FULL PAPER *Surgery*

Retinoids Induce Growth Inhibition and Apoptosis in Mast Cell Tumor Cell Lines

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ABSTRACT. Retinoids are well recognized as promising antitumor agents in humans. However, there have only been a few reports about the effect of retinoids in canine cancers. To investigate the antitumor effect of retinoids on mast cell tumors (MCT), inhibitory effect on cell growth and induction of apoptosis were examined *in vitro*. Although sensitivity of these cells differed among the cells, the growth of three MCT cell lines (CoMS, CM-MC and VI-MC) were inhibited dose dependently when they were treated with retinoids. FACS analysis of PI-stained nuclei revealed an apoptotic fraction in CM-MC cells about 30% when treated with retinoids, while those of control cells were less than 5%. Caspase-3 activation was observed after retinoid treatment in CM-MC cells. This was confirmed by inhibiting the retinoid-induced apoptosis using the pan-caspase inhibitor, ZVAD-FMK. Both retinoid receptors, RARs and RXRs, were detected by immunoprecipitation followed by western blot analysis in all the three MCT cells. These data suggests that retinoids inhibit the growth of MCTs partly through apoptosis, and this growth inhibition by retinoids may be mediated by RARs and RXRs. We conclude that retinoid may be a potential adjunctive chemotherapeutic agent for the treatment of canine MCT.

KEY WORDS: canine, mast cell tumor, retinoid.

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Mast cell tumors (MCTs) represent the most common cutaneous tumor in the dog, accounting for between 16 to 21% of all cutaneous tumors [28]. Histologic grading, determined by the morphologic characteristics of the neoplastic cells, has been established as the most consistent prognostic factor highly predictive of biological behavior and clinical outcome [19]. The majority of dogs that suffer undifferentiated MCTs carry a poor prognosis [19]. Surgical excision and radiation therapy have been the most successful treatment modality for MCTs described to date, and the use of adjuvant chemotherapy is indicated after the excision of grade 3, metastatic and nonresectable MCTs [23]. However, chemotherapy for canine MCT has been unrewarding, and long-term responses have not been demonstrated in well-controlled clinical trials. Therefore, there is a need for chemotherapy to be improved in order to obtain a better prognosis in undifferentiated MCTs.

Retinoids are active metabolites of vitamin A and modulate various biological functions such as cell differentiation, proliferation and embryonic development in vertebrates [11]. It has been well established that retinoidal activities result mainly from the transcriptional regulation of specific gene programs. Retinoids bind to and activate two classes of receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which belong to the steroid/thyroid hormone receptor superfamily [4, 13, 16, 20]. Preclinical and clinical studies suggest that retinoids induce the growth inhibition of various kinds of cancers in human through differentiation and apoptosis. All-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9cRA) are natural retinoids, and Am80 is a synthetic retinoid. ATRA activates only RARs, while 9cRA activates both RARs and RXRs [4, 13, 16, 20]. Am80 can only bind to RAR alpha and beta, though their binding affinities are more potent than ATRA [27].

The potential interaction between retinoids and cancer therapy has been recognized for decades, acute promyelocytic leukemia (APL) being the most notable example. APL can be effectively eradicated by retinoids, and therefore forms the prototype for retinoid-based therapies [22]. In contrast, retinoids have been used mainly in dermatology as a differentiating agent for sebaceous adenitis in dogs [30]. There have only been few studies that have discussed the effect of retinoids on canine cancers [17, 29]. In our laboratory, we have previously shown the effect of retinoids in canine osteosarcoma and melanoma cells [6, 7, 18]. Osteosarcoma cells showed significant growth inhibition when treated with retinoids, although significant growth inhibition was only seen when the cells were treated with the highest dose, 10^{-5} M [6, 7]. No effects on the growth rate or differentiation of melanoma cell lines were observed [18].

It has recently been reported that treatment with ATRA results in the decrease of mast cells when ATRA is added to the human mast cell progenitor cells [12]. The purpose of this study was to investigate the antitumor effect of retinoids in MCT cell lines using three kinds of natural and synthetic retinoids, in order to further evaluate the potential efficacy of retinoids on canine cancers.

