Retinoid Receptors and the Induction of Apoptosis in Canine Osteosarcoma Cells

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ABSTRACT. Retinoids, all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid (9-cis-RA), induced morphological changes and apoptosis-like cell death characterized by cell shrinkage, chromatin condensation and nuclear disintegration in three canine osteosarcoma cells, OOS, HOS and POS, at a concentration of $10^{-5}$ M. Both retinoid receptors, RARs and RXRs, were identified in these cells. 9-cis-RA bound to both the RXRs and the RARs, whereas ATRA bound to only the RARs in these cells. Those results indicate that the induction of apoptosis in canine osteosarcoma cells may be mediated by the specific control of RARs and RXRs.—Key words: osteosarcoma, receptor, retinoids.


Retinoids, the active metabolite of vitamin A, are known to have profound effects on vertebrate development and differentiation in vitro [6, 18]. They also induce differentiation and maturation and inhibit proliferation of malignant tumors of epithelial or non-epithelial origins in vitro [12, 13]. In addition, they have been reported to induce differentiation and apoptosis of a large number of cells in vitro [1, 3]. We also reported on the growth inhibition, morphologic differentiation [10] and changes in osteoblast phenotypic markers [9] of canine osteosarcoma cells precipitated by retinoids in vitro.

The biological effect of retinoids is mediated by two distinct families of transcription factors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which belong to the steroid-thyroid hormone receptor superfamily [5]. RARs and RXRs form heterodimers (RAR/ RXR) or homodimers (RXR/RXR) and stimulate gene transcription by binding to cis-acting enhancer elements, termed RA responsive elements (RAREs), in a variety of target genes [14]. The RAR family is activated by both ATRA and 9-cis-RA, whereas the RXR family is activated exclusively by 9-cis-RA [17]. 9-cis-RA is up to 40 times more potent than is ATRA on RXRα, and it binds to RXR protein with nanomolar affinity [7]. The induction of apoptosis is under the specific control of RARs and RXRs and it is reported that the antitumor activity is mediated by these receptors [15].

This study was designed to examine the expression of RA-binding receptors in canine osteosarcoma cells and their relationship to apoptosis and responses to retinoids.

Three osteosarcoma cells, OOS [8], HOS [8] and POS [11], were used in this study. Cells were maintained in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (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.

All-trans-[11, 12-3H]retinoic acid (1110 GBq/mmol; New England Nuclear, Boston, MA, U.S.A.), 9-cis-[20-methyl-3H]retinoic acid (2756.5 GBq/mmol; New England Nuclear), unlabeled ATRA (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 9-cis-RA (Sigma Chemical Co.) were used for the analysis of the receptors according to the method by Clagett-Dame and Repa [4]. Cytosol preparation and saturation binding analysis was performed by the method described by Clagett-dame and Repa [4]. Briefly, for one-point binding assays various concentration of labeled retinoids and a 200-fold excess of unlabeled retinoids are used. The cell extract/retinoid mixture was incubated on ice for 3 hr while being shielded from the light. Specific ligand binding to receptors was determined by a hydroxylapatite (HAP) separation method with modifications [4]. $K_d$ values and number of binding sites were calculated according to the Scatchard analysis.

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 fresh control medium or medium containing $10^{-5}$ M of ATRA or 9-cis-RA. The cells were incubated with retinoids for 72 hr at 37°C in a humidified atmosphere of 5% CO₂. The cells were washed with physiologic-buffered saline (PBS) and stained with Giemsa solution and their morphological responses to retinoids were determined by light microscopy.

To analyze the effect of retinoids on the change in cell size, cells were seeded in 90-mm-diameter dishes. After 3 days of incubation the cells with or without retinoids, they were fixed with 5% paraformaldehyde PBS and the cell size was measured using FACSScan (Becton Dickinson Immunocytometry System, San Jose, CA, U.S.A.).
Table 1. Binding dissociation constants and total binding sites for retinoid receptors with ATRA and 9-cis-RA

<table>
<thead>
<tr>
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<th>RARs</th>
<th>RXRs</th>
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<tr>
<td></td>
<td>$K_d$ (nmol)</td>
<td>Binding sites (pmol/mg prot.)</td>
</tr>
<tr>
<td>ATRA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OOS</td>
<td>0.39 ± 0.10</td>
<td>3.00 ± 0.89</td>
</tr>
<tr>
<td>HOS</td>
<td>0.06 ± 0.01</td>
<td>1.09 ± 0.28</td>
</tr>
<tr>
<td>POS</td>
<td>0.29 ± 0.08</td>
<td>2.90 ± 0.95</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OOS</td>
<td>0.20 ± 0.11</td>
<td>12.15 ± 4.10</td>
</tr>
<tr>
<td>HOS</td>
<td>0.07 ± 0.01</td>
<td>4.96 ± 1.60</td>
</tr>
<tr>
<td>POS</td>
<td>0.22 ± 0.03</td>
<td>10.60 ± 0.69</td>
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a) Dissociation constants ($K_d$) were determined by Scatchard analysis. Numbers represent the mean ± SD of at least three individual experiments done in duplicate.
b) NB indicates no measurable binding detected.

For determination of deoxyribonucleic acid (DNA) fragmentation, cells were seeded as described for the examination of morphological changes. The cells were incubated with the retinoids for 12, 18, 24 or 48 hr at 37°C in a humidified atmosphere of 5% CO$_2$. After the selected incubation time (18 hr) had passed, the cells 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 II (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 comparisons between groups, one-factor ANOVA followed by the Sheffe’s F test was used. Values of $P<0.05$ were considered as significant.

