Apoptosis Induction of POS Canine Osteosarcoma cells by Vitamin D and Retinoids
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ABSTRACT. Vitamin D₃; 1-α,25(OH)₂D₃ (calcitriol), 22-oxa-1,25(OH)₂D₃ (OCT), cholecalciferol (vitamin D₃), and retinoids: all-trans retinoic acid (ATRA) and 9-cis retinoic acid, induced morphological changes in POS canine osteosarcoma cells into elongated, spindle or fibroblast like-shaped cells, and apoptotic like cell death characterized by cell shrinkage, condensation and margination of the nucleus for all drugs at 10⁻⁶M-10⁻⁹M after 72 to 120 hr culture. Apoptosis as shown by DNA laddering was induced at 48 hr by all drugs at 10⁻⁶M, 10⁻⁸M at 96 hr, 10⁻⁸M and 10⁻⁹M at 120 hr respectively. These vitamins are suggested to adjunct antineoplastic agents in canine osteosarcoma therapy by induction of apoptosis. — KEY WORDS: apoptosis, canine osteosarcoma, vitamin.

Mortality due to tissue toxicity, is usually observed when antineoplastic agents are used at high dosages. This is one limitation in cancer therapy at the present. Success in cancer therapy depends on identifying ways to induce selective cell death in tumor cells while sparing the normal tissues. The possibility of exploiting the cell death program, and the identification of less toxic drugs which can induce tumor cells to commit themselves to die, presents a promising adjunct for cancer therapy. Osteosarcoma is one of the most malignant tumors of animals and humans, and has a high percentage of pulmonary metastasis in its early stages [18]. Canine and human osteosarcoma closely resemble each other in their histological appearance, biological behavior and pathogenesis, hence canine osteosarcoma becomes an excellent model for studying antitumor responses for human osteosarcoma [2, 12].

While vitamin D and retinoids have been shown to increase the rate at which transformed cells express complex patterns of gene expression, to control cell cycle that leads to differentiation, loss of proliferative capacity and loss of oncogenicity [9, 15, 17, 19] we investigated in the present study whether vitamin D₃ and its derivatives, and the retinoids have the potential for inducing apoptosis in canine osteosarcoma using the POS osteosarcoma cell line.

POS canine osteosarcoma cell line, established and characterized directly from a spontaneous osteosarcoma on a femur of a 1.5 year old male mongrel dog [8] were used for this study. These cells were maintained in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS), HEPES (2.383 mg/ml), L-glutamine, and antibiotics penicillin (62.5 mg/ml) and streptomycin (100 mg/ml) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. OCT (22-Oxa-1,25-dihydroxyvitamin D₃) was a courtesy from Chugai Pharmaceuticals Co., Ltd. (Tokyo, Japan). Calcitriol (1-α,25-Dihydroxyvitamin D₃), cholecalciferol (vitamin D₃); all-trans retinoic acid (ATRA) and 9-cis-retinoic acid were purchased from Sigma Chemicals Co. (St.Louis, California, U.S.A.). Stock solutions of the drugs were prepared in 100% ethanol, with the final concentration of ethanol not exceeding 0.1%, and were stored at -20°C. The drugs were diluted to the required concentrations with 100% ethanol immediately before use.

For cell morphology, POS cells were seeded into 8-well chamber slides (NUNC Inc., Naperville, Illinois, U.S.A.) at 1 x 10⁵ cells/chamber at 200 µl of RPMI-1640 supplemented with 10% FCS. At near confluency growth, each cell type was treated with varying concentrations of the drugs ranging from 10⁻⁶M-10⁻⁹M. The cells were incubated with the drugs for 48, 72 and 120 hr at 37°C in a humidified atmosphere of 5% CO₂, after which the cells were stained with giemsa solution and their morphological responses to the drugs were studied by light microscope at 200X and 400X magnifications.

