Establishment of a Melanin-Producing Cell Line Derived from a Canine Malignant Melanoma

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Abstract. A canine malignant melanoma cell line (CMMe) was established from the metastatic foci of a malignant oral melanoma. The cell line has been subcultured 130 passages during 3.5 years, and has continued to produce melanin pigment in monolayer cultures. The cell line generally consists of triangular and fusiform cells with numerous melanin granules. Melanosomes in various stages of maturation were detected in most of the cultured cells by electron microscopy. The population doubling time of the cell line was 68 hours at the 25th passage, and chromosome number showed wide distribution from 44 to 106 with a mode of 52. Heterotransplantation of cultured cells into the nude mice produced black nodules and histological appearance of the nodule was similar to that of the original tumor.

Some pigmented cell lines were established from murine and human melanomas [8, 10, 11, 15-17, 19, 22]. Cultured pigmented cells are valuable for many fields of research, and have been used particularly for studies of tumor cell maturation and regulation of melanin synthesis in vitro [1, 2, 5, 6, 13, 14, 24, 28].

Although many melanoma cell lines have been established from the melanotic tumors, some of cell lines had gradually lost ability to produce pigment with passage of time [11, 12, 18, 21, 22]. There are a few canine cell lines derived from spontaneous melanomas. These cell lines have a little or no melanin-producing ability [12, 21]. In our laboratory, two cell lines were established from the canine pigmented tumors. The one, which is present reported case has stably produced melanin pigment, but the other had gradually lost the ability to form pigment over 21 transfers in vitro and thereafter not produced pigment [9].

The purpose of present report is to describe the establishment of a new melanin-producing cell line derived from a spontaneous canine melanoma.

Materials and Methods

Source of tumor specimen: A 12-year-old, male German shepherd with neoplastic dark-greyish masses on the mucosa of left upper lip was received for pathological examination. Necropsy was performed on November 13, 1976, and gross examination revealed widespread metastasis. The metastatic foci smaller than 0.5 cm in diameter appeared in various visceral organs such as the lungs, liver, kidneys and pancreas. Histologically the tumor was composed of polyhedral and fusiform cells with various amount of black-brown pigment in the cytoplasm and was diagnosed as pigmented malignant melanoma. The mandibular lymph nodes, from which the cell line was established, showed marked enlargement due to predominant proliferation of pigmented tumor cells and loss of the characteristic structure of lymph node (Fig. 2).

Cell culture: The black regions in the metastatic lesion of tumor were selected, minced and trypsinized. After centrifugation of cell suspension at 800 rpm for 5 minutes, cell pellet was resuspended

in Fisher's medium supplemented with 0.15% NaHCO_3 , 15% calf serum, kanamycin ($50 \mu\text{g/ml}$) and streptomycin ($50 \mu\text{g/ml}$), and cell suspension transferred into glass culture bottles was incubated at 37°C .

Light microscopy: The cultured cells were viewed with a phase-contrast microscope. When necessary, cover-slip cultures were stained with Hematoxylin-Eosin and Masson-Fontana method, and were examined by light microscope.

Electron microscopy: The cultured cells were fixed in 2% glutaraldehyde, centrifuged into a pellet, post-fixed in 1% OsO_4 and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by a Hitachi HS-8 electron microscope.

Chromosomal study: The cells were exposed to colchicine in a final concentration of $0.5 \mu\text{g/ml}$ for 3 hours. After the cells were resuspended in a hypotonic solution, they were fixed in Carnoy's solution, dropped on wet slide glass (50% ethanol) and stained with Giemsa solution.

Heterotransplantation: The cell suspension containing 1.4×10^7 cells was transplanted subcutaneously or intraperitoneally into the six BALB/cA nude mice at 6 weeks of age.

Results

Cell culture: On the 9th day after seeding, cover-slip cultures consisted of triangular and polyhedral cells with numerous black-brown granules in the cytoplasm. A few fibroblastic cells scattered. The primary culture propagated slowly and showed dark-grey color in each of 4 culture bottles. On the 42nd day after cultivation, the cells in the most dark colored bottle were trypsinized and subcultured. The first and subsequent subcultures grew favorably and since then these pigmented cells have been subcultured 130 passages during 3.5 years. When the cells became crowded, the cultures showed dark-grey in color.

