Establishment of a New Canine Cell Line (CCT) Originated from a Cutaneous Malignant Histiocytosis

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ABSTRACT. A new canine cell line, named CCT, was established from the cutaneous malignant histiocytosis in a 4-year-old male Borzoi. CCT proliferated with loose adherence and doubling time was approximately 30 hr. When co-cultured with latex beads, CCT phagocytized beads vigorously. Lysozyme and vimentin were positive by immunostaining, and non-specific esterase and acid phosphatase were positive by cytochemical staining. These features indicated the cells had a histiocytic nature. Furthermore, by subcutaneous injection to nude mice CCT could successfully form tumors with the morphological and immunohistochemical features similar to the original tumor.

KEY WORDS: canine, cell line, malignant histiocytosis.

Malignant histiocytosis (MH) is the most aggressive syndrome in the spectrum of histiocytic diseases although its origin has been still under debate [5]. The first report of MH was described in the Bernese mountain dog [11]. This uncommon but highly malignant round cell neoplasm has since been reported in various dog breeds such as rotweiler and golden retriever [8]. Typically, MH involves the spleen, liver, lung, kidney, lymph nodes, and bone [5], but skin lesions can occur [13], either alone or as part of the multiorgan lesions. In this study, we describe the successful establishment of a new canine cell line, named CCT, originated from cutaneous MH.

The CCT was established from one of multiple skin nodular lesions in a 4-year-old male Borzoi. Dozens of various sized lesions (0.5–2 cm) were distributed throughout the skin of his body. Several cutaneous tumors were excised surgically for histopathological diagnosis. The lesions were not responsive to corticosteroid therapies but number of lesions even increased. Consequently, this dog was euthanized ten months after the initial admission. Unfortunately, necropsy could not be performed. Histological examination of the biopsy specimens revealed a diffuse infiltrative proliferation of pleomorphic atypical cells in the dermis and subcutis. Large round and polygonal neoplastic cells contained abundant eosinophilic cytoplasm with ovoid nuclei (Fig. 1). Some neoplastic cells resembled normal macrophages, but others showed marked variation in size and shape and their nuclei were also varied in size and hyperchromatic containing several prominent nucleoli. Marked atypia and numerous mitoses were observed, many of which were bizarre. Binucleated giant cells were scattered about. In some neoplastic nodules, severe hemorrhages were observed, and occasionally neoplastic cells phagocytized erythrocytes (Fig. 1), hemosiderin, or cellular debris. Inflammatory cells, mainly neutrophiles, could be seen scattering between the neoplastic cells.

The neoplastic tissues used for histopathological examination were minced finely in a RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing 2 mg/ml sodium bicarbonate, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The tissues were washed twice in the medium. The isolated cells were then suspended and incubated in the above medium with a 20% heat inactivated fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2. Passages were performed every 3 to 5 days. The established cell line named CCT was maintained in continuous culture for over 70 passages. In the subsequent experiments we used the cells processed through at least 60 passages. Established CCT cells loosely attached to the plastic dish (Fig. 2) and multinucleated giant cells were occasionally seen between them. For electron microscopic observation, CCT cells were removed by 0.05% trypsin/0.02% ethylenediaminetetraacetic acid disodium salt dihydrate (TE, Gibco BRL, New York, U.S.A.) and pelleted by centrifugation. Subsequently, pellets were fixed by a 4% paraformaldehyde/ 2.5% glutaraldehyde solution and 2% OsO_4, then dehydrated, and embedded in epoxy resin (Epon 812 resin embedding kit, Taab Laboratory Equipments, Berkshire, UK). Ultrathin sections were stained with uranyl acetate and lead citrate and then observed under H-8100 (Hitachi, Tokyo, Japan) at 75 kV. Ultrastructurally, many cells had villous cytoplasmic projections on surface and possessed markedly convoluted nuclear membranes. There were several mitochondria and abundant rough endoplasmic reticula in the cytoplasm. The Golgi's apparatus was well developed in some cells (Fig. 3).