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MATERIALS AND METHODS

Cell culture: Three canine MCT cell lines (CoMS, CM-MC and VI-MC) were used in this study [9, 21]. CoMS and VI-MC cells were cultured in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), 2 mM L-glutamine, 50 mg/L gentamycin sulfate and 1.5 mg/L amphotericin B. CM-MC cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 U/m*l* penicillin G, 10 μg/m*l* streptomycin and 50 mM 2-mercaptoethanol. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Reagents: ATRA and 9cRA were purchased from Sigma (St Louis, MO, U.S.A.). Am80 was kindly provided from Dr. Hiroyuki Kagechika. Retinoids were dissolved in 100% ethanol at a concentration of 10^{-2} M and stored in the dark at –20°C under nitrogen. Stock solutions were diluted to the final concentration of $1 \times 10^{-5} - 1 \times 10^{-10}$ M with culture medium immediately before use. The same amount of ethanol [(less than 0.1% (vol/vol)] was added to the control medium. A pan-caspase inhibitor, ZVAD-FMK, was obtained from Takara Bio Inc (Shiga, Japan).

Antibodies: To detect RARs and RXRs, rabbit polyclonal antibodies for RARs and RXRs (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, U.S.A.) were used in this study. For detection of caspase-3, polyclonal antibodies for pro- and active caspase-3 antibodies were obtained from Cell Signaling Technology, Inc (Beverly, MA, U.S.A.). A horse radish peroxidase (HRP) conjugated anti-rabbit antibody (Bethyl, Inc, Montgomery, TX, U.S.A.) was used as a secondary antibody in both studies.

Assays for inhibition of cell growth: The inhibitory effect of retinoids on cell growth was evaluated using the Cell Counting Kit (Dojindo Co., Osaka, Japan). The cells were plated in 96-well plates in quadruplicate, and treated with or without 1×10^{-5}–1×10^{-10} M of retinoids for three days. Relative viable cells were obtained according to the manufacturer's protocol. The percentage of viable cell numbers of the treated groups was compared to that of the control (100%).

Measurement of cell cycle distribution and sub G1 fraction: Cells were treated with 1×10^{-5} M ATRA for three days, fixed with 70% ethanol and incubated with RNase and propidium iodide (PI). Then, the cells were analyzed on a FACS Calibur (Becton Dickinson, Le Pont de Claix, France) using Cell Quest software for cell cycle distribution (sub G1, G1, S and G2). For treatment with the combination of ATRA and ZVAD-FMK, cells were treated with 1×10^{-5} M ATRA and/or 5×10^{-5} M ZVAD-FMK for four days. On the third day of incubation, the half of the spend medium was replaced with fresh control medium or medium containing ATRA and/or ZVAD-FMK.

Detection of the expression and activation of caspases: Total cell extracts from CM-MC with/without 1×10^{-5} M ATRA were prepared using Chaps cell extract buffer (Cell Signaling Technology, Inc) according to the manufacturer's protocol. Twenty mg protein per sample was loaded onto a 15% sodium dodecyl sulfate (SDS) acrylamide gel and fractionated by electrophoresis, then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Pall Co, NY, U.S.A.) and immunoblotting was performed according to the manufacturer's protocol. Bands were visualized with a chemiluminescence-based procedure using the Phototope-HRP Western Blot Detection kit (Cell Signaling Technology, Inc).

Detection of RARs and RXRs: Nuclear extracts were prepared as described elsewhere [1, 5]. Protein concentrations were determined by protein assay kit (Bio-Rad Laboratories, Inc, Tokyo, Japan) based on the method of Bradford [2]. Immunoprecipitation of nuclear extracts (3 mg protein) was performed using the immunoprecipitation kit, according to the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). Immunoprecipitates were loaded onto 10% SDS acrylamide gel, fractionated by electrophoresis and electrotransferred onto a PVDF membrane (Bio-Rad Laboratories, Inc). Primary antibodies were incubated with membranes for 1 hr at room temperature in Tris-Buffered Saline (TBS), then secondary antibodies were incubated with membranes for 1 hr. Blot was detected by diaminobenzidine solution (Sigma).

The specificity of the reaction was checked using samples immunoprecipited with control anti-rabbit IgG, and subsequently immunostained with the specific rabbit polyclonal antibodies.

