FULL PAPER

Surgery

Biological features of canine cancer-associated fibroblasts and their influence on cancer cell invasion

Ayano KUDO[1,3], Sho YOSHIMOTO[1,3], Hiromitsu YOSHIDA[1], Yusuke IZUMI[1] and Satoshi TAKAGI[2,3]*

[1]Laboratory of Advanced Veterinary Medicine, Department of Veterinary Clinical Sciences Faculty of Veterinary Medicine, Hokkaido University, Hokkaido, Japan
[2]Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Hokkaido, Japan
[3]Laboratory of Small Animal Surgery, School of Veterinary Medicine, Azabu University, Kanagawa, Japan

ABSTRACT. Cancer-associated fibroblasts (CAFs) play an essential role in tumor invasion and metastasis. In dogs, the biological features of CAFs have not been well characterized. The purpose of this study was to investigate differences in the biological activities of canine CAFs and normal fibroblasts (NFs), and their influence on the migration and invasion of cancer cells. Canine CAFs and NFs were harvested from surgically-resected malignant epithelial tumor tissues and skin tissues of dogs. A wound-healing assay was conducted to compare the migratory and invasive abilities of canine CAFs and NFs. The results of this study showed that canine CAFs have a greater migratory and invasive ability than NFs. To observe the indirect and direct interactions between fibroblasts and cancer cells, Boyden chamber assay and 3D co-culture with collagen gel were conducted. The number of migrated and infiltrated cancer cells co-cultured with canine CAFs was greater than that with NFs. In the 3D co-culture, cancer cells showed noteworthy proliferation on the surface of gels containing canine CAFs and invasion into the gel. On the other hand, no infiltration of cancer cells into the gel containing NFs was observed. It was suggested that canine CAFs activate migration and invasion of cancer cells and promote the infiltration of cancer cells into collagen gels.

KEYWORDS: cancer-associated fibroblast, canine tumor, invasion assay, tumor microenvironment

Tumor tissue is composed of not only cancer cells but also stromal cells, including immune cells, endothelial cells, and fibroblasts. These components comprise a tumor microenvironment wherein cancer and stromal cells interact to promote the proliferation and invasion of cancer cells [9].

Cancer-associated fibroblasts (CAFs) are a major component of the tumor stroma. Many types of cells can be predecessors of CAFs, including resident tissue fibroblasts, epithelial cells, endothelial cells, bone marrow-derived mesenchymal stem cells, and hematopoietic stem cells [3, 17]. CAFs are biologically similar to activated myofibroblasts during inflammation; therefore, high expression of α-smooth muscle actin (α-SMA) is considered a feature of CAFs [19].

Several studies in humans and mice have suggested that direct and indirect co-activation of CAFs and cancer cells promotes cancer malignancy. For example, cancer cells cannot invade type I collagen gels that contain extracellular matrix; however, they can infiltrate the gel when they are co-cultured with CAFs [6, 12, 13]. In the process of cancer cell proliferation, CAFs have also been reported to be associated with angiogenesis, resistance to several treatments, invasion, and metastasis by releasing various types of humoral factors and cytokines [14]. In a tumor transplant mouse model, inhabitation of stromal derived factor-1 (SDF-1), a humoral factor released from CAFs, suppressed tumor growth [5]. Based on these findings, the potential for therapeutic strategies targeting CAFs, such as the administration of inhibitors of the fluid factor secreted by CAFs and its potential as a target for immune therapy [1], are promising.

In veterinary medicine, studies on CAFs are limited. In one study, canine CAFs were separated from epithelial malignant tumors, and the expression of genes and proteins associated with their activity was examined [22]. In another study, genes related to adhesion and angiogenesis in canine CAFs were upregulated when co-cultured with canine mammary cancer cells [10]. According to these previous reports, the role of CAFs in canine cancer may be similar to that of CAFs in human cancer. However,

*Correspondence to: Takagi, S.: s-takagi@azabu-u.ac.jp, Laboratory of Small Animal Surgery, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

no studies have compared the biological characteristics of canine CAFs and NFs. To assess the properties of CAFs in the tumor microenvironment, a comparison with NFs as a normal symmetry seemed necessary.

The purpose of this study was to examine the differences in the biological activities of CAFs and NFs in dogs and their effects on tumor migration and invasion. In this study, we investigated the influence of CAFs in canine cancer cells by co-culturing them with canine cancer cell lines and CAFs and NFs.

