



Trimer form of tumor necrosis factor-related apoptosis inducing ligand induces apoptosis in canine cell lines derived from mammary tumors

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ABSTRACT. We evaluated the cytotoxic effect of isoleucine-zipper tumor necrosis factor-related apoptosis inducing ligand (izTRAIL) against cell lines, B101592, Cha, and C090115, derived from canine mammary gland tumors. These cells were derived from three dogs diagnosed with mammary adenoma or carcinoma. All three cells were positive for vimentin, while B101592 and C090115 were positive for cytokeratin (CK) AE1/AE3 and CK CAM5.2. Treatment with izTRAIL decreased the viability of the three cell lines. The proportion of annexin V+/propidium iodide-cells increased in all three cell lines after treatment with izTRAIL. Additionally, cell cycle analysis revealed that izTRAIL treatment increased the number of cells in sub-G1 phase. Moreover, izTRAIL treatment activated caspase-8 and caspase-3 and enhanced the levels of cleaved poly (ADP-ribose) polymerase. The cytotoxic effect of izTRAIL was mitigated upon co-treatment with caspase-8 or caspase-3 inhibitor. These results indicated that izTRAIL induces apoptosis in cell lines derived from canine mammary tumor, which was also previously reported in canine hemangiosarcoma cell lines. This suggested that canine tumor cells have conserved TRAIL receptors. This study will provide the basis for further studies on TRAIL receptors and TRAIL-related molecules.

KEY WORDS: apoptosis, canine, mammary tumor, small animal, tumor necrosis factor-related apoptosis-inducing ligand

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Canine mammary tumors (CMTs) are among the most common neoplasms in female dogs [24]. Histopathological examination is the gold standard diagnostic method for CMT, and in 2011, a new classification for CMTs was proposed [14]. Based on this classification, the survival time for most aggressive tumor subtypes is about 3 months [36]. In addition, several studies indicate that lymphatic vessel infiltration and regional lymph node metastasis of tumor cells are important for determining the tumor prognosis, and the new classification of CMTs is useful to predict the metastatic potential [35, 41, 44]. Currently, a combination of surgical resection and chemotherapy was used for treating mammary carcinomas, but this was ineffective against highly malignant tumors exhibiting lymphatic vessel infiltration [44]. Globally, human breast cancer (HBC) is the major cause of cancer death in women, and there are various therapeutic strategies to treat HBC [13]. HBC and CMT have similar risk factors, clinical features, and molecular players, such as hormones; therefore, CMT is considered as a model for HBC [1, 33]

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also called Apo2 ligand, was first discovered as a member of tumor necrosis factor family protein in 1995 [53]. The expression of TRAIL protein is reported in the normal spleen, prostate, lung, tonsillar T cell, and lymphoma cell lines (K299 and Raji) [53]. TRAIL is reported to induce apoptosis in various tumor cells, but not in normal cells [2, 31, 49, 53]. In addition, some studies reported that TRAIL exhibits anti-tumor effects without marked systemic side effects in mouse xenotransplantation models and in cynomolgus monkeys [2, 20, 49]. Therefore, TRAIL can be a potential therapeutic agent for cancer. The mechanism of action of TRAIL has been well studied, and studies on the mechanism

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underlying TRAIL resistance in cancer cells are ongoing [26, 46]. Several studies investigating the TRAIL pathway in HBC and other tumors have designed various TRAIL receptor-specific antibodies, and some antibodies are undergoing clinical trials [4, 5, 19, 26, 32, 43, 45]. Furthermore, to enhance the sensitivity of the TRAIL-resistant tumors, many combinations with other molecules, such as MS-275 and caudatin, have also been studied [12, 38, 40, 46].

However, these studies are conducted mainly in the medical field, and there is limited information on TRAIL in veterinary medicine. Four studies reported the effect of TRAIL on canine tumor cells, including mast cell tumor, lymphoma, osteosarcoma, cholangiocarcinoma, mammary tumor, and hemangiosarcoma [10, 15, 30, 39]. Among these studies, only two studies reported the anti-tumor effect of TRAIL after a single administration [10, 15]. Our previous study revealed that the isoleucine zipper recombinant human TRAIL (izTRAIL), which is forming trimer stably was more effective in inducing apoptosis than the TRAIL monomer [15]. This was the first study to report that TRAIL trimer is more effective in dogs; this was similar to reports in humans [15, 49]. Although a study reported the cytotoxic effect of TRAIL on canine epithelial tumors, no studies have evaluated the cytotoxic effect of izTRAIL on canine epithelial tumors [39]. In veterinary medicine, studies on such basic features of TRAIL are few, and no advancements have been seen in the identification of receptors. Therefore, it is necessary to evaluate TRAIL sensitivity, presence of TRAIL receptors, and TRAIL pathways in dogs, which will aid in targeting the TRAIL receptors that chemotherapeutic agents target/bind to in humans. As a first step in such research, it is important to confirm the conservation of the sensitivity to TRAIL in dog tumors. In this study, we investigated the cytotoxic effect of izTRAIL on CMT to accumulate the basic information of the effect on various tumors. This will provide the basis for future studies on TRAIL receptors and the TRAIL pathway in dogs.

MATERIALS AND METHODS

Case information

Three canine mammary tumors were collected from the Veterinary Teaching Hospital of Gifu University (VTH-GU), in Gifu city, Japan. The characteristics of dogs with tumors are listed in Table 1. In B101592, the dog was consulted with a private animal hospital for a mass in the mammary gland. Half a month later, an excision was performed in the VTH-GU. In Cha, the right mammary gland was swollen 10 days after excision of the left mammary tumor at a private animal hospital. At the VTH-GU, punch biopsy was performed 15 days after the swelling developed. On computed tomography, lung metastasis was suspected at this time. The dog died 38 days after the biopsy. In C090115, the dog was treated for mastitis due to swollen mammary glands. However, there was no improvement; thus, she was referred to the VTH-GU for a detailed examination 20 days later, and fine needle biopsy was performed with a 23 gauge-needle. Unfortunately, the prognosis details of B101592 and C090115 cases were lost. The two tumors (B101592 and Cha) obtained by biopsy were fixed with 10% buffered neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). These tumors were histologically classified into subtypes based on the 2011 classification [14]. Only cytological diagnosis was done for C090115 using a Romanowsky-based stain (Hemacolor; Merck KGaA, Darmstadt, Germany), and no histopathologic examination was performed as an owner's intention.

Immunohistochemistry of original tumors

For immunohistochemical staining, sections of B101592 and Cha were heated for 15 min at 121°C and 110 kPa in a target retrieval solution (pH 6.0, Dako, Glostrup, Denmark). Next, the sections were incubated overnight at 4°C with the following primary antibodies: mouse anti-human cytokeratin (CK) monoclonal antibody (clone AE1/AE3, 1:10, Dako), mouse anti-vimentin monoclonal antibody (clone V9, 1:20, Dako), and murine anti-CK monoclonal antibody (clone CAM5.2, pre-diluted, BD Biosciences, Franklin Lakes, NJ, U.S.A.). The positive signal was visualized using 3, 3'-diaminobenzidine solution (Histofine SAB-PO (M) kit, Nichirei, Tokyo, Japan). Finally, the sections were counterstained with hematoxylin.

Cell culture

The B101592 and Cha tumor tissues were minced and sequentially digested with 0.1% collagenase Type I (Gibco, Carlsbad CA, U.S.A.) at 37°C for 15 min and 0.25% trypsin-EDTA (Gibco) at 37°C for 15 min respectively. The cell suspension was subsequently filtered through a 70- μ m cell strainer (BD Biosciences) and resuspended in high-glucose Dulbecco's modified Eagle medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B (Penicillin-Streptomycin-Amphotericin B Suspension, Wako Pure Chemicals), and 100 μ g/ml kanamycin (Wako Pure Chemicals) (10% FBS/D-MEM). In C090115, after cytological examination, the cells that remained in the needle and syringe were directly seeded in 10% FBS/D-MEM. All cells were cultured in a humidified incubator at 100% humidity, 37°C, 20% O₂, and 5% CO₂. Sub confluent cells were passaged after digestion with 0.25% Trypsin-1 mmol/L EDTA•4Na solution (T/E solution, Wako Pure Chemicals). The cells were cultured with more than 60 passages.