Statistical analysis: All experiments were carried out at least 3 times, and were shown to be reproducible. The difference in cell growth within each retinoid-treated group was analyzed using one-factor ANOVA for repeated measure, followed by the Sheffe's F test. For comparison of the changes in cell viability between each retinoid-treated group, one-factor ANOVA followed by the Scheffe's F test was used. Values were expressed as means ± SD, and $P<0.05$ was considered significant.

RESULTS

Assays for inhibition of cell growth: Cell viability was measured after three days of exposure to ATRA, 9cRA and Am80 at the dose of 1×10^{-5}–1×10^{-10} M (Fig. 1). Doses that caused 50% inhibition (IC50 values) are shown in Table 1. Although sensitivity to retinoids varied between the cell lines, dose-dependent growth inhibition was observed in all the three cell lines. CM-MC cells showed the highest sensitivity among the cells, with the IC50 being 9.7×10^{-8} M and 2.2×10^{-9} M in ATRA and 9cRA, respectively. IC50 of ATRA in VI-MC cells and Am80 in all the three cell lines could not be calculated, because the growth inhibition rate was less than 50% even at the highest dose (1×10^{-5} M).

Measurement of cell cycle distribution and sub G1 fraction: Flow cytometry analysis of PI-stained nuclei was performed to examine the cell cycle distribution and cell cycle arrest imposed by ATRA treatment. The nuclei in apoptotic

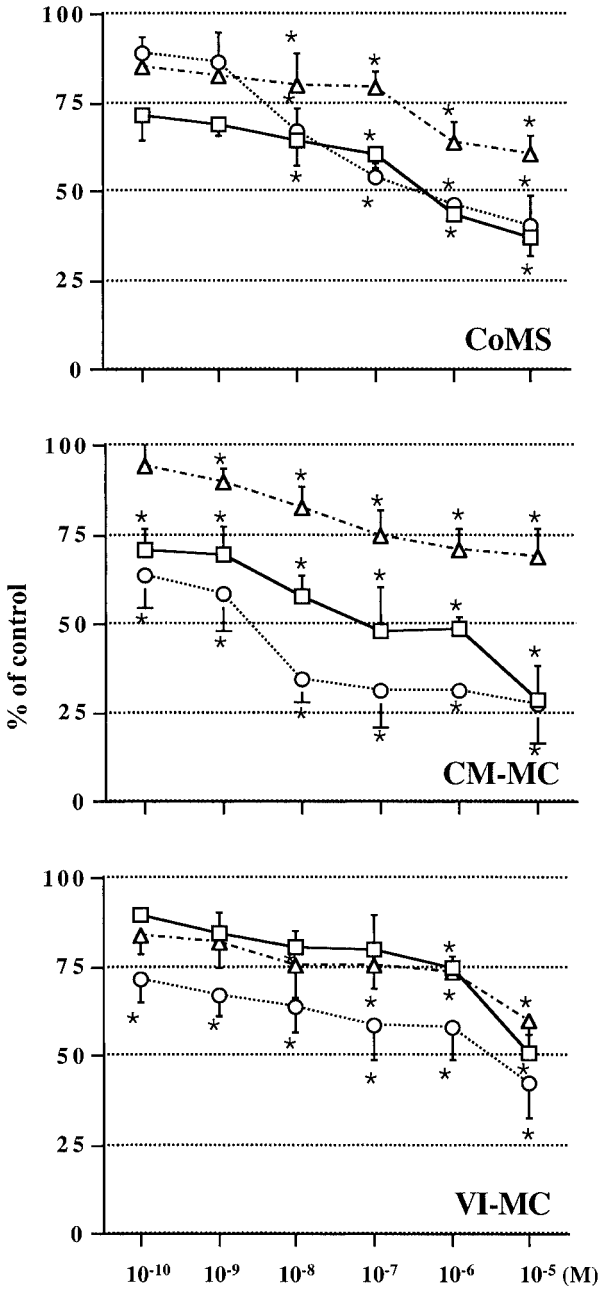

Fig. 1. Effects of retinoids on three canine MCT cell lines. Dose response growth inhibition was observed by ATRA (square), 9cRA (circle) and Am80 (triangle). The percentage of viable cell numbers of the treated groups was compared to that of the control (100%). The results shown are from 1 representative experiment of 3 conducted. Data are expressed as mean ± SD. *: Significantly different from control cells (P<0.05).

