Effect of Retinoids on Growth Inhibition of Two Canine Melanoma Cell Lines

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ABSTRACT. Two new canine melanoma cell lines (CMM1 and CMM2) were established from the patients with oral malignant melanomas. Histopathological type of both CMM1 and CMM2 was a mixed cell type consisted of spindle-shaped cells, polygonal cells, and oval cells. Doubling time of CMM1 and CMM2 were 18.4 ± 1.96 hr and 21.0 ± 0.73 hr, respectively. The effect of two kinds of retinoids (all-trans retinoic acid and 9-cis retinoic acid) on the proliferation of these cells were examined by morphological changes, proliferation assay and apoptosis assay. However, the retinoids did not suppress growth rate of these cells. This result suggests that retinoids used in this study did not induce differentiation, apoptosis, and growth inhibition of the canine melanoma cell lines.

KEY WORDS: canine, melanoma, retinoid.

Canine malignant melanomas are very common [9], and it accounts for 4% to 6% of all canine malignant tumors [24]. Surgical excision with wide margin is the treatment of choice, however local recurrence and/or lung metastasis often occurs postoperatively. Even though adjunctive chemotherapy has been conducted, effective protocol has not yet been established.

Various melanoma cell lines have been established from human [12, 19] and animals such as mice [20] and rabbits [11]. But, there have been few reports on the establishment of canine melanoma cell lines [14, 29].

Retinoids, synthetic derivatives of vitamin A, have been shown to modulate differentiation and proliferation of normal and malignant cells [3]. Recently, it has been recognized that retinoids induce apoptosis [5, 25, 26]. Retinoids as an anticancer agent have been studied in vivo and in vitro using various murine and human cancer cell lines including melanomas for more than twenty years, where many melanoma cell lines of various species showed suppressed growth by retinoids [16, 19, 24]. Although their clinical usefulness has so far been limited by various adverse effects, retinoids are of increasing importance for the development of therapeutical strategies in cancers. These effects may be mediated by various actions, such as direct toxicity on the tumor cell membrane, induction of tumor suppressive mediators and/or modulation of tumor receptors [5, 9, 21, 25]. Specific receptors, the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs) have been reported to bind to retinoids, which is thought to be the major process for retinoids to act on tumor cells [24].

The purpose of this study is to establish and characterize two canine malignant melanoma cell lines, and to evaluate the potential efficacy of two retinoids, all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-CRA), on the differentiation and growth inhibition of these cell lines.

One sample (CMM1) was obtained from an autopsy specimen of a 12-year-old male Toy Poodle with malignant melanoma of the oral cavity. This dog underwent mandibulectomy as an initial treatment. The tumor stage according to WHO staging system at the time of diagnosis was T2bN1M0 (clinical stage 3). The other (CMM2) was obtained from a 13-year-old mongrel dog with malignant melanoma of the oral cavity. The tumor stage at the time of diagnosis was T2aN0M0 (clinical stage 2). These specimens were dissected, digested with collagenase, washed with phosphate-buffered saline (PBS) for 3 to 5 times and incubated in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 20% fetal bovine serum (FBS) (Equitech-Bio Inc., Ingram, TX, U.S.A.), L-glutamine (Nissui Pharmaceutical Co.), and antibiotics (50 mg/L gentamycin sulfate and 1.5 mg/L amphotericin B) at 37°C in a humidified atmosphere of 5% CO2. After the second passage, the concentration of FBS was reduced to 10%.

Morphologic observation was performed as follows. Cells were cultured until semi-confluent at a density of about 2 × 106 cells per ml in 6-well tissue culture plates. The cells were monitored on a phase-contrast microscope (Olympus, Tokyo, Japan). Doubling time at a logarithmic growth phase was calculated as described before [13].

Transplantation of the cells into nude mice was performed as described before [13]. Briefly, five-week-old BALB/c female nude mice (Nippon SLC, Hamamatsu, Japan) were irradiated with a dose of 4 Gy of X-ray. After 3 days of irradiation, the cell suspension (3 × 106 cells) of each cell line was transplanted subcutaneously into the back of the nude mice. When tumor mass developed to a certain size, nude mice were euthanized. Masses were fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with May-Gimsa solution for microscopic evaluation.

ATRA and 9-CRA (both Sigma Chemical Co., St Louis, MO, U.S.A.) were dissolved in 100% dimethylsulfoxide (DMSO) (Wako Pure Chemical Inc., Osaka, Japan) at a concentration of 10–2 M and kept as a stock solution up to 3 weeks at –70°C under nitrogen. The stock solution was
added to the medium to achieve the desired concentration (10^{-5} to 10^{-7} M). The final DMSO concentration never exceeded 0.1%. The control culture medium contained 0.1% (vol/vol) DMSO alone.

To evaluate changes in cell number by addition of various concentrations of retinoids, CMM1 and CMM2 cells (both 2 \times 10^5/ml) in a logarithmic growth phase were cultured in the above medium and were plated in 12-well plates in duplicate with/without retinoids (10^{-6} M). After 24 hr, and at every 72 hr thereafter, the spent medium was replaced with fresh control medium or medium containing retinoids. The total viable cells were counted daily by trypan-blue dye exclusion method for 5 days after the medium was changed.