MATERIALS AND METHODS

Separation and cultivation of CAFs and NFs

Malignant epithelial tumor tissues and skin tissues were collected from dogs diagnosed with epithelial tumors who underwent surgical resection at the Hokkaido University Veterinary Teaching Hospital between May 2016 and August 2017. Written informed consent was obtained from all owners. CAFs and NFs were harvested from the tumor and skin tissues of these dogs, according to a previous study [22].

Canine epithelial tumors and skin tissues were minced into small pieces and digested with 1 mg/ml collagenase IV (Sigma Aldrich, St. Louis, MO, USA) with 5% bovine serum albumin (BSA; Wako, Osaka, Japan). These tissues were kept at 37°C for 2 hr and vortexed every 30 min. After removing the undigested tissue by filtration, the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Wako) containing 10% fetal bovine serum (FBS; Biosera, Nuaille, France), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Wako) at 37°C with 5% carbon dioxide (CO_2). Fibroblasts and other cells were separated on the basis of the difference in the time required for detachment by 1 mM EDTA-4Na and 0.25% trypsin. After fibroblast detachment, FBS was added to terminate the action of trypsin. The isolated cells were cultured and used for each experiment after to 3–5 passages following the primary culture [4, 22].

Identification of fibroblasts

Fibroblasts were identified using immunofluorescence staining. Cells were seeded 1.0×10^5 on poly-L-lysine-coated coverslips for 24-well plates (Matsunami Glass, Osaka, Japan), cultured for 48–72 hr, and washed by phosphate-buffered saline (PBS; Wako). The cells were fixed with paraformaldehyde at room temperature for 10 min. After fixation, the cells were permeabilized with 0.5% Triton X-100 (Wako) in PBS, blocked with 10% goat serum albumin (Wako) for 30 min, and immunostained overnight at 4°C with primary mouse antibodies for vimentin (a marker for mesenchymal cells) (clone V9, Dako, Tokyo, Japan), cytokeratin (a marker for epithelial cells) (clone AE1/AE3, Dako), and α-SMA (clone 1A4, Sigma Aldrich) diluted 1:2,000 in PBS. The cells were incubated at room temperature for 1 hr with fluorochrome-conjugated secondary goat anti-mouse antibody (Alexa-Fluor-488, catalog number A21202, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS, and nuclei phenylindole (DAPI; Dojindo, Kumamoto, Japan) at room temperature for 15 min. The coverslips were mounted on glass slides. Images were obtained using a confocal microscope (LSM700; Zeiss, Oberkochen, Germany).

Comparison of migratory and invasive ability of CAFs and NFs

To compare the migratory ability of canine CAFs and NFs, a wound-healing assay was conducted. Fibroblasts were seeded at 5.0×10^4 in 24-well plates, cultured until they reached 70% confluence, and straight scratches were made with pipette chips. Cells that were scraped off were removed by washing 2–3 times with PBS. Cell movement was microscopically evaluated immediately and at 36 hr after scraping. Cell movement was calculated by measuring the width of the uncovered region.

To investigate the invasive ability, 24-well plates were coated with collagen gel (Cell matrix type IA, Nitta Gelatin, Osaka, Japan), five-fold concentrated DMEM (Nitta Gelatin), and reconstitution buffer (Nitta Gelatin) according to the manufacturer's instructions. Fibroblasts were seeded on collagen gel and covered with the same gel after scratching. The invasion of fibroblasts into gels was evaluated immediately and at 48 hr after scraping [6, 11, 12].

Care was taken to ensure that the passage number between CAFs and NFs did not differ by more than one in subsequent experiments.

Co-culture of fibroblasts and cancer cells

To observe the indirect interaction between fibroblasts and cancer cells, we used the Boyden chamber assay. Canine CAFs and NFs were seeded at 2.0×10^5 in a 12-well plate and cultured until they reached a 70% confluent. Cell culture inserts for a 12-well plate (pore size 8 μm / high density pore; Ikeda Rika, Tokyo, Japan) were set up on the plates, and canine mammary grand cancer cells (RCM-SO; Donated by Professor Tuyoshi Kadosawa, Department of Companion Animal Medicine, Faculty of Veterinary Medicine, Rakuno-Gakuen University) were seeded at 1.0×10^5 in each insert. The upper insert was filled with serum-free culture medium, and the lower part was filled with 10% FBS culture medium to promote cell migration. After 48 hr of incubation, the membrane was fixed, and non-migrated cells remaining on the top side of the filter were removed with a cotton swab. The migrated RCM-SO cells were stained with Diff quick stain (Sysmex, Kobe, Japan), and the number of cells was counted.