cells show a uniform reduction in DNA staining ability with PI, which is indicated by the appearance of a sub G1 peak on the DNA histogram. As shown in Table 2, percentages of ATRA-treated and intact CM-MC cells in sub G1-phase were 32.5% and 11.1%, respectively, indicating the induction of apoptosis by retinoids. In addition, percentage of S-phase cells decreased from 10.9% to 7.0%. A decrease in the S-phase population, the DNA synthetic phase, indicates cell-cycle arrest. Although CM-MC cells showed the highest sensitivity to retinoids, less potent but similar result was obtained in both CoMS and VI-MC cells.

Table 1. IC50 Values (1×10^{-5} M) of each retinoids on MCT cell lines

Cell Line	Retinoids, IC50 (μM)		
	ATRA	9cRA	Am80
CoMS	4	0.63	>10[a)]
VI-MC	>10[a)]	3.3	>10[a)]
CM-MC	0.097	0.00212	>10[a)]

a) >10 indicates that the growth inhibition rate was less than 50% even at the highest dose, 1×10^{-5} M.

Table 2. Distribution of cells on cell cycle arrest and apoptosis in 3 MCT cell lines

Cell Line	% sub G1	% G1/G0	% S	% G2/M
CoMS C	5.77	65.35	10.29	18.59
CoMS A	21.86	60.05	5.78	10.74
VI-MC C	7.26	66.12	12.01	14.63
VI-MC A	15.65	59.67	11.65	13.13
CM-MC C	11.13	64.89	10.9	12.99
CM-MC A	32.47	51.38	7.04	8.57

The percentages of cells present in sub G1, G0/G1, S and G2/M phases are shown.
The results shown are from 1 representative experiment of 3 conducted.
C: control cells, A: 1×10^{-5} M ATRA-treated cells.

Detection of the activation of caspases: To analyze the signaling pathway of retinoid-induced apoptosis, immunoblotting of pro- and activated caspase-3 was performed after treatment of ATRA. The presence of proteolytic degradation bands (active caspase-3) was observed when CM-MC cells were treated with ATRA (Fig. 2). The expression of these pro- and active caspase-3 correlated to those detected in MDCK cells, indicating that these antibodies used in this study are cross reactive to these in dogs (data not shown). We confirmed the involvement of caspase in ATRA-induced apoptosis by using ZVAD-FMK, and measuring cell cycle distribution. As shown in Table 3, the proportion of sub G1-phase cells in ATRA-treated CM-MC cells were 31.8%, whereas addition of ZVAD-FMK resulted in decrease of sub G1-phase down to 10.3%. ZVAD-FMK also increased the proportion of S-phase cells from 7.1% to 9.1%.

Expression of RARs and RXRs: Figure 3 shows the immunoblotting of RARs and RXRs. Three MCT cell lines expressed all the subtypes of RAR and RXR. The data presented here showed that, although there was an apparent difference in sensitivity to retinoids, all the subtypes of RARs and RXRs were detected at the similar level in 3 MCT cell lines.

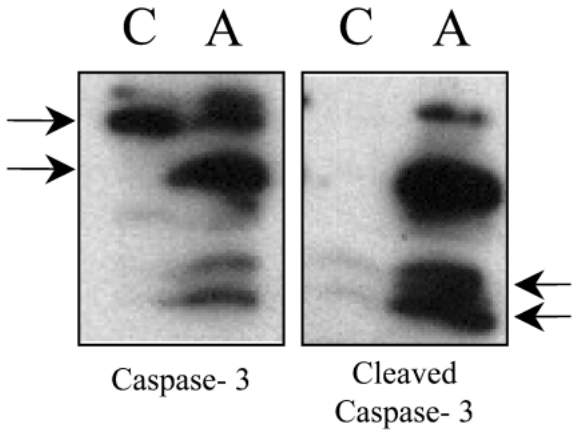

Fig. 2. Induction of caspase-3 activation by retinoids. Western blotting of pro-caspase-3 antibody (left panel) and active caspase-3 antibody (right panel) in the ATRA-treated (A) and untreated (C) CM-MC cells. Active caspase-3 was detected when cells were treated with ATRA for three days. Antibody for caspase-3 detects two bands at molecular mass of approximately 35 kDa and 25 kDa, while antibody for active caspase-3 detects band at 19 kDa and 17 kDa.

