NOTE

Surgery

Effect of Natural and Synthetic Retinoids on the Proliferation and Differentiation of Three Canine Melanoma Cell Lines

Emi OHASHI1), Kaori INOUE1), Hiroyuki KAGECHIKA2), Sung-Hyeok HONG1), Takayuki NAKAGAWA1), Tomoko TAKAHASHI1), Manabu MOCHIZUKI1), Ryuhei NISHIMURA1) and Nobuo SASAKI1)

1)Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences and 2)Graduate School of Pharmaceutical Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo, 113–8657, Japan

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ABSTRACT. The effect of two natural retinoids and synthetic retinoids with or without retinoid synergists on the proliferation and differentiation of 3 melanoma cell lines were investigated in vitro. No retinoids showed significant growth inhibitory effect on these cell lines when used alone, however, cell differentiation and significant growth inhibition were observed when treated with a combination of retinoids and a retinoid synergist. This study may suggest that, though the cells showed low susceptibilities when retinoids were treated alone, the combination of retinoids and a retinoid synergist may be effective to control the growth of canine melanoma cell lines.

KEY WORDS: canine melanoma, retinoid.


Melanoma is a common neoplastic disease of dogs with variable biological behaviors. Melanomas account for 9–20% of skin tumors in dogs, and they are one of the most common malignant tumors of the oral cavity and of the digits in dogs [14]. Melanomas of the oral cavity are aggressive tumors that commonly metastasize to the regional lymph nodes and lung, and are poorly responsive to conventional therapy by the time they are discovered [14]. The retinoids are a class of pharmacological agents consisting of vitamin A (retinol) and its derivatives. They have long been known to play a pivotal role in the development and maintenance of the normal epithelial tissue as well as growth, reproduction, immune function, and vision [23]. They have also been well recognized as promising anticancer agents. Preclinical studies, both in vitro and in vivo, suggest that retinoids may be effective in growth inhibition of many kinds of cancers [1, 4, 7, 23], including melanoma [12, 15, 19, 22]. The effects of retinoids are mainly mediated by two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Both RARs and RXRs are encoded by three distinct genes (α, β, and γ) and are members of the steroid/thyroid hormone receptor superfamily that function as ligand-activated transcription factors [3, 5, 21]. The RXR-RAR heterodimers have significant roles in most of the retinoidal activities by binding to a variety of retinoic acid receptor elements (RAREs) and regulate their trans-activation activities. The RAR affinities, or the activation abilities of retinoids, correlate well with their biological activities [6, 20]. Specific ligands for RXRs alone cannot activate the RXR-RAR heterodimers, but they do regulate the activities of retinoids allosterically by binding the RXR site of the heterodimers [6].

All-trans retinoic acid (ATRA) and 9-cis retinoic acid (9CRA) are natural retinoids, and Am80 and HX630 are synthetic retinoids. ATRA activates only RARs, while 9CRA activates both RARs and RXRs [9]. Am80 can only bind to RARα and β, though their binding affinities are more potent than ATRA [9]. HX630 is a RXR-selective compound, which is so called retinoid synergists. Retinoid synergists are thought to be inactive alone because they cannot activate RXR-RAR heterodimers, but can enhance the growth inhibition and differentiation-inducing activities of retinoids [24].

We have previously reported the effect of natural retinoids on two canine melanoma cell lines in vitro [16]. This study was designed to evaluate the susceptibility of three other canine melanoma cell lines to various kinds of retinoids (two natural retinoid acids, synthetic retinoids, and the combination of retinoids and a retinoid synergist) on the differentiation and growth inhibition of these cell lines.

Three cell lines, CMeC, LMeC, and KMeC, established in our laboratory from the spontaneous canine cases of melanoma, were used in this study [8]. CMeC was originated from an 11-year-old male Chow with a subcutaneous mass on the right shoulder area. KMeC was originated from a 14-year-old male mongrel dog with a mass on the left oral gingiva. LMeC was from a 9-years-old female beagle dog with a mass on the left oral mucosa. These cells were cultured in RPMI-1640 medium supplemented with heat-inactivated (56°C, 30 min) fetal bovine serum (Equitech-Bio, TX, U.S.A.), L-glutamine (Nissui Pharmaceutical Co.), gentamycin sulfate (50 mg/L), and amphotericin B (1.5 mg/L) at 37°C in a humidified atmosphere of 5% CO2.

Natural retinoids (ATRA and 9CRA) and synthetic retinoids (Am80 and HX630) were used in this study. The retinoids were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10–2 M and stored in the dark at –20°C under nitrogen. Stock solutions were diluted to the appropriate concentration (10–3 M, 10–6 M, 10–7 M) in culture medium immediately before use. The same amount of DMSO (0.1% (vol/vol)) in the retinoid-treated medium was added to the culture medium of the control group. This
DMSO concentration did not affect the characteristics of the canine melanoma cells.

To analyze the morphological change, cells were seeded on the cover glass in a 6-well plate. At near semi-confluent growth, the spent medium was replaced with medium with/without the indicated retinoids (10⁻⁵ M). The cells were incubated with retinoids for 72 hr, then washed with phosphate-buffered saline (PBS) and stained with Giemsa solution and their morphological responses to retinoids were determined by light microscopy.

MTT dye reduction assay was used to examine the inhibitory effect of retinoids on the metabolic activity of melanoma cells. Cells were seeded at a density of 5 × 10⁴ cells/well in triplicate in 96-well plates, and were treated for 3 days in total with the indicated retinoids. Medium with/without retinoids were changed after 24 hr of incubation. On the fourth day, 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 630 nm as the reference wavelength. The percentage of viable cell number of the treated groups was compared to that of control (100%).