To investigate the invasion ability, Matrigel (Corning, New York City, NY, USA) was added to the surface of the insert membrane before seeding RCM-SO cells. RCM-SO cells were cultured for 96 hr and the number of infiltrating cells was counted using the same procedure.

Three-dimensional co-culture model of cancer cells and fibroblasts

To investigate the direct interaction between fibroblasts and cancer cells, a three-dimensional (3D) co-culture was performed. Collagen gels were prepared by mixing 400 µl of cell suspension of canine CAFs or NFs (5.0×10^5/ml) in FBS, 1.8 ml of collagen gel (Cell matrix type IA, Nitta Gelatin), 540 µl of five-fold concentrated DMEM (Nitta Gelatin), and 260 µl of reconstitution buffer (Nitta Gelatin) for each well of the 12-well culture plate, following the manufacturer's recommendation. The solution was then incubated at 37°C for 30–60 min. RCM-SO cells were seeded on collagen gels at 2.0×10^5 in each well. As control groups, collagen gels containing only fibroblasts and collagen gels with only RCM-SO cells were prepared.

After incubation overnight, each gel was detached and cultured in growth medium for five additional days. The diameter of the gel was measured for five consecutive days, and the relative value compared to the initial size was determined.

The gels were exposed to air by placing them on a mesh in a new plate with a growth medium, and an air-liquid interface culture was used to stimulate cancer cell invasion into the gel. After five days of incubation, the gel was fixed in formalin solution, embedded in paraffin, and the vertical sections were stained with hematoxylin and eosin.

Data analysis

The results were confirmed by performing experiments in triplicates. Data are expressed as the mean ± SD. Analyses were performed using Microsoft Excel 2016 (Microsoft, Redmond, WA, USA), and statistical significance was set at $P<0.05$. The significance of differences was analyzed using the Student's t-test for comparison of two groups and the Tukey honestly significant difference test for comparisons of three groups.

RESULTS

Canine CAFs showed a higher migratory and invasive ability than NFs

Canine CAFs and NFs were successfully isolated from the cancer and skin tissues of the five dogs (Table 1). Each cell displayed elongated, spindle-shaped fibroblast features. Immune fluorescence was conducted to ascertain that the obtained cells were fibroblasts before proceeding with the experiment. The images of Dog 1 are shown in Fig. 1. Fibroblasts were identified by positive staining for vimentin and negative staining for cytokeratin. Both CAFs and NFs were positive for α-SMA. These fibroblasts were used in the subsequent experiments.

To evaluate the migratory and invasive abilities of canine CAFs and NFs, wound-healing assays were conducted (Fig. 2A). The percentage of wounded area filled with CAFs was significantly higher than that filled with NFs in every experiment (Fig. 2B, 2C).

Next, to investigate the infiltration ability of canine CAFs and NFs, we added type I collagen gel to surround both fibroblasts (Fig. 2D). Fibroblasts were observed immediately after scraping and 48 hr later, and invasion into the collagen gel of both fibroblasts was observed. The re-population of the cell-free area of CAFs was greater than that of NFs in Dogs 1 and 3. In Dog 3, the percentage of wounded area filled with CAFs was significantly greater than that filled with NFs. In contrast, there was no difference in the wounded area filled between CAFs and NFs in Dog 2 (Fig. 2E, 2F).

Cancer cells showed greater migratory and invasive ability when co-cultured with fibroblasts

To observe the indirect effects of co-culture with canine fibroblasts on cancer cells, we performed a Boyden chamber assay (Fig. 3A). The number of migrated RCM-SO cells co-cultured with CAFs was greater than that co-cultured with NFs. All cases used in this experiment showed a significantly greater number of migrated cells when cultured with fibroblasts. When RCM-SO cells were co-cultured with CAFs, their migratory ability tended to be greater compared to when they were co-cultured with NFs (Fig. 3B, 3C).

To compare the invasive ability of RCM-SO cells co-cultured with CAFs and NFs, collagen gels were coated on the bottom of the insert. Then, RCM-SO cells were cultured on the collagen gels as shown in Fig. 3D. Every case that was co-cultured with fibroblasts showed a significant increase in the number of invasive cells compared to that without fibroblasts. There were significantly more infiltrating cells when RCM-SO cells were co-cultured with CAFs compared to when they were co-cultured with NFs (Fig. 3E, 3F).