For calculation of the population-doubling time, cells were seeded in 24 well multiplate at 2×10^4/ml/well in the growth medium. Cell growth and viability were measured every 24 hr by a trypan blue dye exclusion test using a
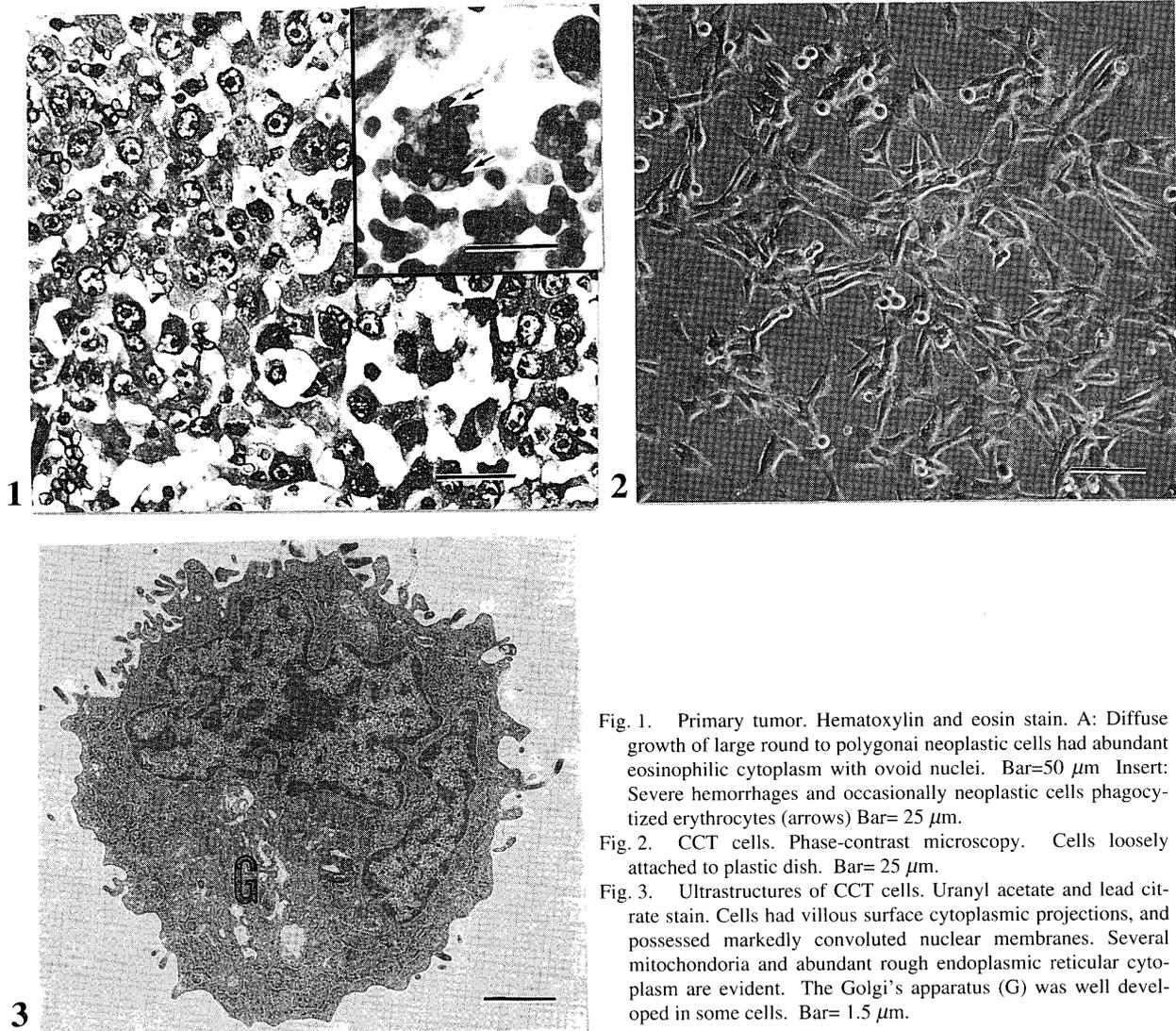
Fig. 1. Primary tumor. Hematoxylin and eosin stain. A: Diffuse growth of large round to polygonai neoplastic cells had abundant eosinophilic cytoplasm with ovoid nuclei. Bar=50 μm Insert: Severe hemorrhages and occasionally neoplastic cells phagocytized erythrocytes (arrows) Bar= 25 μm.

Fig. 2. CCT cells. Phase-contrast microscopy. Cells loosely attached to plastic dish. Bar= 25 μm.

Fig. 3. Ultrastructures of CCT cells. Uranyl acetate and lead citrate stain. Cells had villous surface cytoplasmic projections, and possessed markedly convoluted nuclear membranes. Several mitochondoria and abundant rough endoplasmic reticular cytoplasm are evident. The Golgi's apparatus (G) was well developed in some cells. Bar= 1.5 μm.

hemocytometer, and the growth curve was described (Fig. 4). The data shown are means of triplicated measurements, population-doubling time of CCT cells was assessed approximately 30 hr.