For the DNA preparation and agarose gel DNA electrophoresis [16], POS cells were cultured to near confluency in a 75 cm² cell culture flask (IWAKI, Tokyo, Japan), and treated with the drugs at 10⁻⁶M-10⁻⁹M for 24–120 hr. Control cells were cultured similarly without drugs. Isolation of genomic DNA was done by the addition of guanadine-detergent lysing solution (DNAZOL™ reagent, Wako Pure Chem., Tokyo, Japan). DNA precipitation and washing were done using 100% and 95% ethanol respectively. DNA solubilization was done by air drying and dissolution in 30 µl Tris-Ethylene diamine tetra acetic acid (TE-EDTA) buffer (PH=7.5). DNA quantitation was done using a A260 spectrophotometer (Beckman DUR 640, Fullerton, CA, U.S.A.). Equal amounts of DNA at 0.2 µg/well were run in 2% agarose for 60 min at 100 V. DNA ladders were visualized by transmitted ultraviolet illumination (302 nm), and photographed with a polaroid camera.

POS cells have been characterized as medium sized cells composed of a mixture of cells from spherical, polygonal and multinucleated giant cells (Fig. 1–1) [8]. In the presence of vitamin D₃ and retinoids at 10⁻⁶M-10⁻⁹M, majority of the cells morphologically changed into elongated, spindle or fibroblast like shaped cells, some cells showed the generation of thin cytoplasmic like processes or branchings, while other cells showed apoptotic-like cell death characterized by cell shrinkage, condensation and margination of the nucleus. These structural changes were
Fig. 1. Photomicrographs of canine osteosarcoma POS cell line mixed type preclonal cells, which shows apoptotic-like cell death characterized by shrinkage and nuclear condensation or margination after various treatments at $10^{-6}$M for 120 hr of culture (200X) of vitamin D$_3$ or retinoids. 1-Control, 2-22-Oxa-Calcitriol(OCT), 3-Calcitriol, 4-Cholecalciferol, 5-All-transRetinoic Acid (ATRA), 6-9-cis Retinoic Acid (9-cis RA).
recognizable after 48 hr of treatment and became more pronounced after 72 hr and 120 hr of treatment (Fig. 1). There were no morphological changes in the control cells as treated with the same amount of the drugs using ethanol.

Analysis of the DNA extracted from POS cells treated with OCT, calcitriol, cholecalciferol, ATRA and 9-cis retinoic acid at $10^{-6}$M-10$^{-9}$M showed positive DNA fragmentation which is the biochemical hallmark of apoptosis. The laddering in POS was induced the earliest at 48 hr culture by OCT, calcitriol, cholecalciferol, ATRA and 9-cis retinoic acid at $10^{-6}$M, followed by $10^{-7}$M at 96 hr culture, and $10^{-8}$M and $10^{-9}$M concentrations at 120 hr culture respectively. OCT brought the most apparent laddering intensity, followed by calcitriol, then ATRA, then cholecalciferol, and finally 9-cis retinoic acid at 48, 96 and 120 hr cultures.

The changes showing morphological differentiation and apoptotic-like death of cells suggest that these vitamin D and retinoids have the capacity to modulate POS cell growth and proliferation and presents its potential use for the differentiation therapy of canine osteosarcoma. Vitamin D and retinoids acts through nuclear vitamin D receptor (VDR) and retinoid A receptor (RAR)/retinoid X receptor (RXR) respectively, resulting into a complex cascade of events leading to changes in the cell differentiation, with parallel inhibition of cell proliferation [15, 19]. Although hypervitaminosis D and A causing hypercalcaemia and hyperkeratosis respectively results from their long term administration in excessive amounts, this new novel vitamin D$_3$ analogue OCT, is more potent than calcitriol in terms of its differentiation inducing properties, antiproliferative and immunoregulatory activities, but has virtually no calcemic activity [7]. These vitamins therefore are implied not to be solely administered for long terms in excessive amounts, but are suggested to be used as an adjunct or in combination to osteosarcoma chemotherapy or radiotherapy.

As a preliminary finding, we demonstrate that these drugs can induce POS cells suicide pathway, and the formation of DNA fragmentation [6, 13]. Since one cause of neoplasms proliferation is the inhibition of their natural apoptotic cascades, these drugs present a critical point for the cellular control of POS. In conclusion, induction of apoptosis by OCT, calcitriol, cholecalciferol, ATRA and 9-cis retinoic acid in POS osteosarcoma cells suggests the possibility of exploiting a new therapy through the use of these vitamins in making canine osteosarcoma cells more sensitive to chemotherapy or radiotherapy.

REFERENCES