Table 1. Summary of canine epithelial tumor samples

No.	Breed	Age (years)	Sex[a]	Site	Diagnosis[b]
Dog 1	Yorkshire Terrier	6	M	Maxilla	SCC
Dog 2	Pomeranian	11	MC	Mandible	SCC
Dog 3	Labrador Retriever	12	FS	Lung	LC
Dog 4	Miniature Dachshund	13	MC	Lung	LC
Dog 5	Shiba dog	14	FS	Mammary grand	MGT

a) MC: male castrated, M: male, FS: female spayed. b) SCC, squamous cell carcinoma; LC, lung adenocarcinoma; MGT, mammary gland tumor.
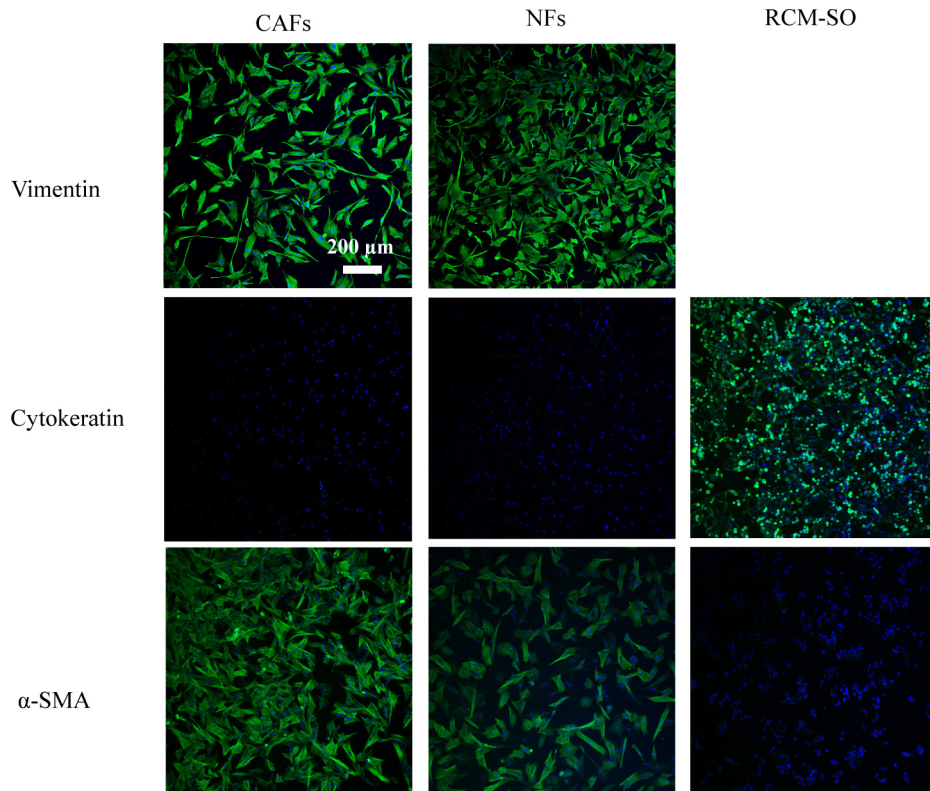
Fig. 1. Identification of fibroblasts. Immunofluorescence staining of cancer-associated fibroblasts (CAFs), normal fibroblasts (NFs), and RCM-SO (mammary gland tumor cell line). Canine CAFs and NFs stained for vimentin but not cytokeratin (confocal microscope, ×100). Fibroblasts stained for α-smooth muscle actin (α-SMA).
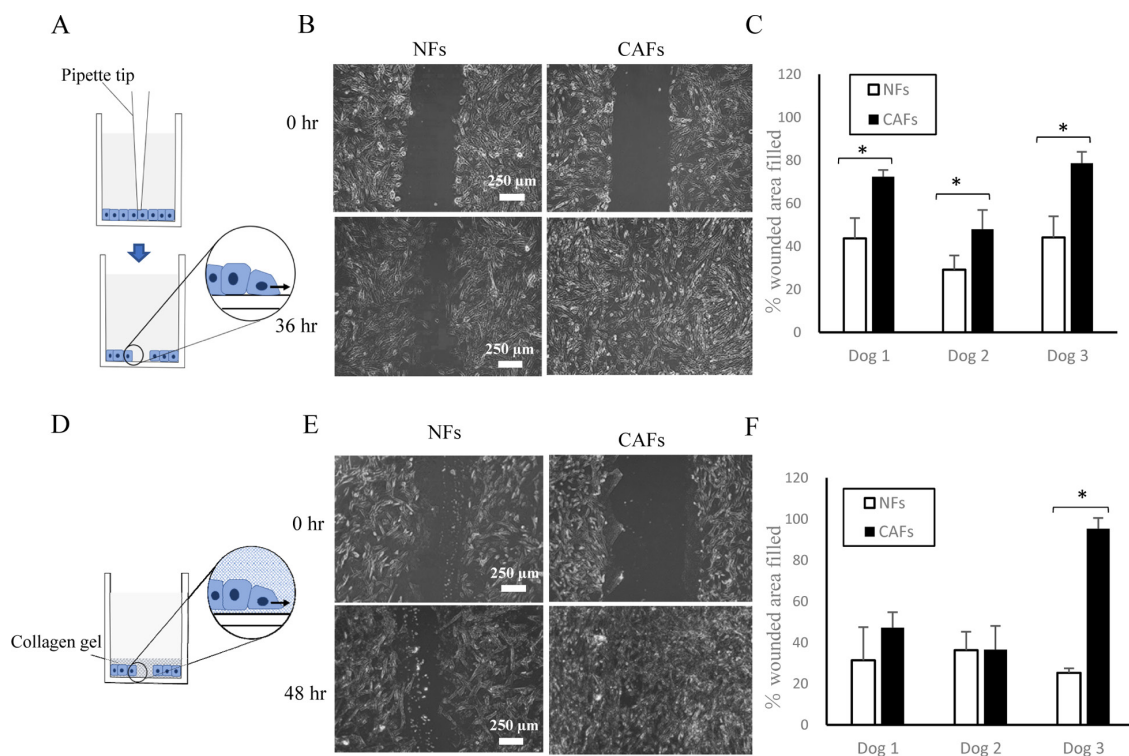