For measuring the growth curve and doubling time, all cells were plated in 24-well plates (ThermoFisher Scientific, Waltham, MA, U.S.A.) at a cell density of 5,000 cells/well in 1 ml of 10% FBS/D-MEM. The cells were collected using T/E solution and counted once every 12 hr using trypan blue in a Countess™ Automated Cell Counter (Thermo Fisher Scientific). Triplicate wells were used for counting each cells.

Table 1. An information of original tumors.

	Breed	Age	Sex
B101592	Toy poodle	9y7m	F
Cha	Beagle	13y2m	F
C090115	Labrador retriever	10y9m	SF

F; Female, SF; spayed female.

Immunocytochemistry of cell lines

The cells were cultured at a cell density of 2.0×10^4 cells/well in a chamber slide for 12 hr before immunofluorescence analysis. The cells were fixed with 100% methanol and incubated overnight at 4°C with the following primary antibodies: mouse anti-human CK monoclonal antibody (clone AE1/AE3, 1:20, Dako), mouse anti-vimentin monoclonal antibody (clone V9, 1:40, Dako), and murine anti-CK monoclonal antibody (clone CAM5.2, 1:10, BD Biosciences). Next, the cells were probed with anti-mouse IgG Fab2 Alexa Fluor® 488 (1:500, Cell Signaling Technology, Danvers, MA, U.S.A.) secondary antibody. The slides were mounted with ProLong™ Diamond antifade Mountant containing 4', 6-diamidino-2-phenylindole (DAPI) nuclear stain (ThermoFisher Scientific). The cells were analyzed under a fluorescence microscope (IX73, Olympus, Tokyo, Japan).

Cell viability assay

Cell viability assays were performed using the premix WST-1 cell proliferation assay system (TaKaRa, Kusatsu, Japan). Three cell lines, TRAIL/izTRAIL-resistant Madin-Darby canine kidney (MDCK) cells [10, 15], and TRAIL/izTRAIL-sensitive HeLa cells [15, 31] were used in this study (both from JCRB Cell Bank, Osaka, Japan). MDCK cells were used as negative control, while HeLa cells were used as positive control. The cultured cells and HeLa cells were cultured in 96-well plates at a density of 1.0×10^4 cells/well. The MDCK cells were seeded at a density of 2.5×10^3 cells/well as they have a fast doubling time. The cells were cultured for 12 hr. The cells were then cultured in 10% FBS/D-MEM containing 0.01, 0.1, 1.0, 10, or 100 ng/ml of izTRAIL (Adipo Gen Life Sciences Inc., San Diego, CA, U.S.A.) resolved with sterile distilled water for 24, 48, and 72 hr. As a negative control (0 ng/ml of izTRAIL), 10% FBS/D-MEM supplemented only with sterile distilled water was used. Next, the cells were incubated with 10 µl WST-1 reagent for 1 hr. Cell viability was quantified as the relative absorbance values of treated wells compared to those of the control (0 ng/ml izTRAIL) wells using the iMark™ microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The half-maximal inhibitory concentration (IC₅₀) of izTRAIL was calculated in Image J 1.51K (National Institutes of Health, Bethesda, MD, U.S.A.) based on the results of the viability assay.

Flow cytometric analysis of apoptosis

To detect changes in the cytoplasmic membrane that indicates early apoptosis, the cultured cells were treated with 100 ng/ml izTRAIL for 18 hr. The cells were collected using T/E solution and washed with Dulbecco's phosphate-buffered saline (D-PBS, Wako Pure Chemicals). The cells were stained with annexin V/ propidium iodide (PI) (Alexa Fluor 488 Annexin V/Dead cell Apoptosis Kit, ThermoFisher Scientific).

For analysis of the cell cycle, the cell lines were treated with 100 ng/ml izTRAIL for 48 hr. The supernatant and cells were collected using T/E solution and washed with D-PBS. The collected cells were then incubated with PI (PI/RNase staining solution, Cell Signaling Technology).

The cells were counted using BD FACSCanto™II (BD Biosciences) and analyzed using BD FACSDiva 6.1 software (BD Biosciences).

Analysis of nuclear fragmentation

The effect of izTRAIL on nuclear fragmentation was analyzed using fluorescence microscopy. The cultured cells were plated in 24-well plates (ThermoFisher Scientific) at a density of 2.0×10^4 cells/well in 1 ml of 10% FBS/D-MEM for 12 hr. The cells were treated with 100 ng/ml izTRAIL for 48 hr and the cells were collected using T/E solution. The cells were washed with D-PBS and fixed in 4% paraformaldehyde (Wako Pure Chemicals) for 30 min. Next, the cells were placed on the slide and mounted with ProLong™ Diamond antifade Mountant containing DAPI nuclear stain (ThermoFisher Scientific). The DNA fragmentation was observed under a fluorescence microscope (IX73, Olympus).

Western blotting

The effect of izTRAIL treatment on apoptosis-related proteins was evaluated by western blotting. Three cell lines were collected using a cell scraper at 1, 3, 6, 12, and 24 hr after treatment with 100 ng/ml izTRAIL. The cells were then lysed with RIPA buffer (ThermoFisher Scientific) containing a proteinase inhibitor cocktail and EDTA (ThermoFisher Scientific). The concentration of the extracted protein was measured using the DC Protein assay kit (Bio-Rad). The protein concentration was determined from a standard curve of bovine serum albumin (Thermo Fisher Scientific).

The protein samples were incubated with 4X Laemmli buffer (Bio-Rad Laboratories) and supplemented with 5% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 95°C for 5 min. The proteins (15 µg/lane) were resolved by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) (4–12.5% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad). The resolved proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Hybond P0.45 PVDF, GE Healthcare Bioscience, Pittsburgh, PA, U.S.A.) at 60 V for 1.5 hr at room temperature (20–25°C: RT).

The PVDF membrane was incubated with 5% blocking reagent (GE Healthcare Bioscience) for 1 hr at RT. Next, the PVDF membrane was probed with the following primary antibodies: mouse anti-human caspase-8 (1:2,000, BD Biosciences), rabbit polyclonal anti-caspase-3 (1:1,000, Cell Signaling Technology), mouse anti-poly (ADP-ribose) polymerase (PARP, 1:2,000, BD Biosciences), or β-actin (1:1,000, Cell Signaling Technology) for 1 hr at RT. The primary antibodies were used after confirming that they reacted with the proteins of the same size as reported in the previous study for both HeLa and canine hemangiosarcoma (HSA) cells established in our laboratory [15].

The membrane was washed with TBS-T (0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, and 0.05% Tween 20; Sigma Aldrich) and

incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit, 1:5,000, Cell Signaling Technology or anti-mouse, 1:5,000, Cell Signaling Technology) for 1 hr at RT. The proteins were developed using Lumina Forte Western HRP Substrate (Merck Millipore, Darmstadt, Germany). The target protein bands were visualized using C-DiGit Blot scanner (LI-COR Biotechnology, Lincoln, NE, U.S.A.) and Image Studio Digits ver 4.0 (LI-COR Biotechnology).

Inhibition of caspase-8 or caspase-3

The cell lines were cultured in 96-well plates for 12 hr. The cells were treated with 0, 2.5, 10, and 40 μ M of caspase-8 inhibitor (Z-IETD-FMK, MBL, Nagoya, Japan) or caspase-3 inhibitor (Z-DEVD-FMK, MBL) for 2 hr. The caspase inhibitors were dissolved in dimethyl sulfoxide. Subsequently, the cells were treated with izTRAIL (at a final concentration of 100 ng/ml) for 24 and 48 hr. The WST-1 assay was performed as described in the cell viability assay.

