma acuminatum. *Proc. nat. Acad. Sci.*, 52, 1561-1564.
 - [6] Tokita, H., and Konishi, S. (1975). Studies on canine oral papillomatosis. II. Oncogenicity of canine oral papilloma virus to various tissues of dog with special reference to eye tumor. *Jap. J. vet. Sci.*, 37, 109-120.

Explanation of Figures

- Fig. 1. Nodular proliferation of basal cells at stage 2. Hematoxylin and eosin staining (HE). $\times 200$.
- Fig. 2. Dome-shaped papillomas. They are white solid tumor with a smooth surface.
- Fig. 3. Basal cells at stage 3. Overlaid basal cells are cubic in shape and have a hyperchromatic nucleus. HE. $\times 200$.
- Fig. 4. Flat polygonal epithelial cells in 2-day culture of papilloma. Giemsa stain. $\times 400$.
- Fig. 5. Epithelial cells in 3-day papilloma culture. HE. $\times 400$.
- Fig. 6. Basal cell layer at stage 5. Basal cells show destructive changes. HE. $\times 200$.
- Fig. 7. Keratinization of epithelial cells in 7-day culture of papilloma. Giemsa stain. $\times 100$.
- Fig. 8. Fibroblastic cells in 4-day culture of papilloma. Giemsa stain. $\times 100$.