Chromosome analysis was simply performed after treated the cells with colcemid (KaryoMAX, Gibco BRL) (0.1 μ/ m*l*) for 7 hr. The modal chromosome numbers detected in 50 metaphase cells were 113–128, and the karyotype was triploid.

Phagocytic activity of CCT cells was tested by co-culture with 1.72 μm polystyrene latex beads (Flouresbrite Carboxylate Microspheres YG, Polysciences, Warrington, U.S.A.) in RPMI 1640 containing 20% non-heat inactivated FCS [12]. Twenty-four hr after incubation with latex beads, the cells were removed by TE and smeared on slide glass. Phagocytized beads in CTT cells were observed with a fluorescent microscope. Moreover, under electron microscopy, several beads could be observed in the cytoplasm together with phagocytic cell debris (Fig. 5).

For non-specific esterase (NSE) stain, CCT cells smeared on slide glasses were stained by an NSE stain solution consisting of an acetone- alpha-naphtyle butyrate (Sigma) mixture and a parasosaniline (Sigma)/sodium nitrate-phosphate buffer. The cytoplasm of CCT cells was stained reddish-brown diffusely or spottily (Fig. 6A).

For acid phosphatase (ACP) stain, smeared CCT cells were stained using an ACP staining kit (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) according to the manufacturer's procedure. Positive reaction was observed granularly in the cytoplasm (Fig. 6B), and became to fade out after addition of L-tartaric acid.

Tumorigenesis of CCT cells was evaluated by xenograft to two strains of nude mice (5 week-old, KSN/Slc and BALB/c-nu/nu Slc, Japan SLC, Inc., Shizuoka, Japan). Each mouse was injected 10^6 CCT cell-suspensions into the subcutis. Tumors appeared grossly one week after the injec-
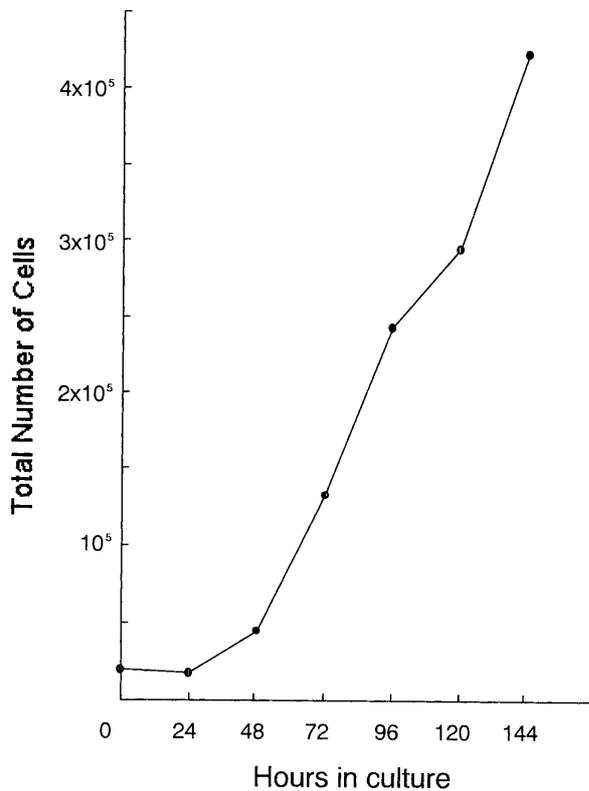
Fig. 4. Growth curve of CCT cells. Doubling time is approximately 30 hr.

tion and attained their maximal diameter by 1.5 cm at 6 weeks after the injection. Histopathological findings of xenografted tumor tissues were similar to those of the original tumor; however, some tumor cells appeared spindle shaped with an arrangement of interwoven patterns. Metastasis was not observed in any organs.

Immunostainings were performed on the original canine MH tissues, CCT cells and xenografted tumor tissues by an avidin-biotin complex (ABC) procedure (Vecterstain Universal Elite ABC kit, Vecter Laboratories, Burlingame, U.S.A.) using anti-vimentin monoclonal (DAKO, Corp., Glostrup, Denmark), anti-lysozyme polyclonal (DAKO), anti-cytokeratin AE-1/AE-3 monoclonal (Zymed Laboratories Inc., South San Francisco, U.S.A.) and anti-S-100 protein polyclonal (Zymed) antibodies. All sample were positive for vimentin (Figs. 7A and 8A) and lysozyme (Figs. 7B, 8B and 9), but negative for cytokeratin and S-100 protein.