Characteristics of the cell line: The cultured cells were generally triangular or fusiform (Fig. 3). The cells had a large, round or oval nucleus with prominent nucleoli varying from two to four numbers and contained numerous black-brown

granules in the cytoplasm (Fig. 4). Black granules were demonstrated in the cytoplasm by Masson-Fontana stain. The cells had long cytoplasmic projections which elongated toward the adjacent cells. A few mono- or multi-nucleated giant cells with large amount of pigment were also present. On the 3-day-old cover-slip culture, the culture consisted of about 80% fusiform and 20% triangular cells, and more than 90% cells contained melanin granules. The rate of cells which contained more than 10 melanin granules was 55.8% on the 3-day-old culture and on the 7-day-old culture its rate elevated to 69%, and many heavily pigmented cells with obscuration of nucleus appeared.

In ultrastructure of cultured cells at the 25th and 41st passage, the cell surface was irregular with cytoplasmic and microvillous projections. The mitochondria were numerous and mainly round or ovoid. The cytoplasm contained many dense melanin granules with distinct limited membrane. Some melanosomes with fibrillar structure and coarse granules were seen. Melanosome complexes were occasionally present in large membrane bounded vacuoles. The development of lamellae and vesicles in the Golgi apparatus was seen. Many small melanosomes with fine granules were observed in the vicinity of Golgi apparatus (Fig. 6 and 7).

Studies on the growth rate of the cells were made by inoculating $1.8 \times 10^5/\text{ml}$ cells into each of culture flasks. At 24 hours intervals, the cells were counted with hemocytometer during 11 days after subculture. The population doubling time of cells was approximately 68 hours at the 25th passage.

Chromosome counts of the cells were made at the 24th passage. One hundred metaphase plates were counted. These counts revealed wide range in chromosome

Fig. 1. Distribution of chromosome number of 100 CMMe cells at the 24th passage.

number from 44 to 106 with a mode of 52 (Fig. 1).

Transplantation of the cells into the nude mice: Inoculation of the cells into the subcutis of four nude mice produced black nodules. A miliary sized nodule was first seen at 7 days after inoculation and reached a size of 3.5 cm in diameter after 75 days. Histologically the nodules consisted of polyhedral and fusiform cells with various amount of melanin pigment, and numerous mitotic figures were present. These findings were similar to that of original tumor (Fig. 5). At 73 days after intraperitoneal inoculation into two nude mice, numerous small black nodules appeared in all areas of peritoneum. Histologically invasive growth of pigmented cells were seen in the tunica muscularis or lamina propria of intestines and pancreas. Pigmented cell clumps were present in the interlobular vein of liver.

Discussion

A few cell lines were established from canine melanomas. The canine cell line, established from the primary lesion of skin melanoma by Kasza [12], had produced abundant pigment until the 30th passage, but thereafter, the pigment appeared in smaller quantities and was not produced during 10-day-old culture about the 50th

passage. Oughton and Owen [21] established cell lines from two canine oral melanomas, but both cell lines were amelanotic form.

Some established cell lines had failed to produce pigment with passage of time [11, 12, 18, 22]. Many reasons have been considered for the loss of pigment formation in vitro, including factors such as the selective and more rapid growth of non-pigmented cells [14], and the inhibition of melanin-producing ability [13, 22, 24, 26, 28].

Our new melanin-producing cell line was successfully established from the metastatic lesion of canine melanotic tumor in the lymph node. The cells have continuously produced pigment in monolayer cultures for more than 3.5 years. The CMMe cell line, which has maintained stable melanin-producing ability, will be available for researches of cytodifferentiation and regulation of melanin synthesis in vitro.