Table 3. Distribution of cells on cell cycle arrest and apoptosis of CM-MC cells after treatment of ATRA (1×10^{-5} M) and ZVAD-FMK (5×10^{-5} M)

	% sub G1	% G1/G0	% S	% G2/M
Control	18.88	60.83	10.2	10.39
ATRA	31.84	53.29	7.05	8.09
ATRA+ZVAD-FMK	10.32	68.4	9.11	12.29
ZVAD-FMK	5.74	70.02	12.26	12.24

The percentages of cells present in sub G1, G0/G1, S and G2/M phases are shown.
The results shown are from 1 representative experiment of 3 conducted.

DISCUSSION

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 *in vivo* and *in vitro*. In this study, we investigated the antitumor effects of three kinds of retinoids on canine MCT cells *in vitro*. The data presented in this paper showed that retinoids induced growth inhibition in all three MCT cell lines, with CM-MC showing the highest sensitivity. In addition to the growth inhibition effect, retinoids also induced apoptosis and cell cycle arrest in all the three MCT cells and the ATRA-induced apoptosis was shown to be caspase-dependent. The growth inhibition study shows that the effect of 9cRA was more potent than ATRA in the inhibition of cell growth. Activation of RARs or RXRs requires the dimerization of these receptors. While RARs may only dimerize with RXRs, RXRs can homodimerize as well as dimerize with other nuclear receptors. Thus activation of RXRs can result in the signaling among numerous pathways. This can be the reason why 9cRA showed more potent growth inhibition compared to ATRA.

Caspases, a family of cystein proteases, play a critical role in the execution of apoptosis. They are activated by proteolytic cleavage from inactive pro-caspases. A member of this family, caspase-3, has been identified as being a key mediator of apoptosis of mammalian cells [24]. In this study, the presence of proteolytic degradation bands resulting from the activation of caspase-3 was observed. This was confirmed by using a pan-caspase inhibitor, ZVAD-FMK. By adding ZVAD-FMK, ATRA-induced apoptosis was decreased down to 10.3%, even lower than that of the control cells. These results indicate that retinoids induce caspase-dependent apoptosis in MCT cells, and this retinoid-induced apoptosis is partially responsible for growth inhibition of MCT cells by retinoids.

Mast cells are derived from immature hematopoetic cells [10]. Under physiologic conditions, circulating mast cell progenitors reach diverse tissues, where they undergo proliferation and maturation. Uncontrolled proliferation and the accumulation of mast cells occurs in MCTs, whereas the normal process of proliferation and differentiation of mast cells are under the control of cytokines, particularly stem cell factor (SCF), also termed as a Kit ligand [8]. Several years ago, point mutations in c-kit that lead to constitutive activation of Kit in the absence of ligand binding were identified in three malignant mast cell lines (human HMC-1, RBL and mouse P815) [3, 25, 26]. This provides an indication that dysregulation of Kit may promote uncontrolled growth of survival of mast cells. It has been also shown that some malignant canine MCTs express c-kit mutations [14, 15]. Most of these mutations lead to the constitutive phosphorylation of Kit in the absence of ligand binding, resulting in unregulated activation of Kit with subsequent MCT formation. It has recently been reported that ATRA negatively regulates the SCF-dependent differentiation of human mast cells *in vitro*, and thus results in the decrease of mast cells when ATRA is added to the mast cell progenitor cells [12]. Although the mechanisms by which retinoids act on MCT cells are still unknown, the role retinoids play in regulating the constitutive activation of Kit may be partially responsible for the growth inhibitory effect of retinoids on canine MCT cells.