The results of Scatchard analyses are shown in Table 1. In OOS, HOS and POS cells, mean $K_d$ values in the binding of [3H]ATRA to RARs were 0.39 nM, 0.06 nM and 0.29 nM, respectively, and in the binding of [3H]9-cis-RA to RXRs were 0.67 nM, 0.22 nM and 0.67 nM, respectively. In addition to binding to the RXRs, 9-cis-RA also bound to all three members of the RARs with high affinity (Table 1); in OOS, HOS and POS cells, Scatchard analyses of the binding of [3H]9-cis-RA to RARs in each cell yielded $K_d$ values of 0.20 nM, 0.07 nM and 0.22 nM, respectively. Therefore, the RARs as a group showed three-fold lower dissociation constants for 9-cis-RA ($K_d$=0.07–0.22 nmol) than did the RXRs for 9-cis-RA ($K_d$=0.22–0.67 nmol). The $K_d$ values for RXRs was three-fold lower than the value for RARs in the three canine osteosarcoma cells we tested. The $K_d$ values of HOS cells was three-fold lower than those of other 2 cells. In OOS, HOS and POS cells, the numbers of the binding sites of [3H]ATRA to RARs were 3.00, 1.09 and 2.90 pmol/mg prot., respectively. Those of [3H]9-cis-RA to RARs were 12.15, 4.96 and 10.60 pmol/mg prot., respectively, and those of [3H]9-cis-RA to RXRs were 15.17, 5.60 and 19.33 pmol/mg prot., respectively.

By retinoid treatments, all the cells exhibited an altered morphology. Retinoids enhanced cell flattening and spreading on the substratum as well as causing a reduction in cell overlapping. Some cells showed apoptotic-like cell death characterized by cell shrinkage, condensation and margination of the nucleus (Fig. 1). There were no morphological changes in the control cells.

In addition, the number of small-sized cells considerably increased in OOS and POS in the presence of retinoids (Fig. 2). HOS did not show remarkable changes in cell size.

Fluorescence microscopy revealed that a number of intact osteosarcoma cells treated for 18 hr with retinoids displayed a small, brightly stained and highly condensed nucleus. Chromatin was aggregated into dense masses at the periphery of the nucleus and, in some cases, fragmented. In OOS and POS cells, the index of apoptosis increased significantly ($P<0.01$) after treatment with both retinoids compared with that of the control group (Fig. 3). In HOS cells the index of apoptosis increased significantly after treatment with only ATRA ($P<0.05$) compared with that of the control group.

The results of this study demonstrated that the retinoids, ATRA and 9-cis-RA, induced differentiation accompanied with apoptosis in three types of canine osteosarcoma cells used in this study. Apoptosis is characterized by the generation of fragmented nuclei with highly condensed chromatin, the protrusion of cytoplasm and the formation of apoptotic bodies, all of which were observed in all the cells. In addition, the number of small-sized cells considerably increased in OOS and POS cells, suggesting that apoptotic bodies increased as a result of retinoids treatment in these cells.

In addition, RARs and RXRs were detected in all three types of canine osteosarcoma cells; however, there was an apparent difference in sensitivity to retinoid receptors among the cells. The receptors’ ligand-binding ability was proved...
Fig. 1. Photomicrographs of three types of canine osteosarcoma cells (1: OOS, 2: HOS, 3: POS) cultured for 72 hr in the absence (a) or presence of $10^{-5}$ M ATRA (b) or $10^{-5}$ M 9-cis-RA (c) (May-Giemsa stain, × 100). Some cells showed apoptotic-like cell death characterized by cell shrinkage, condensation and margination of the nucleus.

Fig. 2. Changes in cell size after retinoids treatment in three types of canine osteosarcoma cells. The number of small-sized cells considerably increased in OOS and POS. HOS did not show remarkable changes in cell size. *=Significantly (P<0.05) different from control. †= Significantly (P<0.01) different from control.

Fig. 3. Effect of retinoids on the percentage of apoptotic body formation in three types of canine osteosarcoma cells. 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 a percentage of apoptotic nuclei/100 nuclei. *=Significantly (P<0.05) different from control. †= Significantly (P<0.01) different from control.
to be intact; for RARs and RXRs, the number of binding sites was almost equal among these cells. It was interesting to note that the ligand-binding affinity of the HOS cell receptors was three times as high as that of the other osteosarcoma cells, and although the sensitivity of HOS cells to retinoids was higher than that of OOS and POS cells, HOS cells were resistant to the cytolethal effect of retinoids. In other words, a correlation between receptor binding affinity and \textit{in vitro} response was not found in HOS cells, which is contrary to findings with other cell types. Relationships between receptors and responses of tumor cells \textit{in vitro} or \textit{in vivo} have been well investigated in lymphoid cells. Studies using mouse lymphoma cell lines have demonstrated a fairly good correlation between the level of glucocorticoid receptor and the \textit{in vitro} action of steroids [16]. Conversely, no correlation was observed between the level of glucocorticoid binding and cytolethal or cytostatic responsiveness of human lymphoid cell lines to glucocorticoid treatment \textit{in vitro}, and some of the insensitive cell lines exhibited the highest levels of steroid binding [2]. The presence of RARs and RXRs may not always predict the responsiveness of the cell to retinoid treatment. It has recently been shown that the resistance to response is partially attributable to mutations in glucocorticoid receptors [19]. A similar phenomenon might have occurred in the retinoid receptors of the osteosarcoma cells tested in this study.

The complexity of the expression and interaction of RARs and RXRs makes understanding the physiology of the action of retinoids extremely difficult and the results of this study did not show the clear relation of receptors and apoptosis, however, the results of the present study supported the hypothesis that the induction of apoptosis can be induced in canine osteosarcoma cells.

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REFERENCES