There were many reports concerning the description of morphological characteristics of cultured melanoma cells [4, 8, 10–12, 16, 17, 22, 23, 27]. According to these reports, melanin-producing cells are fusiform, triangular (dendritic), polyhedral (epithelioid) or round. The CMMe cell line has mainly consisted of about 80% fusiform and 20%

triangular cells with long cytoplasmic projections on the 3-day-old culture. Melanin content of the CMMe cells increased at the relatively steady rate and many heavily pigmented cells appeared in old culture. Spontaneous maturation of melanoma cells in old culture was observed in other melanoma cells [12, 14, 20]. But melanogenic activity of some sublines, which were isolated from B16 mouse melanoma, rose to a maximum in the growth phase and then decreased to a low level in the stationary phase, and Oikawa et al. [20] surmised that differentiation of cultured melanoma cells was not only induced because of the inhibition of proliferation.

In cultured melanoma cells, exclusive relationship between cell malignancy and immaturation was observed. The 5-bromodeoxyuridine (BrdU) suppressed both pigmentation and tumorigenicity of pigmented melanoma cells in vitro [25, 26].

The CMMe cells grew rapidly on the nude mouse. The expansive growth of pigmented cells was seen in the subcutis of nude mouse. After intraperitoneal inoculation, numerous small black nodules appeared on the serous membrane. Histologically invasive growth of pigmented cells were seen in the intestines and pancreas, and pigmented cell clumps were present in the vein of liver. Formation of metastatic lesions was reported in a few cases of nude mouse with growth of human tumor cells.

The viral particles were observed in a few melanoma cell lines [3, 7, 22], but in the CMMe cells virus-like particles were not found by electron microscopy.

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要 約

イヌ色素産生性黒色腫培養細胞系の樹立: 井上 誠・山手丈至・佐藤昭夫 (山口大学農学部家畜病理学教室)——12歳のイヌの口唇部に原発した悪性黒色腫の下顎リンパ節転移巣よりメラニン産生性の培養細胞系を樹立した。この細胞系は3.5年間、130代の継代培養を経ても安定した色素産生能を有しており、その樹立経過、細胞性状について報告する。培養材料とした下顎リンパ節は黒色に腫大し、組織学的には細胞質内にメラニン顆粒を有する紡錘形および多角形の腫瘍細胞の増殖により占められ、リンパ節固有の構造は失なわれていた。培養には15% 仔ウシ血清を加えたフィシャー培養液を使用し、材料を細切、トリプシン処理後に37°Cで閉鎖系静置培養を行なった。培養開始後42日目に継代を行ない、以後、細胞の増殖状態をみながら7日~14日の間隔で約5倍~10倍に希釈して継代培養を行ってきた。培養細胞は二極性ないし多極性を呈し、これらの細胞は隣接する細胞に長く延びる突起を有しており、継代後3日目のヘマトキシリン-エオジン染色で約90%の細胞が黒褐色のメラニン顆粒を有していた。電顕では培養細胞に種々の成熟段階にあるメラノソームがみられた。25代継代細胞の倍加時間は約68時間で、また細胞の染色体数は44~106と幅広く分布し、52に最頻値を示していた。培養細胞はヌードマウス (BALB/cA系) に移植可能で、皮下接種により黒色の腫瘍をつくり、接種後2カ月半で直径約3.5 cm大に増大し、組織学的に腫瘍はメラニン顆粒を有する紡錘形および多角形の細胞からなっていた。

Explanation of Figures

- Fig. 2. Metastatic neoplastic cells in the mandibular lymph node, from which the cell culture was prepared. Hematoxylin-Eosin stain (H-E stain), $\times 400$.
- Fig. 3. Phase-contrast micrograph of cultured CMMe cells, 52nd passage, 4-day-old culture, $\times 200$.
- Fig. 4. Coverslip culture of CMMe cells, 43rd, 7-day-old culture. Triangular and fusiform cells contain numerous melanin granules. H-E stain, $\times 400$.
- Fig. 5. Histological appearance of the nodule in the subcutis of nude mouse. Pigmented polyhedral and fusiform cells are seen. H-E stain, $\times 400$.
- Fig. 6. Electron micrograph of cultured CMMe cells, 41st passage, 6-day-old culture. The cells contain many melanosomes in various stages of maturation. $\times 7,600$.
- Fig. 7. Electron micrograph of cultured CMMe cells. Some melanosomes enveloped by a limited membrane are present. $\times 19,000$.