Fig. 2. Comparison of migratory and invasive ability of canine cancer-associated fibroblasts (CAFs) and normal fibroblasts (NFs). **A.** Cells were scratched in a straight line. The migration of fibroblasts into the cell free area was observed. **B.** The comparison under the microscopes. **C.** The % wounded area filled of canine CAFs was significantly greater than NFs in every experiment. **D.** Comparison of invasive ability of CAFs and NFs. Type I collagen gel was used to embed the fibroblasts. **E.** In canine CAFs, more fibroblasts invasive into the collagen gel than NFs. **F.** Cancer associated fibroblasts of Dog 3 showed significantly higher % wounded area filled than NFs (*$P<0.05$).
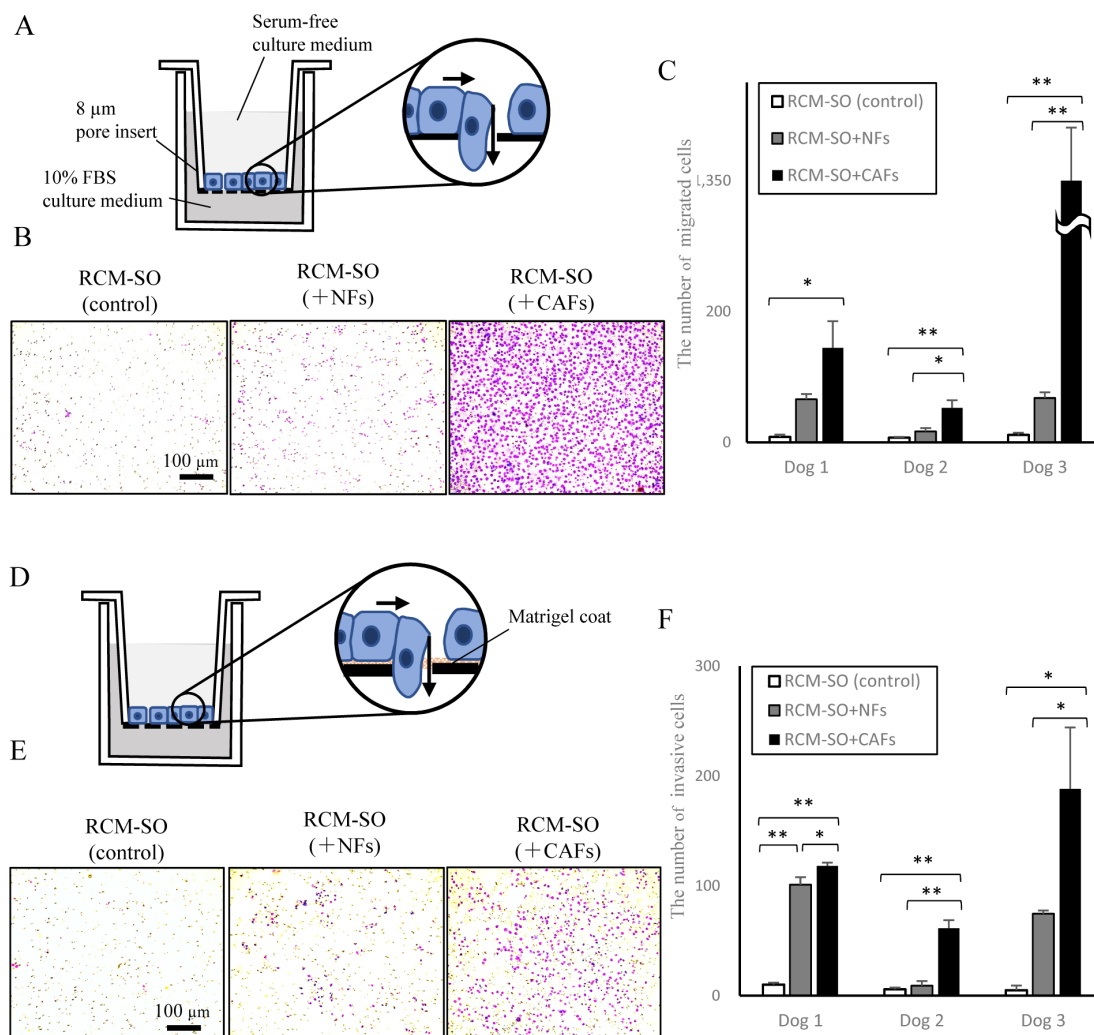
Fig. 3. Influence of fibroblasts fluid factors on the migratory and invasive ability of cancer cells. **A.** A schematic representation of Boyden chamber assay. **B.** The bottom of insert after 48 hr. Each purple dot represents migrated RCM-SO cells. **C.** The number of migrated RCM-SO cells increased significantly when co-cultured with canine CAFs. **D.** The inserts were coated with Matrigel for investing invasive ability. **E.** The bottom of insert after 96 hr. **F.** The number of invaded RCM-SO cells increased significantly when co-cultured with each fibroblast (*$P<0.05$, **$P<0.01$). Control: single culture of RCM-SO cells, + NFs: co-culture with NFs, +CAFs: co-culture with CAFs. RCM-SO, mammary gland tumor cell line; CAF, cancer-associated fibroblasts; NF, normal fibroblasts.