In analyzing the cell cycle, the cell lines were treated with caspase-8 and caspase-3 inhibitors before the addition of izTRAIL. Next, they were collected and stained with PI/RNase staining solution (Cell Signaling Technology). The cell cycle analysis was performed by flow cytometry as described previously.

Statistical analysis

All experiments using cells were performed at least in triplicates. Cell viability assay results were statistically evaluated by one-way analysis of variance and Dunnett's *post-hoc* test. The percentage of annexin V-positive and PI-negative (annexinV+/PI-) cells or sub-G1 phase cells were compared between groups by Student's *t*-test. These tests were performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). All values are expressed as mean \pm standard deviation (SD). The difference was considered statistically significant when the $P < 0.05$.

RESULTS

Tumor diagnosis and characteristics of cultured cells

The B101592 tumor tissue had papillary and ductal growth patterns with single layer of epithelial cells supported by fibrovascular stroma (Fig. 1A). Below the epithelial cells, a layer of myoepithelial cells was identified. These epithelial cells had an oval-shaped nucleus and eosinophilic cytoplasm. The nuclear and cellular pleomorphisms in the epithelial cells were not severe, and mitoses were rare. The epithelial cells were positive for CK AE1/AE3 and CK CAM5.2 (Fig. 1B and 1C). The myoepithelial cells were positive for vimentin, whereas the epithelial cells forming the duct were negative for vimentin (Fig. 1D).

The Cha tumor had solid growth pattern comprising tightly packed cells (Fig. 1E). These cells had round- to oval-shaped nucleus with prominent nucleoli. The cells had lightly basophilic to eosinophilic cytoplasm. Anisokaryosis was moderate, and mitoses were frequent in the Cha tumor cells. The tumor cells were partially positive for CK AE1/AE3 and CK CAM5.2, and positive for vimentin (Fig. 1F–H). Based on the criteria proposed by Goldschmidt in 2011 [14], the B101592 tumor was classified as intraductal papillary adenoma, while the Cha tumor was classified as carcinoma-solid.

The C090115 tumor had epithelial cells arranged in small clusters on cytological specimens (Fig. 1I). These epithelial cells had a round to oval-shaped nucleus and scanty to moderate amount of deeply basophilic cytoplasm. The nucleus-to-cytoplasmic ratio of these cells was high. The nucleoli were large, prominent, and multiple. Based on the cytological diagnostic criteria, the C090115 tumor was diagnosed as a malignant epithelial tumor, suspected as originating from the mammary gland [22].

The doubling times of the cultured cells derived from the B101592, Cha and C090115 tumors were 19.1, 21.5, and 21.3 hr, respectively (Fig. 2). The cultured cells of B101592 had an oval-shaped nucleus and spindle to polygonal cytoplasm (Fig. 3A). Although some cells loosely adhered to each other and many cells were growing independently. The cultured cells of Cha had a round-shaped nucleus and abundant polygonal cytoplasm (Fig. 3B). These cells adhered to each other. The C090115 cultured cells had a round to oval-shaped nucleus and spindle to polygonal cytoplasm that adhered very tightly (Fig. 3C). The C090115 cells had very prominent nucleoli. The cultured cells of B101592 and C090115 were positive for CK AE1/AE3 and CK CAM5.2, whereas those of Cha were negative for CK AE1/AE3 and CK CAM5.2 (Fig. 3D–I). All cultured cells were positive for vimentin (Fig. 3J–L).

Cell viability and IC₅₀ values

Treatment with izTRAIL decreased the cell viability of the three cultured cells, and HeLa cells depended on the concentration and treatment time. However, there was no significant cytotoxic effect of izTRAIL on MDCK cells (Fig. 4). The IC₅₀ values of izTRAIL on B101592, C090115, and Cha cells were 11.6 ± 4.4 , 5.45 ± 2.2 , and 14.9 ± 6.9 ng/ml, respectively at 72 hr post-treatment. The IC₅₀ value of izTRAIL against HeLa cells was 13.76 ± 0.9 ng/ml at 72 hr post-treatment, which was similar to the IC₅₀ value reported in a previous study [15].

The morphological change of cultured cells and analysis of apoptosis

Morphologically, cultured cells were small and round, and were detached from the dishes after treatment with izTRAIL for 18 hr (Fig. 5). Flow cytometric analysis revealed that the proportion of early apoptosis phase cells (annexin V+/PI- cells) increased at 18 hr post-izTRAIL treatment.

DAPI staining revealed nuclear fragmentation in the cultured cells at 48 hr post-izTRAIL treatment. In addition, the cell cycle analysis revealed that the proportion of cells in sub-G1 phase markedly increased, whereas the proportion of cells in G0/G1 phase decreased after treatment with izTRAIL (Fig. 6).

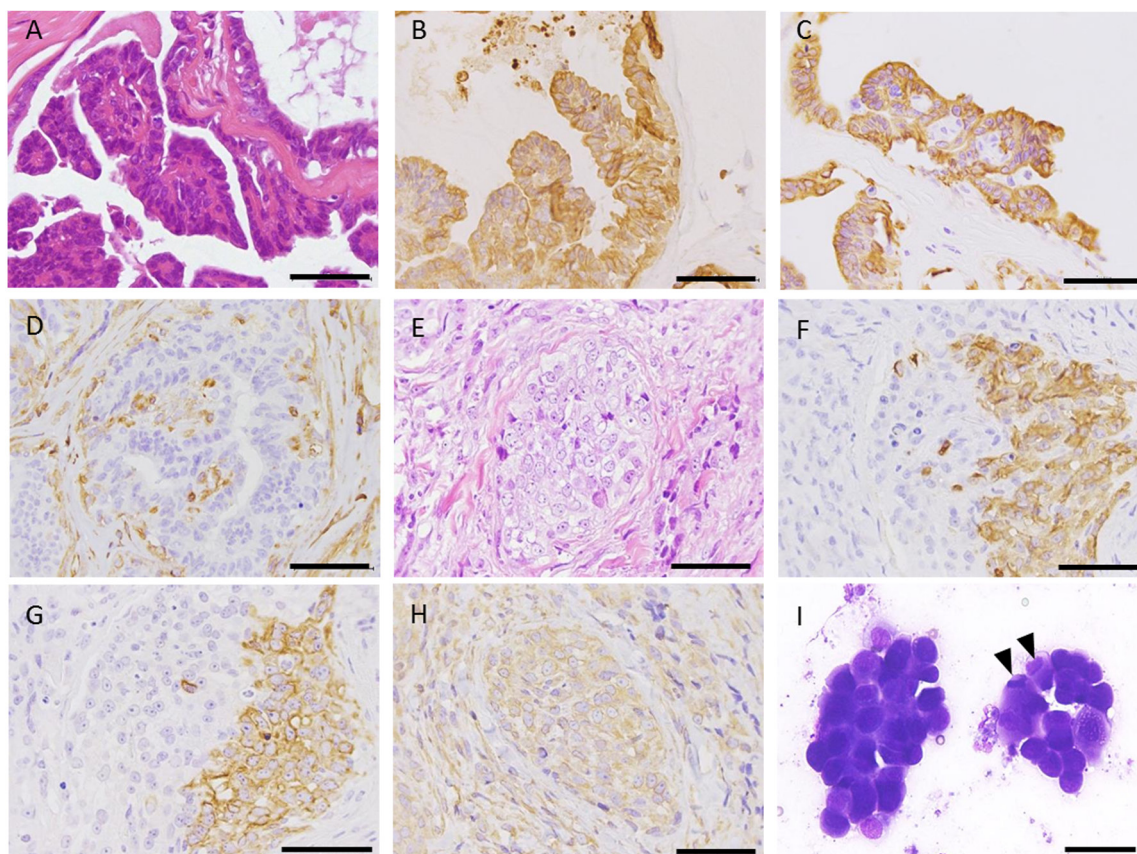


Fig. 1. Biopsy and fine-needle aspiration of original tumors. The papillae are covered with a single layer of epithelial cells in the B101592 tumor. Hematoxylin and eosin (HE) staining (A). Tumor cells are positive for cytokeratin (CK) AE1/AE3 (B) and CK CAM5.2 (C), and negative for vimentin (D). Tumor cells are packed tightly without tubular differentiation in the Cha tumors. HE staining (E). The tumor cells are partially positive for CK AE1/AE3 (F), CK CAM5.2 (G), and positive for vimentin (H). The epithelial cells show round to oval-shaped nucleus and scant to moderate amount of deeply basophilic cytoplasm (I). Arrowheads show mitoses. Hemacolor stain. Bar=50 μ m.

