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Phenotypic Analysis for a Cell Line of Canine Epidermal Keratinocytes

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ABSTRACT. Epidermal keratinocytes have the potential to produce inflammatory mediators that are considered to play an important role in skin diseases such as atopic dermatitis (AD). Thus, cell lines of canine epidermal keratinocytes are useful for studying the biological reactivity of keratinocytes *in vitro*. However, there has been no report on properly analyzing the phenotype of canine keratinocyte cell lines. In this work, we performed phenotypic analysis of CPEK, which was derived from the epidermis of an adult dog in order to examine the phenotypic similarity with epidermal keratinocytes. The present findings indicated that CPEK cells expressed markers for epidermal keratinocytes including cytokeratin 14, α_6 integrin and PCNA. Our findings demonstrated that CPEK could be a useful cell line for investigating the central role of epidermal keratinocytes in the pathogenesis of AD *in vitro*.

KEY WORDS: cytokeratin, integrin, keratinocytes.

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Atopic dermatitis (AD) is one of the most common skin diseases in dogs and is associated with type I hypersensitivity. Previous studies have reported that the thymus and activation-regulated chemokine (TARC/CCL17) is strongly expressed in lesional skin of humans [12] and dogs [10] with AD. Thus, CCL17 is thought to participate in the pathogenesis of canine AD. Recent studies have demonstrated that basal keratinocytes may be a major cell source for CCL17 production in dogs [10]. However, induction factors for CCL17 production in keratinocytes have not been identified because an appropriate canine cell line has not been available. To date, one keratinocyte cell line, CPEK (CELLnTEC Advanced Cell Systems, Switzerland) is commercially available for veterinary research. CPEK was derived from the epidermis of an adult Beagle dog. However, phenotypic analysis of CPEK has not previously been conducted. In the present study, to determine the possibility of CPEK as a model for canine skin-derived basal keratinocytes, we immunohistochemically investigated the phenotype of CPEK using proliferative and differential markers.

A cell line of canine epidermal keratinocyte, CPEK cells were purchased from CELLnTEC Advanced Cell Systems. Fifth- to tenth-passage cells were used for experiments. The cells were initially cultured in 75 cm² flasks (NUNC, Denmark) in 10 ml complete CnT-09 (CELLnTEC) until approximately 80% confluence at 37°C under 5% CO₂. For subculture, the cells were rinsed with 10 ml Versene 1:5,000 (Invitrogen Corporation, U.S.A.) and trypsinized by the addition of 2 ml of TrypLE Express (Invitrogen) followed by incubation at 37°C for 5–10 min. The cell suspension was transferred to a sterile 15 ml centrifuge tube and centrifuged at 180 × g for 5 min at room temperature. The cell pellet was gently re-suspended in CnT-09 and transferred to

75 cm² flasks at a density of approximately 2 × 10⁴/cm² for subculture.

To establish a growth curve, CPEK was seeded to 6-well plates in 2 ml CnT-09. On days 1, 2, 3, 5 and 7, the cells were harvested for cell counting by a hemocytometer. Viability was confirmed by the trypan blue exclusion method. This experiment was replicated three times. The results were plotted semilogarithmically in order to determine population doubling time by saturation density. The population doubling time was 14.8 ± 1.50 hr, and the saturation density was 23.3 × 10⁴ ± 2.49 cells/cm² when the initial cell density was adjusted to 1 × 10⁴ cells/cm².

For morphological and immunohistochemical analyses, CPEK was plated at 2 × 10⁴/cm² in a total volume of 1 ml/chamber of CnT-09 in 4-chamber glass culture slides (BD Biosciences, U.S.A.). When CPEK reached 80% confluency, the cells were fixed with 99% methanol and permeabilized with 0.5% Triton[®]X-100 in PBS followed by blocking with 10% goat serum for 30 min. The cells were incubated with anti-human Proliferating Cell Nuclear Antigen (PCNA, monoclonal, 1:50 dilution, DakoCytomation, Denmark) [3], mouse anti-human cytokeratin 14 (monoclonal, 1:5 dilution, PROGEN, Germany) [14] and anti-human cytokeratin 10 (monoclonal, 1:100 dilution, Abcam, UK) [14] for 1 hr. Mouse IgG₁ (1:100 dilution, Ancell Corporation, U.S.A.) and mouse IgG_{2a} (1:100 dilution, BD) were used as isotype controls. For secondary antibodies, rat anti-mouse IgG₁ FITC conjugate (1:200 dilution, BD) for anti-cytokeratin 14 and 10, rat anti-mouse IgG_{2a} RITC conjugate (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, U.S.A.) for anti-PCNA were used. DAPI (1:500 dilution, Dojindo Laboratories, Japan) was used for nucleus counterstaining at the same time. The morphology of the cells under phase contrast microscopy demonstrated the classical cobble appearance of undifferentiated keratinocytes (Fig. 1A). Immunohistochemical study indicated that CPEK was positive for cytokeratin 14 (Fig. 1B) but negative for cytokeratin