Gel shrinkage and cancer cell invasion were observed in three-dimensional cultures of canine CAFs

To investigate the direct interaction between canine fibroblasts and cancer cells, 3D co-cultures were performed. Collagen gel contraction and invasion of RCM-SO cells into the collagen gels were observed. The collagen gel with CAFs showed a significantly higher percentage of contraction than that with NFs in Dog 4. In addition, collagen gels that were co-cultured with fibroblasts and RCM-SO cells showed a greater contraction percentage than those with fibroblasts in Dog 4. In contrast, there was no remarkable difference between the percentage of contraction of collagen gel with CAFs and that of NFs in Dogs 3 and 5. Gels cultured only with RCM-SO cells showed no shrinkage (Fig. 4A).

A cross-section of the collagen gel after hematoxylin eosin staining is shown in Fig. 4B. RCM-SO cells co-cultured with CAFs showed noteworthy proliferation at the surface of the gel and invasion into the gel. In Dog 3, the invasion of RCM-SO cells into the collagen gel with CAFs was remarkable. In collagen gels with NFs, RCM-SO cells existed only at the surface of the gels.

DISCUSSION

CAFs comprise the majority of tumor stroma and are involved in tumor growth, metastasis, and invasion through direct and indirect effects [14]. In particular, their impact on tumor invasion has attracted significant attention in human medicine. Teng *et al*. isolated CAFs from epithelial tumors and reported that the migration and invasion of cancer cells were activated indirectly by co-culturing with CAFs [18]. In veterinary medicine, there are only a few reports on CAFs, and no studies have examined