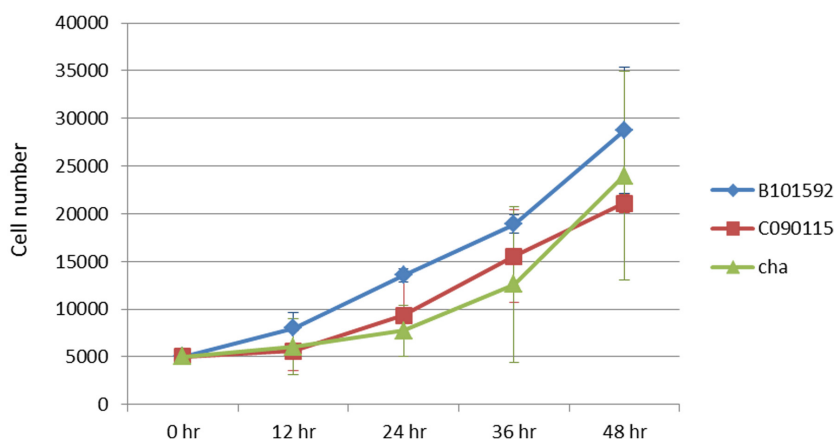


Fig. 2. Growth rate of cultured cells derived from the canine mammary gland. The vertical axis represents the cell number, and the horizontal axis represents the time. The graph is presented as mean \pm standard deviation (SD).

Three cell lines were detected about caspase-8, caspase-3, and PARP after izTRAIL treatment by western blotting (Fig. 7). The expression levels of cleaved caspase-8, cleaved caspase-3, cleaved PARP increased, whereas those of non-cleaved caspase-8, non-cleaved caspase-3, and non-cleaved PARP decreased in the B101592 cells after izTRAIL treatment for 1 hr when compared to those in the controls. Non-cleaved PARP was not detectable at 3–24 hr. The expression levels of cleaved caspase-8 and cleaved

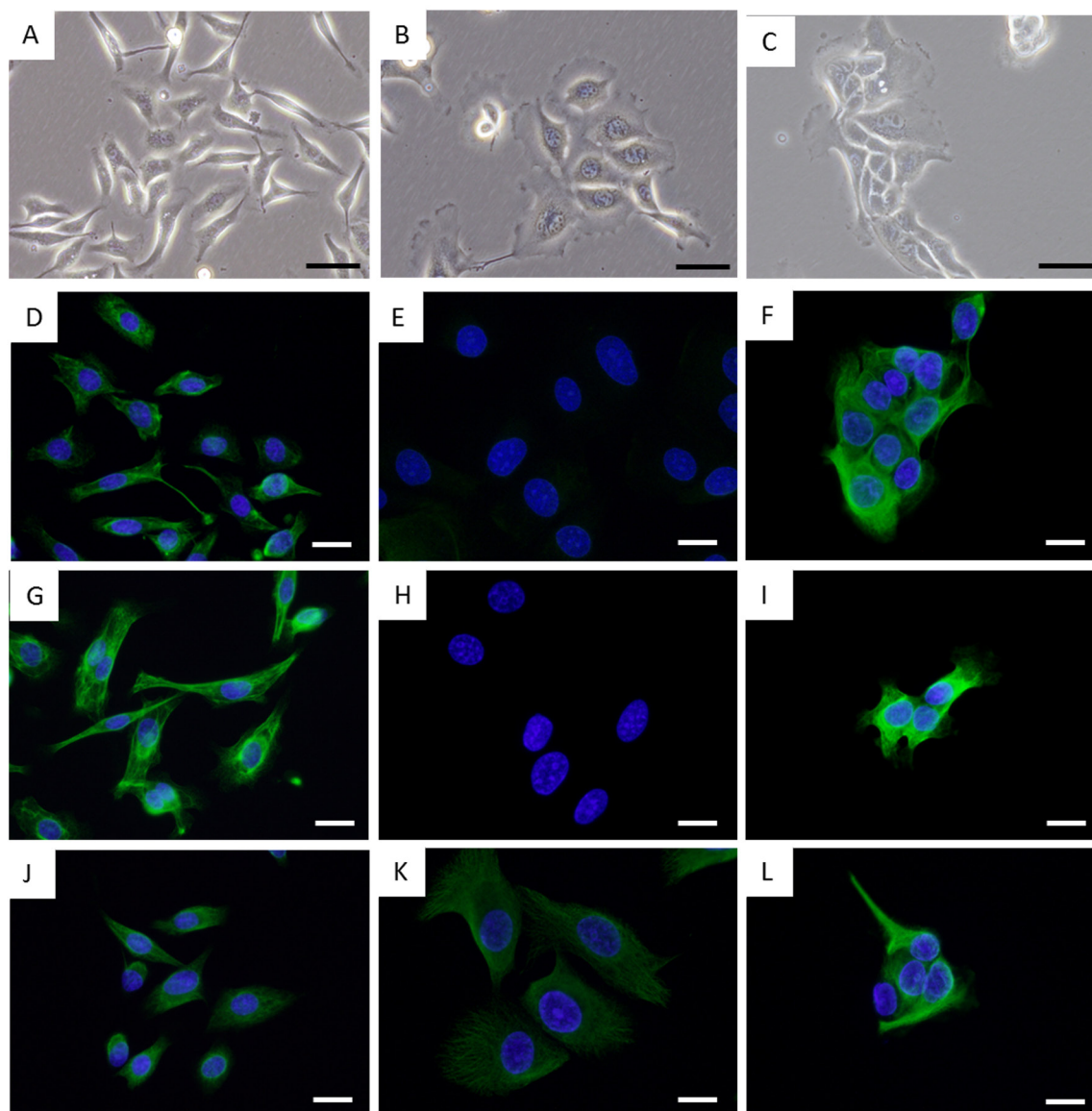


Fig. 3. Morphological and immunocytochemical characteristics of cultured cells. Cultured cells derived from B101592 tumor show round-to oval-shaped nucleus and spindle to polygonal cytoplasm (A). Cultured cells derived from Cha tumor show oval-shaped nucleus and abundant polygonal cytoplasm (B). Cultured cells derived from C090115 tumor show round- to oval-shaped nucleus and polygonal cytoplasm adhered to each other very tightly (C). Bar=50 μ m. In immunofluorescence analysis, B101592 cells are positive for cytokeratin (CK) AE1/AE3 (D), CK CAM5.2 (G), and vimentin (J). Cha cells are positive for vimentin (K), but negative for CK AE1/AE3 (E) and CK CAM 5.2 (H). C090115 cells are positive for CK AE1/AE3 (F), CK CAM5.2 (I), and vimentin (L). Bar=20 μ m.