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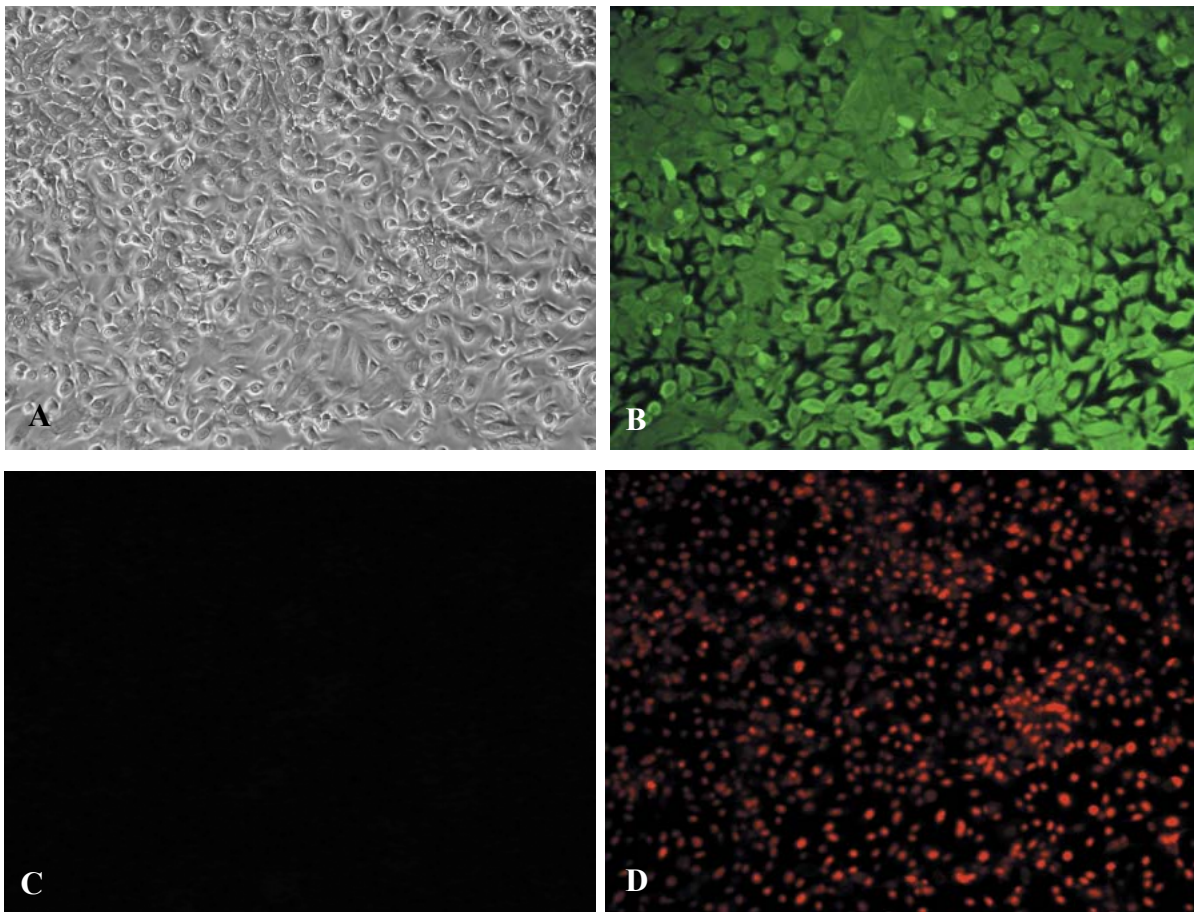


Fig. 1. Morphological and immunohistochemical findings of CPEK cells. A: bright field, B: Cytokeratin 14, C: Cytokeratin 10, D: Proliferating cell nuclear antigen (200 \times).