To detect the apoptotic changes induced by retinoids, the cells were seeded as described above for the examination of morphological changes. The cells were incubated with retinoids for 24 hr, and were washed with PBS and analyzed for the presence of DNA fragments by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method using the MEBSTAIN Apoptosis Kit (MBL Co., Ltd., Nagoya, Japan) following the manufacturer’s protocol. At this incubation time, there were few detached cells in the well when irrigated. Fluorescent nuclei were screened for normal morphology (unaltered chromatin), and apoptotic nuclei comprised of cells with fragmented (scattered) and condensed chromatin were counted. Apoptosis was expressed as the percentage of apoptotic nuclei/100 nuclei.

For comparison of the differences 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.

Figure 1 shows the typical morphological changes of LMeC cells following exposure to HX630 or a combination of Am80 and HX630. While the cells treated with HX630 alone did not show morphological changes as in control cells, the cells treated with a combination of 9CRA or Am80 and HX630 revealed phenotypical differentiation including cell flattening and growth of dendritic extensions. Similarly, CMeC and KMeC treated with ATRA, 9CRA or Am80 alone did not show any morphological changes, while the cells treated with the combination of these retinoids and HX630 showed morphological differentiation.

The growth inhibitory effects of retinoids are shown in Fig. 2. Various inhibitory effects were observed depending on the cell lines and the concentration of the retinoids, however, all the retinoids at any concentrations did not significantly inhibit the cell growth when added alone. When the cells were treated with 9CRA or Am80 combined with HX630, growth inhibition was observed in a dose-dependent manner, and significant growth inhibition was observed when all the three cell lines were treated with these retinoids at the highest concentration. Significant growth inhibition was not observed in any of the cell lines when treated with a combination of ATRA and HX630.

In TUNEL assay, even after 24 hr of incubation with retinoids, only few apoptotic cells were detected and there was no difference in the number of apoptotic cells between the treated and the non-treated control cells (data not shown). Morphological changes, such as chromatin condensation and nuclear segmentation, were not observed in this study.

Retinoids have been shown to inhibit the proliferation of a considerable number of cultured cells, including human and murine melanoma cells [16]. The effect of retinoids are mainly mediated by two classes of nuclear receptors, RARs and RXRs. They exert their anticancer activities by modulating proliferation, differentiation, and apoptosis of cancer cells. A growing literature has demonstrated that retinoid-induced differentiation and cell growth occurs through these
GROWTH INHIBITION OF MELANOMA CELLS BY RETINOIDS

We have previously reported that natural retinoids (ATRA and 9CRA) did not induce growth inhibition and morphologic differentiation of either of the two canine melanoma cell lines, suggesting the low sensitivities of these cell lines to retinoids. In this study, any retinoids including more potent synthetic retinoid, could not significantly inhibit the cell growth.

In our preliminary study measuring RARs of CMeC and KMeC, only few receptors were detected (unpublished). Correlation between receptor binding affinity of the tumor cells and the inhibitory effect of retinoids have been demonstrated in lymphoid cells [20]. Osteosarcoma cell lines also showed the similar correlation between them [6]. The low sensitivity of three melanoma cell lines in this study may have occurred because of the low level of the receptors of these cells.

On the contrary, retinoids combined with the retinoid synergist inhibited the cell growth with morphological change of three melanoma cells in this study. The combination of Am80 or 9CRA and HX630 significantly suppressed the growth in a dose-dependent manner, though the inhibitory effect of ATRA and HX630 was not significant. Retinoid synergists (RXR-specific ligands) are the silent partners of RARs, and the activation of RXR-RAR heterodimers does not require RXR ligand. Retinoid synergists exhibit weak or no retinoidal activities alone, though investigations using RAR- and RXR-selective compounds revealed that the retinoid synergist in RXR homodimers can enhance the growth inhibition and differentiation induction through the activities of RAR ligands in various assay systems [8]. This study also indicated that the simultaneous addition of RAR and RXR ligands renders the cell more sensitive, indicative of a synergistic effect of RXR ligands.

Though it is reported that higher concentration of HX630 suppressed the retinoidal activity of Am80 [24], synergistic efficiency of HX630 was observed dose dependently in this study. RXRs act as heterodimer partners not only with RARs, but also with other nuclear receptors such as vitamin D3 receptors, thyroid hormone receptors, and peroxisome proliferative activated receptors (PPARs) [13]. The effects of RXR ligands in RXR-RAR heterodimers cause unique modulation of retinoid actions depending on the combinations of RAR or RXR ligands, and some of the above hormonal activities can be positively or negatively regulated by RXR ligands [2, 10, 11, 17, 18]. The difference in synergistic activity of HX630 may be resulted from these biological effects of RXR ligands. 9CRA and Am80 have considerably high binding affinities to RARs than ATRA. The treatment of ATRA with HX630 did not show significant growth inhibition as seen in 9CRA and Am80 with HX630. It might be caused by the low binding affinities of ATRA to RARs.

In one study, it is reported that in human melanoma cells, the features of the clones characterized by low biological aggressiveness (high differentiation, low aggressiveness) significantly affected the drug treatment [22]. The high aggressive clone did not show a consistent response to drug treatment [22]. All 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.

The results of this study did not show the clear effects of retinoids to canine melanoma cells. The complexity of the expression and interaction of RARs and RXRs makes understanding and the physiology of the action of retinoids extremely difficult. Although there have been many reports reporting retinoids as an effective therapy for numerous
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tumors including melanoma in human, our previous study and this study proved the low sensitivity of canine melanoma cell lines to retinoids. However, the simultaneous use of retinoids and a retinoid synergist may be the step for the effective treatment of canine melanomas.

REFERENCES