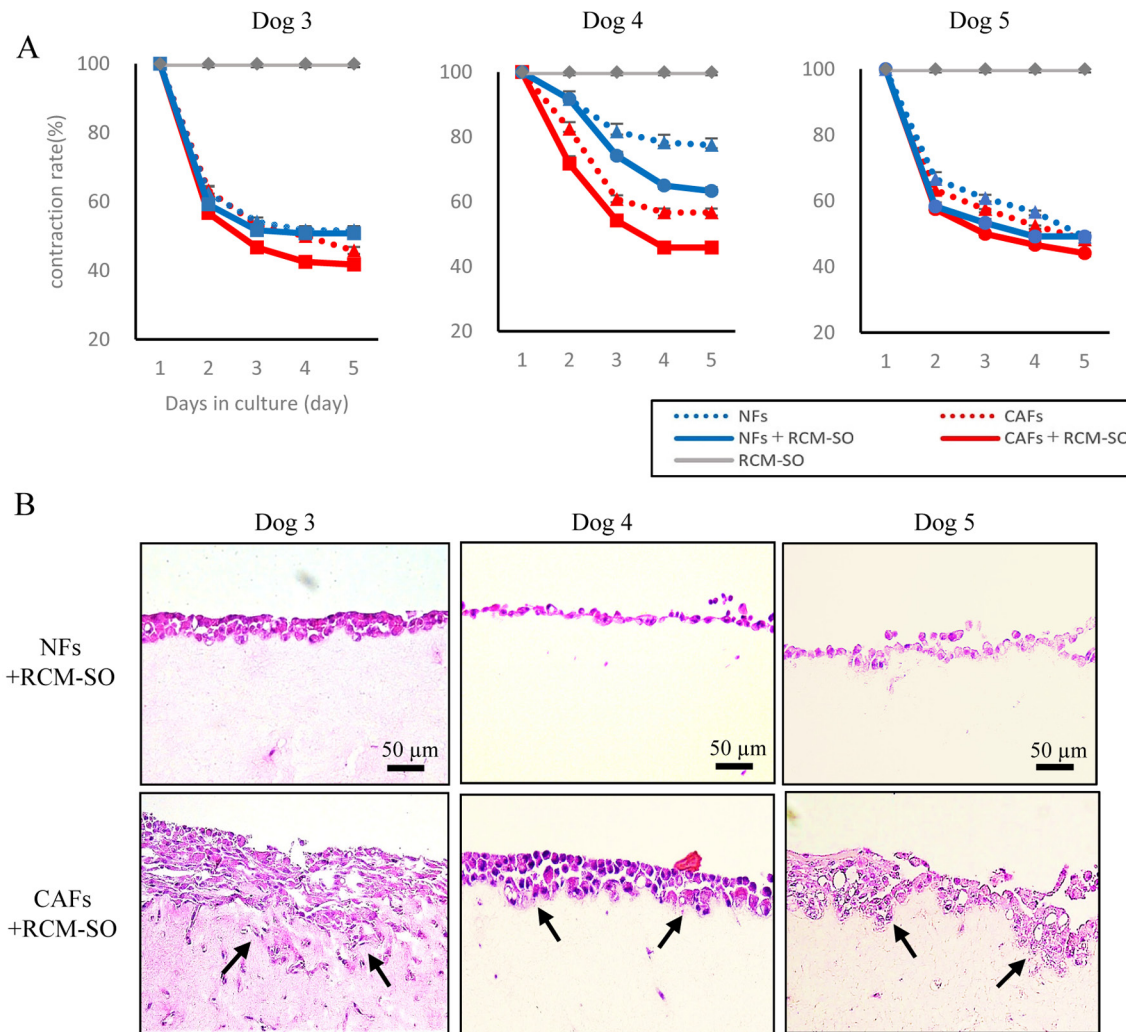
Fig. 4. Observation of changes in three-dimensional co-culture of cancer cells and fibroblasts. **A.** Coefficient of contraction of CAFs and NFs collagen gels. Collagen gels with canine CAFs showed a higher contraction rate than that with NFs. Also, collagen gels that co-cultured with RCM-SO cells increased the contraction when compared to collagen gels only with fibroblasts. Gels containing RCM-SO cells alone did not show shrinkage. **B.** Hematoxylin and eosin stain of collagen gels after co-culturing. When observed the gels contained NFs, RCM-SO cells existed just at the surface of gels. In the contrast, RCM-SO cells showed noteworthy proliferation and invasion into the gels with CAFs (arrows). NFs: collagen gels containing NFs, CAFs: collagen gels containing CAFs, +RCO-CO: co-culture with RCM-SO cells. RCM-SO, mammary gland tumor cell line; CAF, cancer-associated fibroblasts; NF, normal fibroblasts.

the differences in biological characteristics of canine CAFs and NFs or their influences on cancer cells. The comparison of CAFs and NFs as a normal symmetry will help elucidate the role of CAFs in malignancy. In this study, we focused on epithelial tumors because it is difficult to distinguish fibroblasts from mesenchymal tumor cells in the isolation of CAFs.

