Establishment and Characterization of Four Canine Melanoma Cell Lines

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ABSTRACT. Four canine melanoma cell lines were established from the subcutaneous, oral gingival and mucosal melanoma tissues at the primary and metastatic sites. These cell lines were designated as CMeC-1, CMeC-2, KMeC and LMeC. The cells were spindles in shape, similar to that of primary tumor cells. The doubling times of these cells ranged from 34.1 ± 5.61 to 57.9 ± 3.28 hr and their chromosome number ranged from 46 to 80. When transplanted into nude mice, CMeC-1 and LMeC produced tumors, whereas CMeC-2 and KMeC did not. The morphology of the tissue formed by xenotransplantation of these cells was similar to their primary tumors.

KEY WORDS: canine, melanoma, xenotransplantation.

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Melanoma is a relatively common malignant neoplasm in dogs. Primary sites are the skin, oral mucosa and eye. Canine melanoma often invades locally and metastasizes to the lung and other organs in the early stage of the disease, similar to human melanoma. The malignancy of canine melanoma may vary according to the site of the primary lesion. Oral melanomas seem to be more malignant than skin melanomas; however, the difference in biological behavior according to the primary site is not fully understood.

Human, mouse, hamster and rabbit melanoma cell lines have been provided as important tools for various investigations including pigmentation, tumorigenesis and metastasis [2, 4-7]. Several canine melanoma cell lines have also been established. The character of canine melanomas and their cell lines seems to be similar to those of other mammalian melanomas [1, 9], and can therefore be used for various investigations, such as the differences in malignancy according to the primary site. However, few papers have compared the characteristics of cells derived from the different sites of the body in canine melanoma *in vitro*. The purpose of this study was to describe the establishment and characterization of four melanoma cell lines derived from different primary and metastatic sites of spontaneous canine melanoma patients.

Three cell lines were established from 3 dogs with spontaneous melanomas (Table 1). They were derived from primary skin melanoma, primary oral melanoma and metastatic mandibular lymph node of oral melanoma and were named CMeC-1, KMeC and LMeC, respectively. None of the dogs had been subjected to chemotherapy or radiation therapy before admission. They underwent surgery at the Veterinary Medical Center, the University of

Tokyo, and the excised tumor tissue and lymph nodes were used as specimens for histology and cell culture.

The tissues were cut into small pieces and digested into single cells by deoxyribo- nuclease (Sigma, St. Louis, MO, U.S.A.), protease (Sigma) and type IV collagenase (Sigma). The cells were suspended in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo, Japan) medium containing antibiotics (penicillin-streptomycin; Sigma), L-glutamine (Nissui Pharmaceutical Co.), 2-mercapto-ethanol (2-ME, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 10% heat-inactivated fetal bovine serum (FBS) (Equitech-Bio Inc., Kerrville, TX, U.S.A.), and were maintained at 37°C in a humidified atmosphere with 5% CO₂. The cells were sub-cultured every week.

The cells from the primary skin melanoma did not initially grow in the medium. However, the cells developed a mass in nude mice by xenotransplantation. The cells from this mass began to grow stably in the cultured condition (CMeC-1).

When CMeC-1 cells were injected subcutaneously into nude mice again, micro-metastatic lesion was formed in the lung. From this metastatic tissue, spindle-shaped cells began to grow in the culture, and designated as CMeC-2.

When the cells proliferated to confluent, CMeC-1, CMeC-2 and LMeC piled up on each other, whereas KMeC did not pile up, but rather floated into the medium and continued to proliferate. Only CMeC-2 could grow in the FBS-free medium.

The primary tissues which were surgically removed, and the masses removed from nude mice grown from transplanted cultured cells were fixed in buffered formalin or carnoy solution. Cultured cells grown in slide chambers (Nunc, Inc. Naperville, IL, U.S.A.) were fixed with acetic acid (WAKO). These tissues and cells were stained with hematoxylin and eosin and by the modified Fontana-Masson silver method.