10 (Fig. 1C). All cells were stained with anti-PCNA, demonstrating active proliferation of these cells (Fig. 1D).

For flowcytometric analysis (FACS CaliburTM, BD), CPEK were rinsed using Versene 1:5000 and trypsinized by TrypLE Express in order to harvest the cells. The cells were incubated with either rat anti-human integrin alpha 6 (α_6 integrin, monoclonal, 1:100 dilution, Chemicon, U.S.A.) [15] or rat IgG_{2a} (1:100 dilution, BD) as isotype control for 30 min, followed by incubation with FITC conjugated-mouse anti-rat IgG_{2a} (1:100 dilution, BD) secondary antibody for 30 min. To quantify the expression of α_6 integrin, mean fluorescence intensity was calculated by a analyzing software (CellQuestTM, BD). Flowcytometric analysis showed that 99.96% of CPEK were positive for α_6 integrin (Fig. 2).

Although basal keratinocytes may be a major cell source for CCL17 production in dogs, *in vitro* study using canine keratinocytes has not been performed. Canine epidermal keratinocytes, CPEK could be used as an *in vitro* experimental system for studying biological reactivity in response to allergic inflammatory stimuli. Thus, this study investigated whether CPEK would be suitable for research on

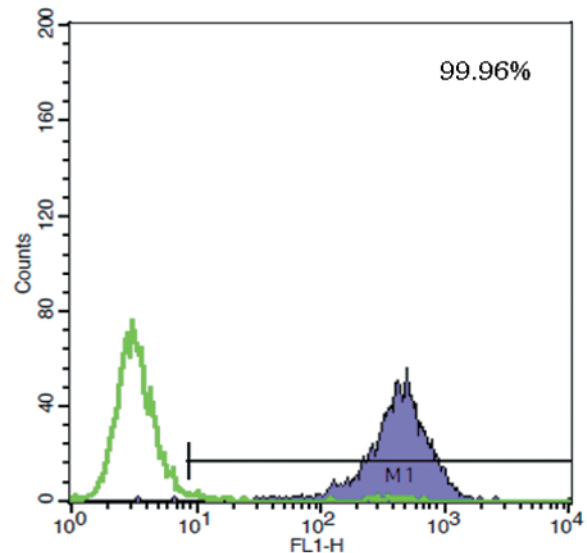


Fig. 2. Flow cytometric detection of α_6 integrin. The histogram shows the staining intensity of isotype (green) and α_6 integrin (purple) monoclonal antibodies.

canine skin diseases.

Cytokeratin 10 and 14 are known as differential markers of epithelial cells. Keratinocytes in the basal layer express cytokeratin 14, but this expression is down-regulated during differentiation, switching to cytokeratin 10 as keratinocytes move into suprabasal layers [7]. The proliferation marker, PCNA identifies a proliferating subpopulation of basal keratinocytes [6]. The present study showed that CPEK was positive for cytokeratin 14 and PCNA but negative for cytokeratin 10, demonstrating phenotypic similarity with basal keratinocytes. For additional characterization of CPEK, we also investigated expression of an adhesion molecule, α_6 integrin, which is exclusively expressed on the epidermal-dermal junction of basal keratinocytes [2, 11, 15]. In basal keratinocytes, flowcytometric analysis could separate two subpopulations into proliferative undifferentiated (α_6^{bri}) or postmitotic differentiating (α_6^{dim}) keratinocytes based on expression of α_6 integrin [9]. In the present study, almost all (99.96%) CPEK expressed integrin, but the expression pattern was not bimodal. Therefore, the present findings strongly indicate that CPEK has a proliferative undifferentiated phenotype as well as HaCaT cells [1]. Although the profile of cytokines and chemokines production remain unclear in CPEK, basal keratinocytes have been reported to produce numerous inflammatory mediators such as CCL2, CCL5, CCL17 and TNF- α [4, 5, 8, 12, 13, 16]. Thus, further study should aim to examine the similarity in the profile between CPEK and basal keratinocytes. In conclusion, we suggest that CPEK should be a useful cell line for investigating the immunopathogenesis of inflammatory skin diseases.

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