The macrophage-monocyte system (MMS) provides the capability to phagocytize large quantities of bacteria, viruses, necrotic tissue, or other foreign particles in the tissues. Phagocytosis is an important function of MMS and known to be a critical marker indicating that the cells is differentiated to MMS from hematopoietic cells under a specific condition to stimulate their differentiation [12]. A canine macrophage-monocyte cell line, named DH82, originated from MH was established by Wellman et al. previously, and this cell line also has a highly phagocytic activity [14]. Furthermore, in DH82 cells, NSE and ACP activities suggested by cytochemical staining were similar to those seen in normal canine monocyte, therefore, DH82 cells pos-
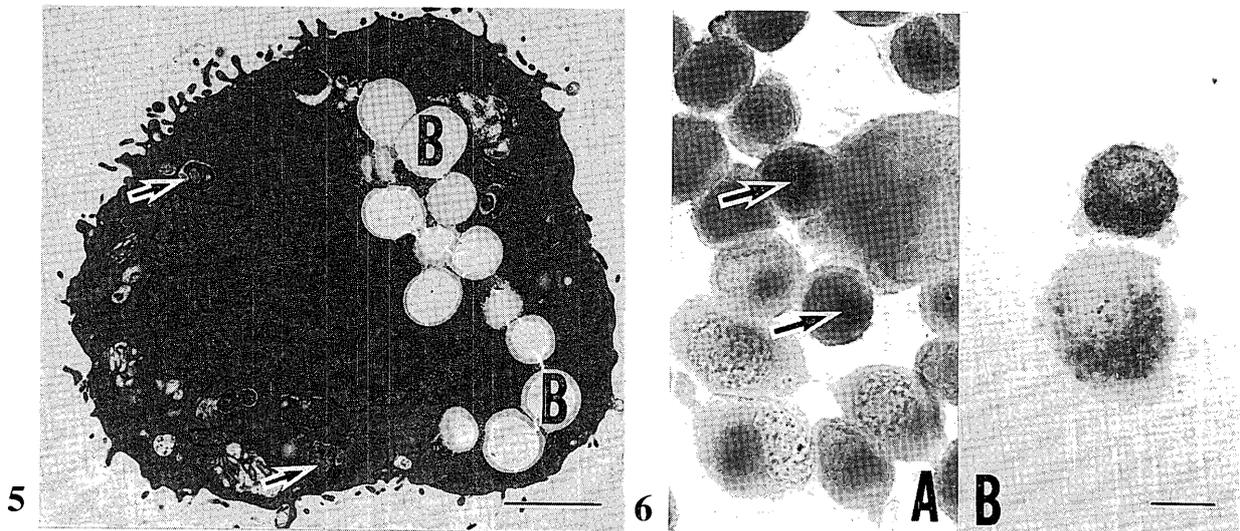

Fig. 5. Phagocytic activity of CCT cells. Uranyl acetate and lead citrate stain. Phagocytized beads (round gray materials indicated as B, approximately 1.7 μm) in CTT cells were observed with phagocytized cell debris (arrows). Bar= 2.5 μm.