The CMeC-1, CMeC-2 and LMeC predominantly con-

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Table 1. Cell lines derived from canine melanomas

Dog			Tumor					Cell Line			
Breed	Age	Sex	Primary site	WHO stage	Clinical Stage	Histologic Pattern	Distant Metastasis	Name	Origin	No. of Subculture	Morphologic cell type
Chow	11	male	right shoulder	T3N1M0	4	Spindle cell	Yes	CMeC-1	Primary	100	Spindle cell
								CMeC-2	Xenotransplantation lung metastasis	35	Spindle cell
Mongrel	14	male	oral gingiva	T3N1M0	4	Spindle cell	ND	KMeC	Primary	90	Spindle cell
Beagle	9	female	oral mucosa	T4N1M0	4	Spindle cell	ND	LMeC	lymph node	100	Spindle cell

ND: not determined.

sisted of spindle-shaped cells (Fig. 1). The morphology of KMeC revealed similar spindle-shaped cells with a slightly thinner shape (Fig. 1-B). The melanin pigment was not observed in CMeC-1, KMeC and LMeC at passage 8 by hematoxylin-eosin staining, but the pigment was observed by the modified Fontana-Masson silver staining method.

The cultured cells were trypsinized and fixed with 1.25% glutaraldehyde in cacodylate buffer. They were then collected into agarose, postfixed with 2% osmium tetroxide in s-collidine buffer, dehydrated in a graded series of ethanol, and embedded in resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and were examined by a transmission electronmicroscope (H-7500, HITACHI Co., Ltd., Tokyo).

Electron microscopy revealed a few pigments and many lysosomes in CMeC-1, CMeC-2 and LMeC (Fig. 2-A). In CMeC-1 cells, little melanin pigment was observed in live cells, but they were observed in dying cells (Fig. 2-B). KMeC cells had many myelin bodies in their cytoplasm (Fig. 2-C). The nuclei were irregular in shape, and the cytoplasm was larger than the nuclei in all the cells. The electron microscopy findings of CMeC-2 and LMeC were similar to that of CMeC-1. In LMeC, there were many phagolysosomes in the cytoplasm.

Doubling time (DT) at a logarithmic growth phase was calculated as described before [3]. The DT of the CMeC-1 (passage 34), CMeC-2 (passage 17), KMeC (passage 16) and LMeC (passage 7) were 37.7 ± 3.44 , 57.9 ± 3.28 , 37.1 ± 1.35 and 34.1 ± 5.61 hr, respectively.

In order to measure the chromosome number, the cells were cultured in small plastic flasks for several days until semi-confluent, when colchicine was added to the medium at a concentration of $1 \mu\text{g/ml}$ and incubated for 20 min at 37°C in a humidified atmosphere of 5% CO_2 . After incubation, the cells were treated with 0.25% (w/v) trypsin EDTA and centrifuged at 1,200 rpm for 5 min. The cells were then suspended in 0.075 M KCl for 20 min at room temperature, and fixed by a mixture of methanol and acetic acid. The chromosomes were stained by Giemsa solution. Fifty cells were counted in each cell lines.

The median chromosome number of the CMeC-1, CMeC-2, KMeC and LMeC were 49 (range 21–101), 46 (range 37–88), 71 (range 23–88) and 80 (range 46–87), respectively.

Fig. 1. Microscopic findings of each cell line. A: CMeC-1 $\times 200$. The cells were spindle in shape and there were many nucleolus in the nuclei. B: KMeC $\times 200$. The cells were spindle in shape but slightly thinner than CMeC-1.

For xenotransplantation, five-week-old female BALB/c nu/nu mice (Nippon SLC Co. Ltd., Hamamatsu, Japan) kept in a specific pathogen-free condition were used. Before transplantation, the mice were irradiated with a dose of 4 Gy of X-rays using an orthovoltage radiation system (MZU-1, Hitachi Medical Co., Tokyo). After 3 days of irradiation, 1×10^7 melanoma cells were injected subcutaneously into the shoulder region of the mice. The size of each mass that developed was calibrated weekly, and the volume of each mass was calculated. After 10 weeks of transplantation, the

Fig. 2. Transmission electron micrographs of CMeC and KMeC. A: CMeC-1 $\times 14,000$. The cells had well developed mitochondria and nucleolus in the irregularly shaped nuclei. B: CMeC-1 $\times 20,000$. The membrane of mitochondria was broken, indicating that this cell was nearly necrotic. There were many melanin particles that stayed many stages of the development. C: KMeC $\times 14,000$. The cells had well developed melanin bodies and the irregularly shaped nuclei.

mice were sacrificed, and the masses were fixed by 10% buffered formalin for histology.