PARP increased after izTRAIL treatment for 3 hr, whereas those of cleaved caspase-3 were detectable after izTRAIL treatment for 6 hr in the C090115 and Cha cells. In C090115 cells, non-cleaved PARP was not detectable after 24 hr. Furthermore, non-cleaved PARP was not detectable after 12–24 hr in the Cha cells.

The WST-1 assay after treatment with caspase inhibitor revealed that both caspase-3 and caspase-8 inhibitors significantly increased the tumor cell viability at 40 μ M (Fig. 8). The flowcytometry analysis revealed that caspase-8 inhibitor and caspase-3 inhibitor decreased the proportion of cells in sub-G1 phase and increased the proportion of cells in G0/G1 phase (Fig. 9).

DISCUSSION

In this study, we evaluated the cytotoxic effect of izTRAIL against cultured cells derived from the mammary gland tissue tumors, including histologically low grade tumor (B101592), histologically malignant tumor (Cha), and cytologically malignant epithelial tumor (C090115). All the three cultured cells were positive for vimentin. The B101592 and C090115 cells were positive for CK AE1/AE3 and CK CAM5.2. This suggested that B101592 and C090115 tumors were derived from the epithelial cells in

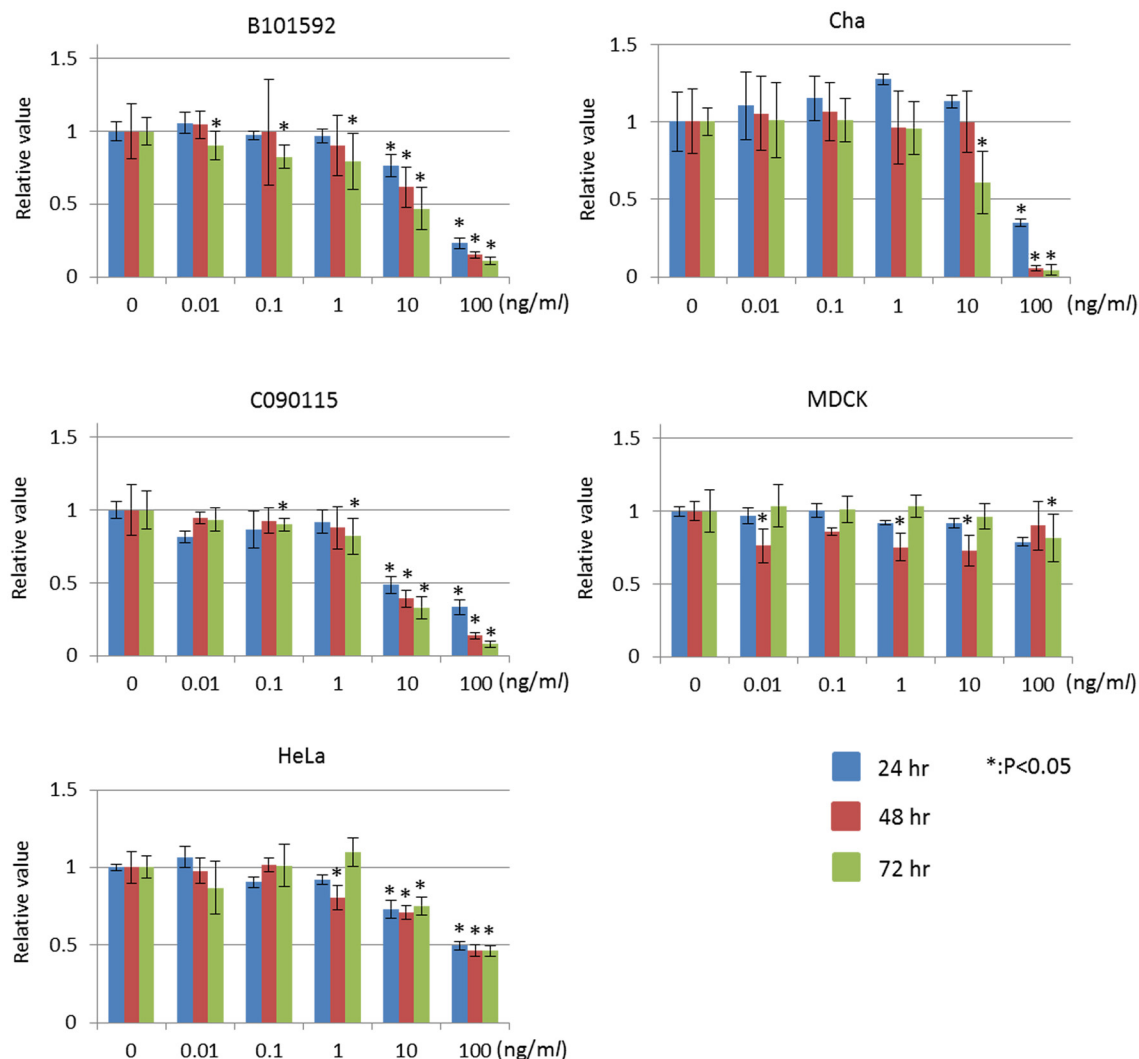


Fig. 4. Cell viability of cultured cells, Madin-Darby canine kidney (MDCK) cells, and HeLa cells after isoleucine-zipper tumor necrosis factor-related apoptosis inducing ligand (izTRAIL) treatment. The vertical axis represents the relative absorbance value compared with the control group (no izTRAIL treatment), and the horizontal axis represents the concentration of izTRAIL. Representative results of more than three independent experiments are presented as mean \pm standard deviation (SD) (* $P < 0.05$).

the mammary gland (except squamous epithelial cells) as the CMT cell lines are reported to be positive for vimentin in several cases [17, 18, 54]. The Cha cells were negative for both of CK AE1/AE3 and CK CAM5.2. However, the Cha cells adhered to each other, which suggested that they were epithelial cells. In biopsy specimens, Cha cells were partially positive for CK AE1/AE3 and CK CAM5.2, while they were homogeneously positive for vimentin. This indicated that the cultured cells were derived from CK AE1/AE3 and CK CAM5.2 negative area of the tumor. There are some established CMT cell lines that are positive for vimentin and weakly positive or negative for cytokeratin depending on their passage number [17, 54]. The results of this study and those of previous reports suggested that the three cultured cells were derived from CMT cells. However, to prove that these cell lines are CMT cells, it is necessary to transplant these cultured cells into mice, evaluate their tumorigenicity, tissue morphology, metastatic ability and confirm their tumor reproducibility. In the future, we are planning to create xenograft model to establish these cell lines as new CMT cell lines.

There are two main signaling pathways that induce apoptosis, the intrinsic pathway and extrinsic pathway [42]. The intrinsic pathway is mainly initiated in the mitochondria and is mediated by p53 as a result of DNA damage [42, 51, 52]. p53 regulates Bax, Apaf-1, and other apoptosis-related molecules [48, 52]. Bax and Bak induce cytochrome c release from the mitochondria, and this released cytochrome c forms apoptosome with Apaf-1 and caspase-9, which subsequently activates caspase-3 [8, 26, 52]. Therefore, p53 mutation results in the loss of apoptotic function [51]. Also, intrinsic pathway is activated by caspase-8 which cleaves Bid, and the cleaved Bid (tBid) activates Bax and Bak [7, 26]. In the extrinsic pathway, the death receptor (DR) on the cell surface activates caspase-8 via Fas-associated death domain protein (FADD), and this initiator caspase activates caspase-3 directly [26, 42]. The cells are classified into two types based on the cellular response after caspase-8 activation: type-I cells mainly activate