Inhibitory effect of retinoids on the metabolic activity of these cells was evaluated by MTT assay [4]. Cells were seeded at a density of 5,000 cells/well in triplicate in 96-well plates, and treated for 5 days in total with indicated retinoids. Media with/without retinoids were changed after 24 hr of incubation. After incubation with/without retinoids, relative viable cells were obtained using the Cell Counting Kit (Dojindo, Osaka, Japan). Optical density (OD) at 490 nm was measured by microplate spectrometer (Bio-Rad Laboratories, Inc., Tokyo, Japan) with 630nm as the reference wavelength. Inhibition Index (I.I.) was calculated by the following equation: I.I.=(a-p)/(a-m) (a=OD of control well, p=OD of treated well, m=OD of background).

To detect apoptotic changes by retinoids, DNA fragmentation was detected by TUNEL (TdT-mediated dUTP-biotin nick end labeling) using the MEBSTAIN Apoptosis kit II (MBL Co., Ltd, Nagoya, Japan) following the manufacturer’s protocol. For comparison of the changes between each group of retinoids, one-factor ANOVA followed by the Scheffe’s F test was used. Values of P<0.05 were considered significant.

The morphology of CMM1 showed mixed cell type figures consisted of spindled-shaped cells, polygonal cells, and oval cells. CMM2 was also a mixed cell type consisted of spindle-shaped cells and oval cells. Melanin granules were recognized at the first few passages in both cells, but they dissapeared through passages. Most of the characteristics such as microscopic appearance and the growth rate of the cells did not change even after 50 passages except melanin.

DT at a logarithmic growth phase of CMM1 (45th passage) and CMM2 (40th passage) were 18.4 ± 1.96 hr and 21.0 ± 0.73 hr, respectively.

Transplantation of CMM1 and CMM2 cells into nude mice induced development of tumor masses in approximately 6 weeks. The masses induced by primary cultured cells showed black in gross appearance, but amelanotic mass was developed when the cells passed over 40 were transplanted. The growth rate did not change. Histologically, the mass showed similar feature to that of primary tumors except for much less intracellular melanin (Fig.1).

Growth curves of the melanoma cells cultured in the absense or presense of ATRA are shown in Fig. 2-A and B.

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Growth curves of the melanoma cells cultured in the absense or presense of ATRA are shown in Fig. 2-A and B.

There was no significant difference in growth rate by treatment with ATRA. Growth rate of both cells treated with 9-CRA was quite similar to that of ATRA (data not shown).
In TUNEL assay, even after 24 hr of incubation with retinoids, only few apoptotic cells were detected and there were no difference in the number of apoptotic cells between the treated and the control cells (data not shown). Morphological changes associated with apoptotic cell death, such as chromatin condensation and nuclear segmentation, were not observed in both cells (data not shown).

Retinoids, naturally occurring and synthetic analogues of vitamin A, exhibit a host of interesting effects on the growth and differentiation of normal, preneoplastic and neoplastic cells \[2, 3, 6, 18\]. In addition to their well-established anti-carcinogenic activity \[2, 3, 6, 18\], retinoids have been shown to inhibit the proliferation of a considerable number of cultured cells, including human and murine melanoma cells \[8, 15, 16, 19, 24, 27, 28\]. However, the data presented in this paper showed that both of the retinoids examined did not induce growth inhibition and morphologic differentiation of either of the canine melanoma cells established in this study.

The cellular responses to retinoic acids are mediated by two families of transcription factors, RARs and RXRs, both of which belong to the steroid-thyroid hormone receptor family \[12, 15, 26, 27\]. From many previous reports, it has been described that retinoid-induced differentiation and cell growth occurs through these receptors \[9, 13, 20, 28\]. In our preliminary study measuring RARs of CMM1 cells, only few receptors were detected (data not shown). The receptors of CMM2 are not measured yet. CMM1 cells had a slight morphologic change without inhibition of the cell proliferation when treated with retinoids. This result indicates that CMM1 cells have a little sensitivity to retinoids. The antitumor activity of retinoids may vary by the level of the receptors, and the low RARs of CMM1 cells may cause in low sensitivity to retinoic acids. In a recent study, it has been shown by an experiment using RAR- and RXR-specific ligands that activation of RXRs is essential and sufficient for the induction of apoptosis \[20\]. This may suggest both CMM1 and CMM2 cells did not have RXRs. Further research to measure RARs and RXRs in both cells must be necessary to clarify the cause of the results in this study.

Previous reports have described differences in responsiveness to retinoic acids among patients with the same cancers and among cultured cells derived from the same cancer \[25\]. Furthermore, in one study, it is also reported that in human melanoma cells, drug treatment is significantly affected by the features of the clones characterized by low biological aggressiveness (high differentiation, low invasiveness) \[29\]. The high aggressive clone did not show a consistent response to drug treatment \[29\]. The cells used in this study were highly aggressive with low differentiation, therefore it may be one factor for these cells to have low sensitivities to retinoids. Several other possible explanations for the diverse response to retinoids may be: different transportations of the drug, variability in the drug conversion to an inactive form or inability of the cells to activate the drug, and the presence of alternative biochemical pathways to overcome the drug-inhibited step \[1\]. All these could also be plausible explanation for the negative response of the canine melanoma cells to retinoic acids.

There are still many unknown factors in mechanism of retinoids to inhibit cell growth induce differentiation and apoptosis of tumor cells. Although there are many papers...
reporting retinoids as an effective therapy for numerous tumors in human, this study here indicates that each tumor may respond differently to this therapy, or it may even cause tumor growth. Further studies on the mechanism of growth inhibition of melanoma cells by retinoids must be required.

REFERENCES