Transplantation of CMeC-1 and LMeC produced tumor masses in nude mice. Initially, CMeC-1 produced a pigmented mass. However, the masses formed by transplantation of the cells at later passages were not pigmented. The masses formed by transplantation of LMeC were not pigmented. The mice injected with CMeC-1 and LMeC had metastases in their lungs about 8 weeks after transplantation. The mandibular and bronchopulmonary lymph nodes were also swollen, but no metastases were confirmed histologically. The masses formed in nude mice showed similar histological findings to those of tumor tissues of their original lesions. The mice injected with CMeC-2 and KMeC did not develop tumor masses.

In this study, four canine melanoma cell lines were established from different primary and metastatic sites. The growth characteristics of all the four cell lines remained stable over multiple culture passages. Spindle-shape cells dominated morphologically in these four cell lines, which is a common feature of canine melanoma cell lines [1, 9]. However, there were few melanin granules in the cytoplasm of these cells. It is known that melanoma cell lines often show the gradual loss of melanin granules after serial passages [4]. The precise mechanism in the synthesis of melanin granules has not yet been clarified, however the potential factors influencing the synthesis of melanin gran-

ules including cyclic adenosine monophosphate (cAMP), tyrosinase and pH of the test medium have been reported [10]. When melanoma cell lines received treatments that would raise the concentration of cAMP of the cells, melanin synthesis would be promoted, and cell proliferation would be suppressed. Interestingly, CMeC-1 cells had many melanin granules immediately before cell death. It is not clear yet, but the phenomenon might be related to the cAMP contents within the cells.

There was a difference in proliferation patterns in the culture condition between KMeC and the other cells, in terms of whether the cells piled up or not at their overconfluent status. This may suggest that inter-cellular adhesion activity or function of these cells was different. Moreover, pH of the medium did not change after a few days of culture of KMeC (data not shown), though pH of the medium generally declined during the culture of cells, in accordance with the production of CO_2 by the cell metabolism of glucose. This may indicate that there was a difference in metabolism between KMeC and the other cell lines. In this study, CMeC-1 and LMeC transplanted into nude mice produced mass lesions, while CMeC-2 and KMeC did not. The reason for the difference is unknown. We did not have any information on the precise disease progress of the patients from which KMeC and LMeC were established. The clinical stage of KMeC and LMeC was the same, with involvement of the regional lymph node. KMeC cells possess a unique

characteristic in their metabolism as described above, which might relate to the differences in their adhesion molecules, such as integrin, matrix metalloproteinase and CD44, compared with other cell lines. This difference may reflect the metastatic ability of KMeC cells.

The CMeC-2 cells were established from the lung metastasis in nude mice transplanted with CMeC-1 cells. This may reflect the difference in metastatic ability of CMeC-1 and CMeC-2, though CMeC-2 showed stable growth in the FBS-free medium, indicating the malignant property of CMeC-2. Tumorigenicity and metastatic ability are sometimes dependent on the position of cell injection. It has been reported that different injection areas and different injection methods may lead to various metastatic ability of B16 mouse malignant melanoma cell lines [8]. The microenvironment of the injection site may influence tumorigenicity. In this study, all of the cells were injected subcutaneously into the shoulder area. A different injection site, such as the footpad, might lead to mass formation in these cells. In addition, the tumor of chow dog was finally metastasized to his lung.

In conclusion, four canine melanoma cells with different primary sites and different biological characteristics were established. Though the details of their characteristics are still not entirely clarified, these cell lines may be quite important investigative tools in the future, especially for the investigation on metastasis or the characteristic of cells from the different primary sites.

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