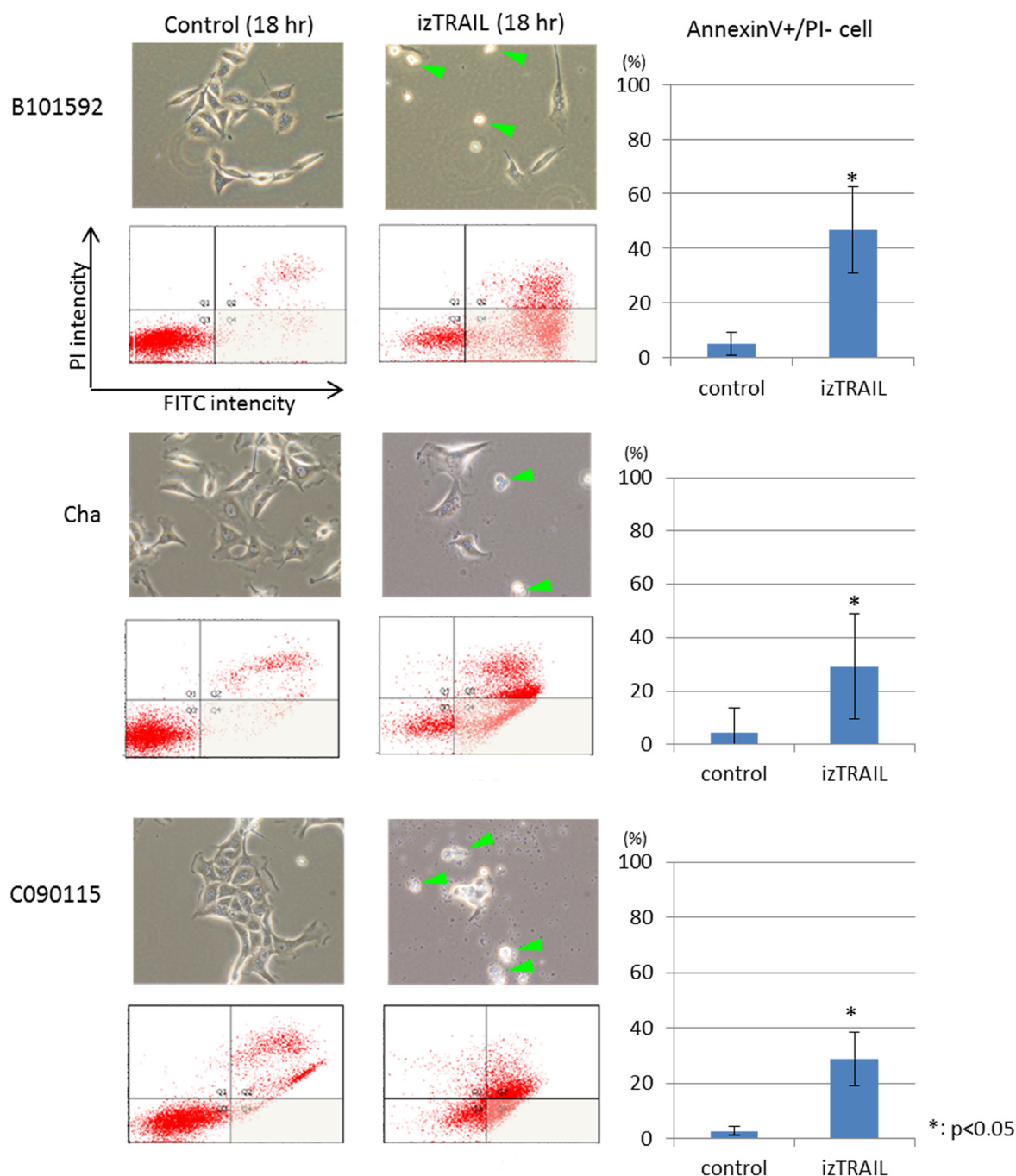


Fig. 5. Cellular morphological changes and percentage of annexin V+/propidium iodide (PI)- cells after incubation with isoleucine-zipper tumor necrosis factor-related apoptosis inducing ligand (izTRAIL). The image shows cultured cells treated with 100 ng/ml of izTRAIL for 18 hr; the cells were detached from dishes as small round (arrowheads) after izTRAIL treatment. The dot plots under the photo show the cells stained with annexin V/PI. The vertical axis represents the PI staining intensity and the horizontal axis represents the annexin V staining intensity. The cells in the gray area are annexin V+/PI- (apoptotic cells). Graph on the right indicating mean percentage of annexin V+/PI- cells from replicate experiments. The annexin V+/PI- cells increased significantly after izTRAIL treatment (* P <0.05).

the extrinsic pathway, and type-II cells rely on the intrinsic pathway [26, 42]. TRAIL activates caspase-8, which leads to the activation of both extrinsic and intrinsic pathways in p53-independent or dependent manner, and expected to exhibit a broad anti-tumor effect even in tumor cells carrying a p53 mutation [7, 26]. p53-independent anti-tumor drugs, such as TRAIL are important for effective treatment of CMT because mutations in p53 are reported in spontaneous CMT and cell lines [1, 6, 25, 34, 47]. Previously, we had demonstrated that canine HSA cell lines carrying p53 mutation were sensitive to TRAIL [15, 16]. In this study, izTRAIL induced apoptosis, as well as the activation of caspase-8 and caspase-3 in cultured cells derived from CMT, which were also observed in the canine HSA cells. In addition, the IC_{50} values of izTRAIL against cultured cells derived from CMT were