To compare the differences in the migratory and invasive abilities of canine CAFs and NFs, wound-healing assays were conducted. In previous reports, wound healing assay has been used to evaluate the migratory and invasive ability of fibroblasts [11, 12]. Wound healing assay was considered to be a suitable experimental method for evaluating the movement of fibroblasts into low cell density areas, as it is not affected by the concentration gradient or fluid factors. In all cases, CAFs showed a significantly greater migratory ability than did NFs. Canine CAFs also tended to have a greater invasive ability than NFs, especially in case 3, which showed a significantly higher percentage of wounded area filled than NFs. In contrast, there was no difference in the wounded area filled between CAFs and NFs in Dog 2. A study using human CAFs and NFs isolated from breast cancer patients showed that CAFs have greater migratory, invasive, and proliferative abilities than NFs [16]. We observed a similar tendency in the present study. Fibroblasts have been reported to dissolve the extracellular matrix by secreting matrix metalloproteinases (MMPs) and remodeling the extracellular matrix [6, 20]. It was suggested that CAFs showed a greater ability to dissolve type I collagen than NFs, depending on MMP activity [21]. These aspects may have contributed to the difference in the invasive ability of canine CAFs and NFs. It is possible that differences in MMP activity and secretions from CAFs led to the differences between the cases shown in Fig. 2F.

Boyden chamber assays were performed to determine the indirect effects of fibroblasts on cancer cells. A significant increase in the number of migratory RCM-SO cells was observed when they were co-cultured indirectly with each fibroblast. There was also a tendency for more RCM-SO cells to migrate when co-cultured with CAFs than with NFs. The inserts were then coated with Matrigel to compare their invasive ability. There was a significant increase in the number of invasive RCM-SO cells when co-cultured with CAFs. A study conducted using Boyden chamber assays with CAFs isolated from human endometrial adenocarcinomas also yielded results similar to those of the current experiments [18]. That study also showed a decrease in the number of migrated and infiltrated cancer cells through the inhibition of SDF-1, a humoral factor secreted from CAFs. Mesenchymalization of cancer cells induced by transforming growth factor-β (TGF-β) and MMPs secreted by CAFs has been reported. These proteins are known to promote tumor invasion and metastasis [1]. Several humoral factors released by CAFs are expected to activate tumor cell behavior, but further experiments are required to elucidate their influence on tumor malignancy.

To observe direct fibroblast-cancer cell interactions, 3D cultures were conducted. RCM-SO cells showed significant proliferation and invasion into collagen gels with canine CAFs. On the other hand, RCM-SO cells existed only on the surface of the gels with NFs. Three-dimensional culture of tumor cells and fibroblasts using type I collagen has been applied in several studies as a culture method reflective of the *in vivo* environment. Human studies that conducted 3D co-culture of cancer cells and fibroblasts isolated from epithelial tumor patients reported cancer cell infiltration into CAF-mixed collagen gels [6, 8]. The formation of guidance pathways, which allow epithelial tumor cells to invade and metastasize without epithelial-mesenchymal transition, into the extracellular matrix by CAFs has been reported [6, 12, 13]. Considering these mechanisms, it is possible that canine CAFs assist RCM-SO cells in infiltrating gels. Furthermore, RCM-SO cells may have been indirectly stimulated by humoral factors secreted from CAFs, as suggested by the Boyden chamber assay.

Collagen gels with canine CAFs showed a significantly greater contraction rate than that of gels with NFs in Dog 4. In contrast, there were no significant differences in Dogs 3 and 5. Several human studies using 3D co-culture of type I collagen reported that CAFs caused stronger gel contraction than did NFs [8, 15]. Myofibroblasts contain an abundance of stress fibers composed of α-SMA, which cause contraction of the gel [2, 7, 19]. The high contraction of the collagen gels with CAFs suggested that the canine CAFs were similar to myofibroblasts. The differences in shrinkage between individuals were attributed to the expression of stress fibers. In addition, the collagen gels co-cultured with RCM-SO cells and fibroblasts tend to show greater contraction than that of gels containing fibroblasts alone. The stimulation of fibroblasts by cancer cells may increase the contraction of gels.

A limitation of this study was that it was difficult to integrate tumor types. The malignancy and behavior of canine epithelial tumors vary according to the type of tumor, which may influence the results. In addition, it was not possible to use the same fibroblasts for all analyses because of the limited number of obtained cells and their proliferative capacity.

The results of the current study showed that canine CAFs have a greater migratory and invasive ability than do NFs. In addition, canine CAFs can activate migration and invasion of cancer cells and promote the infiltration of cancer cells into collagen gels. Although this study revealed some of the biological characteristics of canine CAFs, further studies are required to confirm the impact of CAFs on the mechanism of tumor malignancy.

CONFLICT OF INTEREST. The authors declare that they have no conflicts of interest.

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