Fig. 6. Cytochemical reactivities of CCT cells. A: NSE stain. B: ACP stain. NSE-activities were diffuse or spotty (arrows) in cytoplasm. ACP-activities were granularly in cytoplasm. Bar=10 μm.
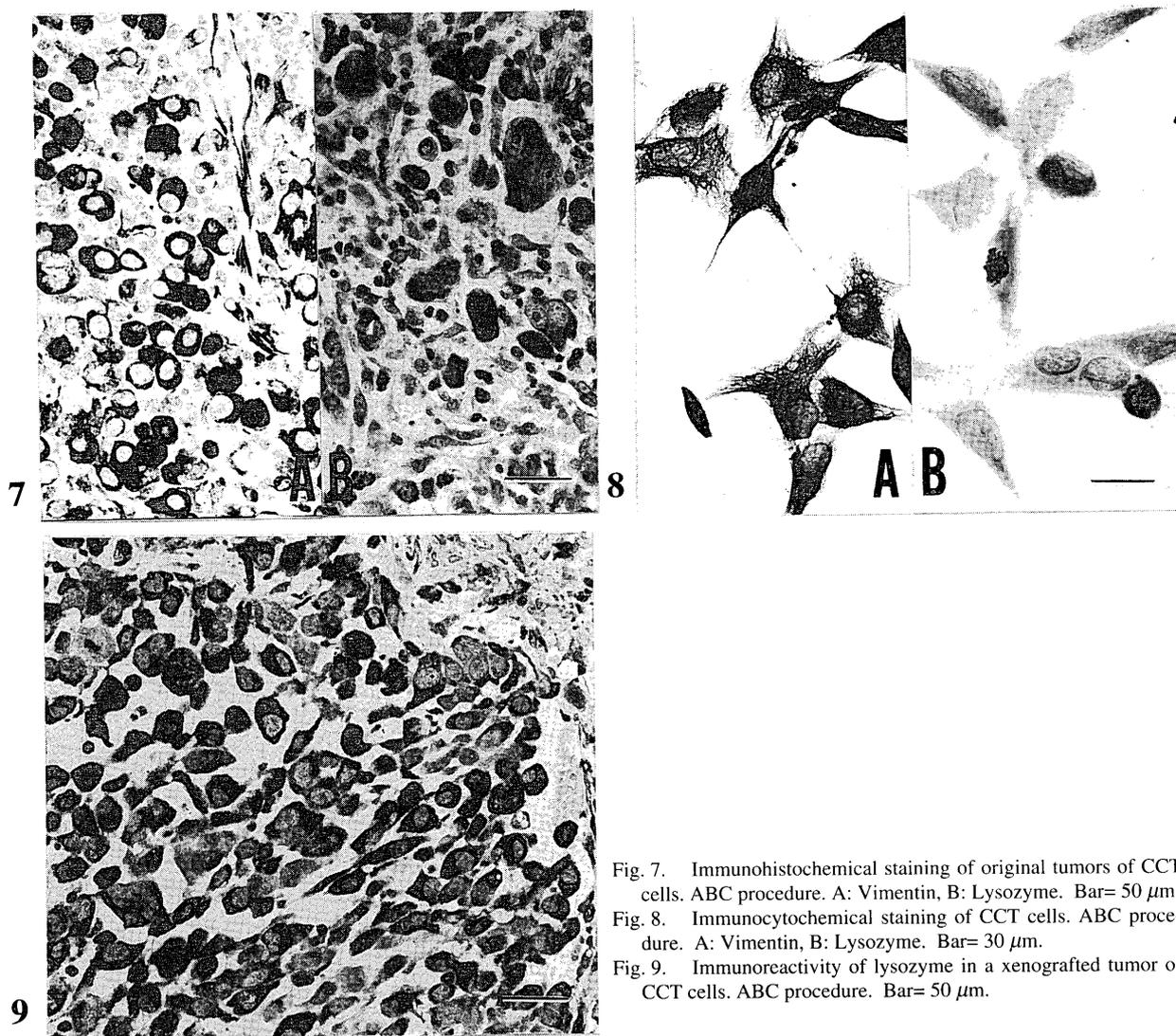
Fig. 7. Immunohistochemical staining of original tumors of CCT cells. ABC procedure. A: Vimentin, B: Lysozyme. Bar= 50 μm.

Fig. 8. Immunocytochemical staining of CCT cells. ABC procedure. A: Vimentin, B: Lysozyme. Bar= 30 μm.

Fig. 9. Immunoreactivity of lysozyme in a xenografted tumor of CCT cells. ABC procedure. Bar= 50 μm.

sessed nature of MMS [14]. Affolter and Moore reported that the common surface antigen phenotype (CD1, CD4–, CD11c+, CD11d–, CD18+, MHC II+, ICAM-1+ and Thy-1±) was observed in canine MH tumor cells, and thus MH is likely a myeloid dendric antigen-presenting cell sarcoma [1]. On the other hand, DH82 expressed CD14, which indicated a macrophage origin [2]. The origin of canine MH is unclear. Because surface antigens of CCT was not analysed, the accurate phenotype was unclear. However, our established CCT cells also have a phagocytic activity, and cytochemical features similar to DH82, suggesting their origin to histiocytic cells, although further studies are required to elucidate the nature of CCT.

DH82 was applied to studies of equine infectious anemia virus [7], *Ehrlichia canis* [4] by utilizing the nature of MMS. Therefore, it may be worthwhile to consider use of CCT cells to the study of infectious diseases.

In the xenograft into nude mice, the tumors developed were similar to the original canine neoplastic tissue. Xenografted cells were also similar to the original cells in their morphology and immunohistological phenotypes. Lysozyme is a useful marker of histiocytic cells [9, 10]. MH with or without visceral lesions was positive for lysozyme [3, 6, 10]; therefore, in addition to the histopathological or ultrastructural features, the immunohistochemistry for lysozyme can be a good tool to diagnose MH. CCT was positive for lysozyme *in vitro* or *in vivo*. Therefore, CCT cell line or CCT cell-xenograft model may be relevant to *in vivo* or *in vitro* analysis of MH.

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