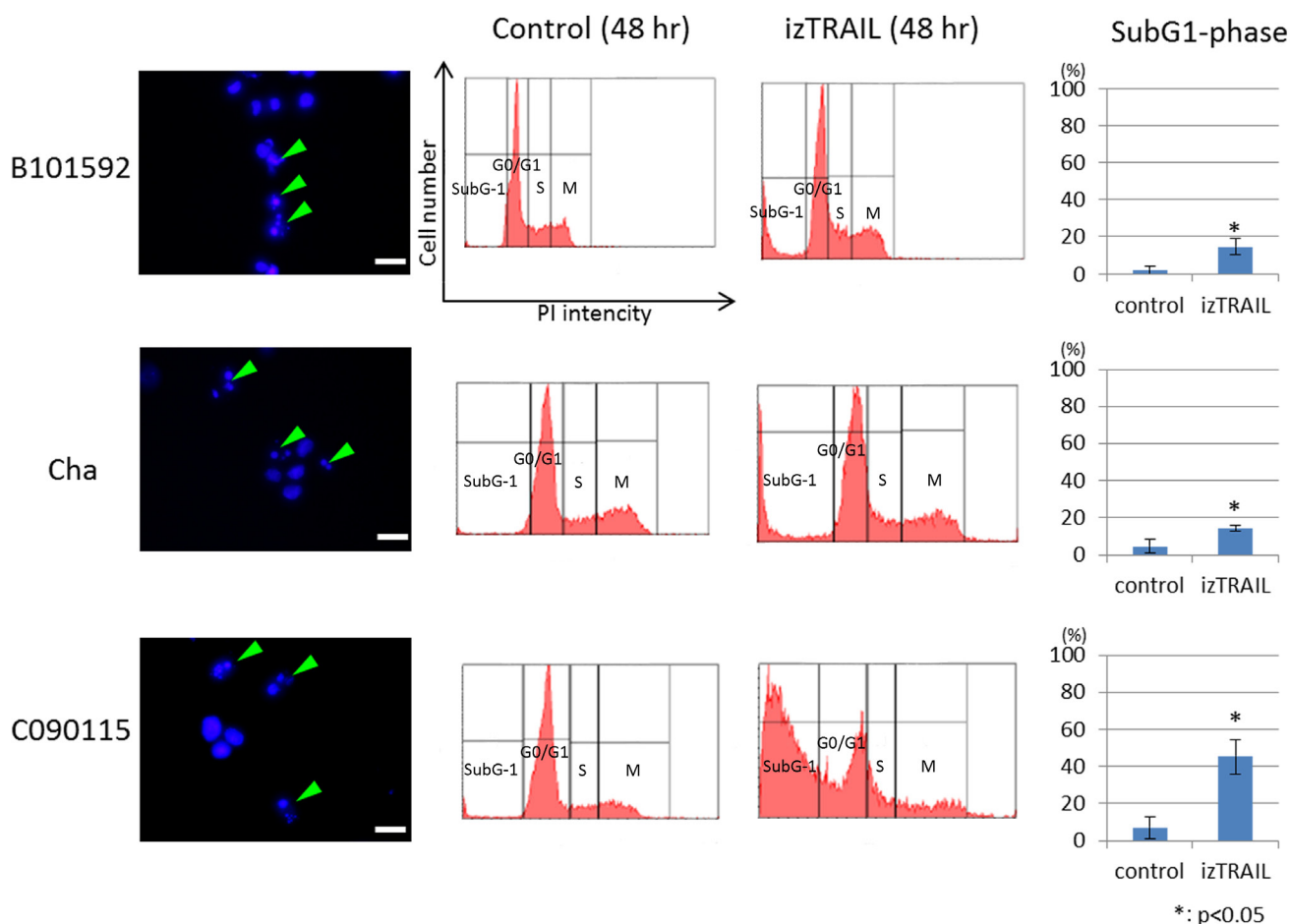


Fig. 6. Nuclear fragmentation induced by isoleucine-zipper tumor necrosis factor-related apoptosis inducing ligand (izTRAIL). The images obtained from 4',6-diamidino-2-phenylindole (DAPI) staining show the nuclear morphology changes after treatment with 100 ng/ml of izTRAIL for 48 hr. Arrowheads show the nuclear fragments. Representative DNA histogram at right showing the cells stained with propidium iodide (PI) for each cell. The vertical axis represents the cell number, and the horizontal axis represents the PI staining intensity. The percentage of cells in the sub-G1 phase is shown in the graph. The proportion of cells in the sub-G1 phase increased significantly after treatment with izTRAIL (* $P < 0.05$).

equivalent to those against canine HSA cell lines [15]. Spee *et al.* reported that the IC_{50} value of TRAIL monomer against CMT cell (P114) was approximately in the range of 5–15 ng/ml; this is consistent with some previous studies which reported that the sensitivity to TRAIL/izTRAIL largely differs depending on the cell lines [7, 46, 49, 53]. Three cell lines can be judged to have sufficient sensitivity to izTRAIL when compared to the HeLa and MDCK cells; and the P114 cells are very sensitive to TRAIL [39]. The differential TRAIL sensitivity of the cell lines derived from breast cancer was previously reported in humans [34]. Spee *et al.* reported that downregulation of X-linked inhibitor of apoptosis (XIAP), which directly binds and inhibits caspase-3, 7 and 9, enhanced the sensitivity of tumors to TRAIL [26, 39, 46]. In this study, the expression of non-cleaved PARP was not detected in all cells at 24 hr post-izTRAIL treatment, which indicated that caspase-3 activity was sufficiently activated. This suggested that XIAP is unlikely to be involved in inhibiting apoptosis in these three cell lines. Additionally, overexpression of survivin, which belongs to the same family as XIAP, was reported in the canine spontaneous mammary tumors [3]. In human medicine, various molecules that suppress the anti-apoptotic factors, such as XIAP and survivin also enhance the sensitivity to TRAIL [46]. Therefore, this suggested that these molecules may increase the sensitivity of canine tumors to TRAIL.

Two TRAIL receptors with death domain, which act as death receptor (DR4/DR5) and three decoy receptors (DcR1/DcR2/Osteoprotegerin: OPG) have been identified in humans [9, 11, 23, 28, 29, 37, 50]. The balance between the expression levels of DR and DcR is correlated with TRAIL sensitivity [21, 46]. In canines, there are very limited the knowledge studies on TRAIL receptor and only OPG was identified, but the function of OPG associated with TRAIL was only predicted based on the gene sequence analysis [10]. Treatment with izTRAIL induces apoptosis in canine HSA and cultured cells derived from CMT. This indicated that canine tumor cells express certain level of conservative TRAIL receptor that has a death domain capable of activating caspase-8. Western blotting analysis revealed that the B101592 cells exhibited the earliest response to izTRAIL. As caspase-8 activation is the first step in the TRAIL pathway, the difference in reactivity may involve competitive factors upstream of caspase-8. One possible

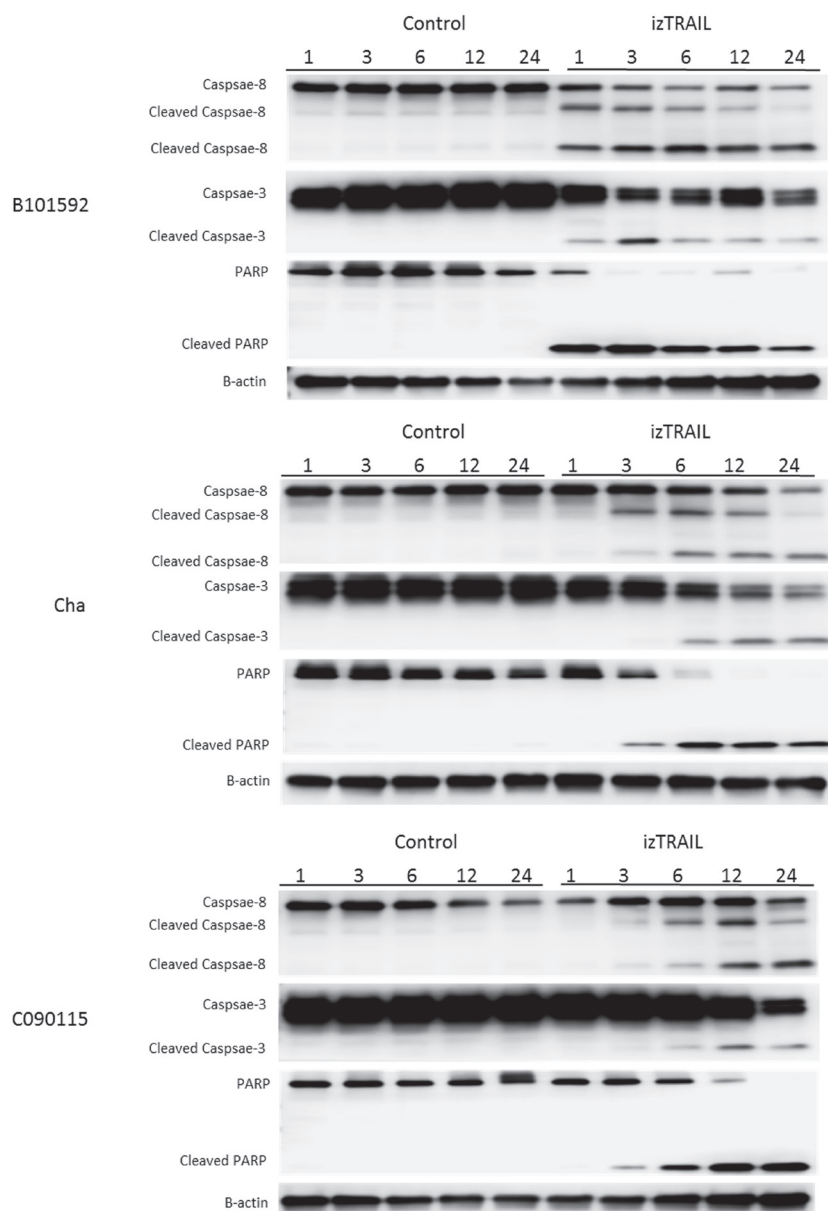


Fig. 7. Expression of caspase-8, caspase-3, and poly (ADP-ribose) polymerase (PARP). Representative western blot bands are shown.

explanation is that the expression level of TRAIL receptors is different among the tumor cells. Further studies on TRAIL receptor are needed to confirm this hypothesis. Another possible explanation is that the function of caspase-8 is inhibited by c-FLIP, which has sequence homology with caspase-8 and competes with caspase-8 to bind FADD [26, 46]. Flavopiridol enhances the TRAIL sensitivity of TRAIL-resistant HBC cell lines by downregulating the expression of c-FLIP, and this molecule is reported to exhibit similar effects in canine lymphoma cell lines [27, 30]. The effect of c-FLIP on other tumor cells must be evaluated after the administration of flavopiridol in the future.