It is well established that the cellular responses to retinoids are mediated mainly through the regulation of two families of transcription factors, RARs and RXRs [4, 13, 16, 20]. In this study, we have detected all the subtypes of RARs and RXRs in three MCT cell lines. Interestingly, the susceptibility of these MCT cells to retinoids was considerably different. One of the purposes of this study was to determine the correlations between the susceptibility to retinoids and the expressions of RARs and RXRs. Since there was no difference in receptor expression between the cell lines, we could not prove the correlation between the expression of receptors and the reaction of tumor cells in this study. The same results were also obtained in the previous study using canine osteosarcoma cells in our laboratory

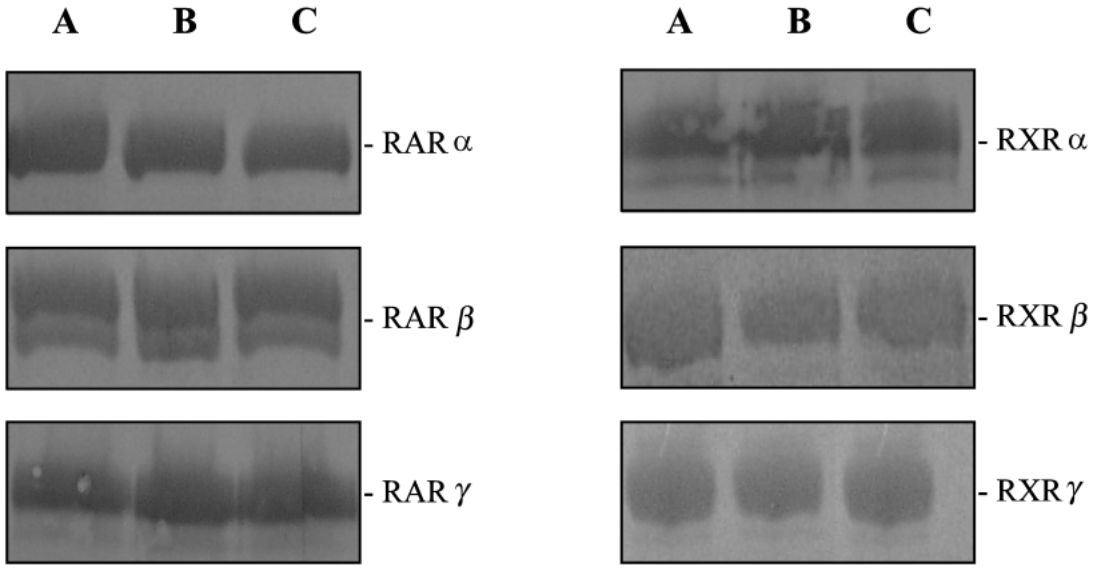

Fig. 3. Western blotting of RARs and RXRs in 3 MCT cell lines. All the subtypes of RARs α, β and γ and RXRs were detected in 3 MCT cell lines (A, CoMS; B, VI-MC; C, CM-MC). The results shown are from 1 representative experiment of 3 conducted.

[7], but the difference in sensitivity to retinoids has not been clarified yet.

This study is not sufficiently conducted to determine the role of each receptor on the effect of retinoids, but the results obtained here indicate that the receptor responsible for the antiproliferative effect of retinoids on canine MCT cells is not equal to those responsible for the effect on human mast cells. In human mast cell HMC-1, the RAR alpha antagonist showed the equivalent potency to ATRA in the inhibition of mast cell growth, concluding that RAR alpha appears to be the major endogenous RAR subtype for the retinoid-dependent regulation of mast cell proliferation [12]. Am80 is a synthetic retinoid that exhibits RAR alpha-selectivity with some RAR beta-binding ability. Although it has been reported that the RAR alpha-binding affinity of Am80 is higher than that of ATRA [27], its inhibitory effect on cell growth observed in this study was lower compared to ATRA. RAR alpha does not seem to be the major RAR subtype for the regulation of cell growth in MCT cells.

The serum ATRA concentration level of APL patients treated with ATRA is about 10 nM. In this study, two of the three MCT cell lines showed significant growth inhibition at this dose. Furthermore, these MCT cell lines were isolated from different primary sites, the skin, gut and oral cavity. CM-MC, isolated from cutaneous MCT, showed the highest susceptibility to retinoids. Although further investigations are needed, this may indicate that cutaneous MCT has high sensitivity to retinoids.

Canine MCTs are often difficult to control by local treatment, and these tumors are resistant to chemotherapy as well. If retinoids inhibit growth of MCT cells *in vivo*, as shown in this study *in vitro*, it is hopeful that the prognosis of MCTs could be improved.

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