In conclusion, we demonstrated the cytotoxicity of izTRAIL against cultured cells derived from CMT and the possibility of conservation of the TRAIL receptor owing death domain, which drives the TRAIL pathway in canine tumor cells. This study supports the versatility of TRAIL, and provides the basis for future studies on TRAIL receptor and TRAIL pathway in dogs.

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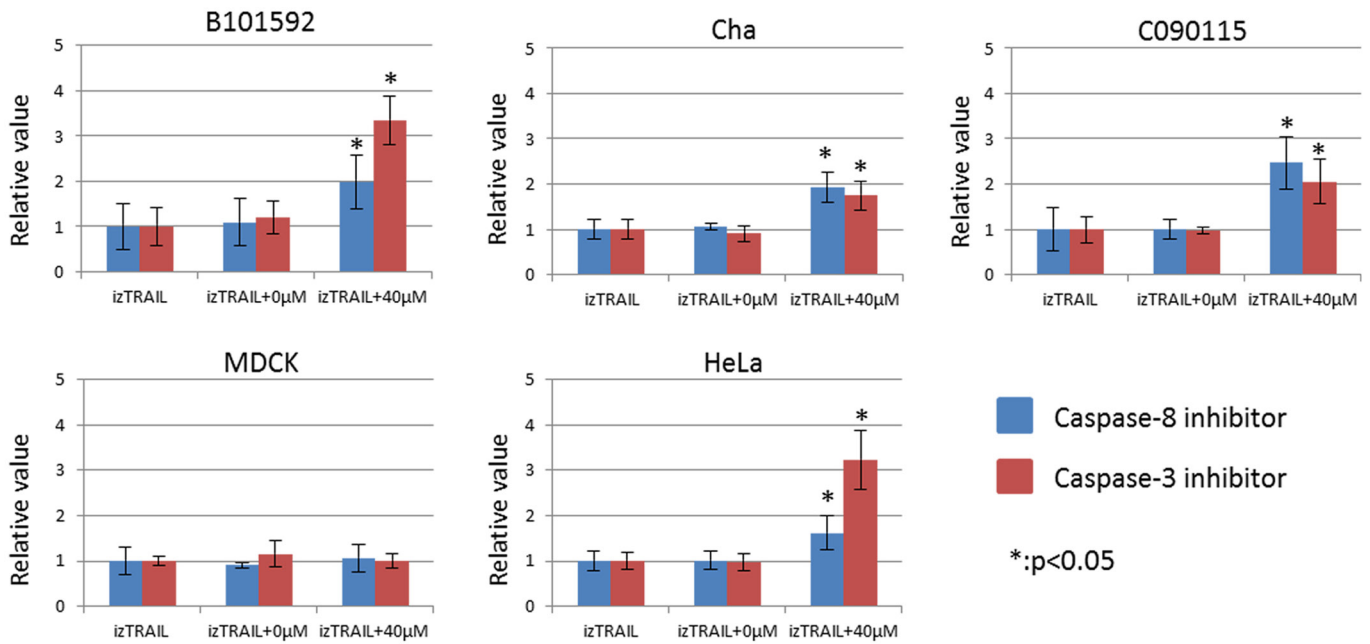


Fig. 8. Viability of cultured cells after treatment with caspase inhibitor and isoleucine-zipper tumor necrosis factor-related apoptosis inducing ligand (izTRAIL). Viability of cultured cells after treatment with caspase-8 or caspase-3 inhibitor and izTRAIL. The vertical axis represents the relative absorbance value compared with the control group, and the horizontal axis represents different treatments: izTRAIL, izTRAIL and vehicle, and izTRAIL and 40 μ M of caspase inhibitor. Representative results of more than three independent experiments are presented as mean \pm positive standard deviation (SD) (* P <0.05).

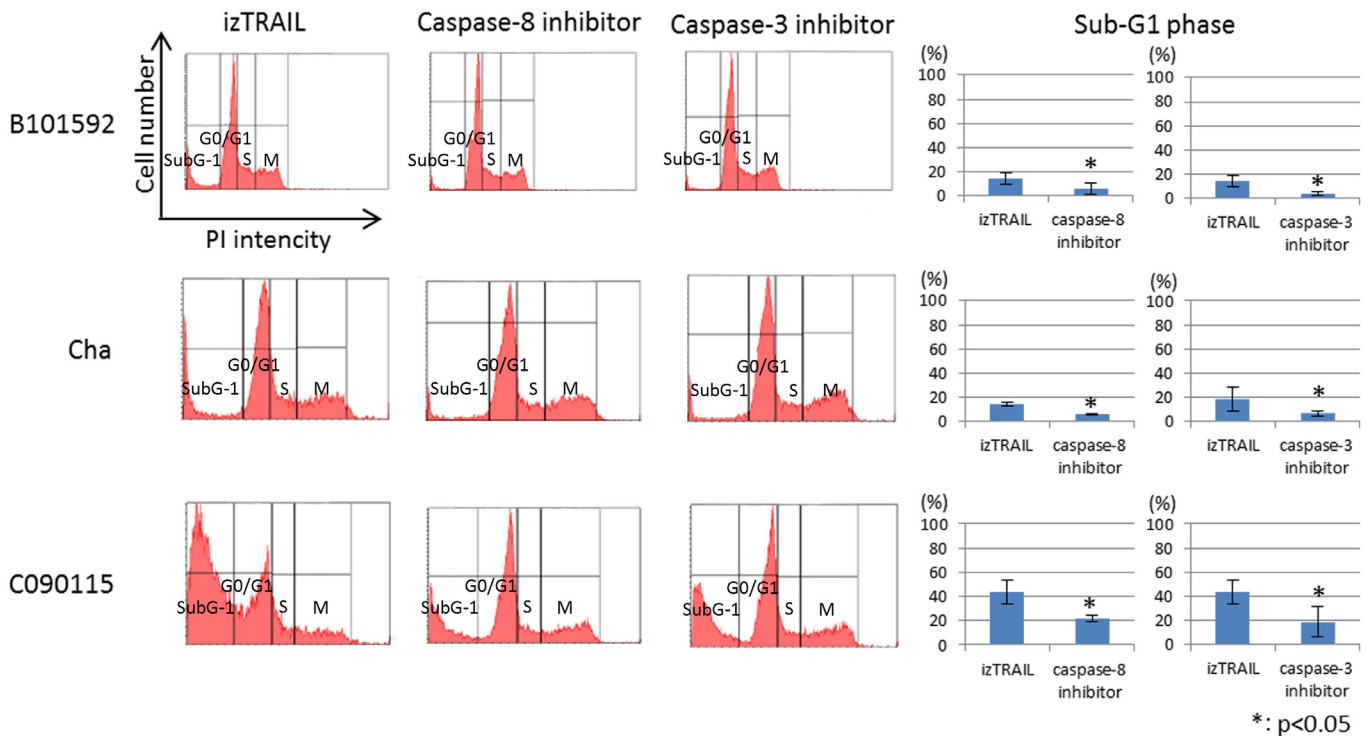


Fig. 9. Effect of caspase inhibitors on the proportion of cells in sub-G1 phase. Three cell lines were treated with caspase inhibitors and isoleucine-zipper tumor necrosis factor-related apoptosis-inducing ligand (izTRAIL) for 48 hr and stained with propidium iodide (PI). The vertical axis represents cell number, while the horizontal axis represents PI staining intensity. The graphs show the percentages of cells in sub-G1 phase (* P <0.05).

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