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RAPID COMMUNICATION

Suppression of vascular endothelial growth factor expression by cannabinoids in a canine osteosarcoma cell line

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Abstract: Vascular endothelial growth factor (VEGF) is a key regulator in both physiologic and pathologic angiogenesis, and cannabinoids decrease VEGF release in human and murine cancer cells. The aim of this study was to assess the in vitro effects of a synthetic cannabinoid, WIN-55,212-2, on the expression of the proangiogenic factor VEGF-A in the canine osteosarcoma cell line 8. After analysis of gene expression by quantitative real-time polymerase chain reaction, the compound decreased VEGF-A expression by 35% ± 10% ($P < 0.0001$) as compared with the control. This synthetic cannabinoid shows promise as a potential inhibitor of angiogenesis, and further studies are warranted to investigate its in vivo effects and to explore the potential of this and related compounds as adjuvant cancer therapy in the dog.

Keywords: dog, cancer, angiogenesis, cannabinoids

Introduction

Vascular endothelial growth factor (VEGF) is a proangiogenic signaling protein that induces proliferation and migration of vascular endothelial cells. The VEGF family comprises several glycoproteins, including VEGF-A, which is a major regulator of normal and abnormal angiogenesis.[1] It plays a key role in tumor angiogenesis and has been found to be overexpressed in malignant tumors.[2] Neoplastic tumors are dependent on angiogenesis because the formation of new blood vessels is required for delivery of the necessary nutrients and oxygen. In the absence of angiogenesis, tumors cannot grow larger than approximately 2 mm in diameter. Cells in rapidly growing masses are triggered by hypoxia to increase cellular VEGF expression in order to induce new vessel growth.[3]

Endocannabinoids, phytocannabinoids, and synthetic cannabinoids bind to cannabinoid 1 (CB_1) and 2 (CB_2) receptors,[4] and can inhibit tumor growth in several cancers, including mouse glioma,[5] and human pancreatic,[6] the uterine,[7] and prostatic cancer.[8] CB_1 and CB_2 receptors are found in many tissues in mammals,[4] including the bone cells of human[9] and mouse[10] species. WIN-55,212-2 is a synthetic cannabinoid that binds to both CB_1 and CB_2 receptors and had been demonstrated to impede angiogenesis, induce apoptosis in mouse cancer cells,[9] and inhibit VEGF expression in both human and mouse cancer cells.[11,12]

Cancer biology and tumor behavior have been shown to be similar in humans and dogs for several common cancers,[13] and the dog is emerging as a valuable naturally occurring cancer model.[14] Examples of these similarities are the canine and human leukemias and lymphomas, that have a similar clinical presentation, tumor biology, and response to therapy, and have been demonstrated to share evolutionary conserved

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chromosomal aberrations and conserved mutations within key oncogenes.[15] Mammary malignancies share the same pattern of deregulated genes.[16] Specifically, studies conducted with canine osteosarcoma were the first to yield results applicable to humans.[17] The similarities between osteosarcoma in both species include a large patient size, 75% or more cases affecting the appendicular skeleton, metaphyseal location, less than 10% of patients having documented metastasis at presentation, over 90% of tumors showing high-grade histology, 75% of tumors showing aneuploidy, the metastatic rate being 80% or more with amputation alone, the lung being the most common site of metastasis, and improved survival with adjuvant chemotherapy,[18] as well as deregulation of key cellular proteins, like STAT3.[19] Recently, the study of molecular profiles derived from canine osteosarcoma helped to group complex human osteosarcoma into biologically and clinically relevant molecular subtypes.[20]

Despite aggressive treatment including surgery and chemotherapy, little improvement in survival time has been achieved in either humans or dogs.[21,22] Added to the great importance of osteosarcoma in both humans and dogs described above is the fact that VEGF levels are predictive of pulmonary metastasis and a poor prognosis in human osteosarcoma,[23] leading to the hypothesis that impairment of the proangiogenic pathway with cannabinoids can improve the prognosis of the disease.

To the best of our knowledge, there are no published studies evaluating VEGF levels in canine osteosarcoma or the effects of cannabinoids in canine cancer or on canine tumor cells. Thus, the aim of this study was to assess the impact of treatment with WIN-55,212-2 on VEGF-A expression in the canine osteosarcoma 8 (OSA-8) cell line.[20] To the authors' knowledge, this is the first study using a canine model to evaluate the possible use of cannabinoids to impair angiogenesis in osteosarcoma.

Materials and methods

The OSA-8 cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 0.2% Primocin (InvivoGen, San Diego, CA, USA), and 1% HEPES (BioReagent, Sigma-Aldrich, St Louis, MO, USA). Twenty-four hours before the experiments, 8×10^6 cells were seeded per well. A stock solution of WIN-55,212-2 (Cayman Chemical Company, Ann Arbor, MI, USA) was prepared in dimethylsulfoxide (Thermo Fisher Scientific Inc, Waltham, MA, USA). The solution was added to OSA-8 cells to a final concentration of 1 μM in supplemented Dulbecco's Modified Eagle's Medium and incubated for 48 hours (n = 8). Control incubations (n = 6) had the same dimethylsulfoxide content of 0.1% (v/v). Three independent experiments were performed.

After total RNA extraction (RNeasy Mini Kit, Qiagen, Valencia, CA, USA), RNA quality and concentration was evaluated by spectrophotometry. Real-time quantitative polymerase chain reaction was performed by a one-step reaction with a primer pair and labeled probe specific for canine VEGF-A (TaqMan Gene Expression Assay, Invitrogen, Carlsbad, CA, USA).

A specific canine GAPDH primer pair and labeled probe (Eurofins MWG Operon, Huntsville, AL, USA) was designed for normalization purposes. It was demonstrated to be a suitable gene for normalization under the experimental conditions after following procedures described elsewhere.[24] GAPDH was considered to be stably expressed during the experiment on the basis of a −1.068-fold change because of treatment and checked by the Student's *t*-test with a *P* value of 0.48.

The comparative quantification method was used for relative quantification[25] and all samples were run in triplicate. The amplification efficiencies were analyzed by 10-fold dilution of a series of pooled RNA samples by 96.3% and 99.9% for VEGF-A and GAPDH, respectively.

Data are presented as the mean ± standard deviation. The statistical analysis was done by analysis of variance followed by the Student's *t*-test[26] using SAS version 9.2 (SAS Institute Inc, Cary, NC, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

As shown in Figure 1, OSA-8 cells incubated with WIN-55,212-2 for 48 hours showed a decrease in VEGF-A expression by 35% ± 10% on average ($P < 0.0001$) compared with the control (dimethyl sulfoxide), which was 100% ± 23%.

Discussion

In this paper, we detail the use of a highly sensitive and specific quantitative real-time polymerase chain reaction method for initial assessment of VEGF-A mRNA levels in a canine tumor cell line following treatment with a cannabinoid. We have demonstrated that WIN-55,212-2 decreased VEGF-A expression in the cultured OSA-8 cells by 35%. This finding is in accordance with other reports of the antiangiogenic effect of WIN-55,212-2, with a greater than 25% decrease in VEGF release from human and rat glioma cells,[12] a 40% decrease from human prostate cancer cells,[8] and marked inhibition of VEGF expression in human skin carcinoma.[11]

In addition to the antiangiogenic effects of cannabinoids observed here and previously, these compounds have been

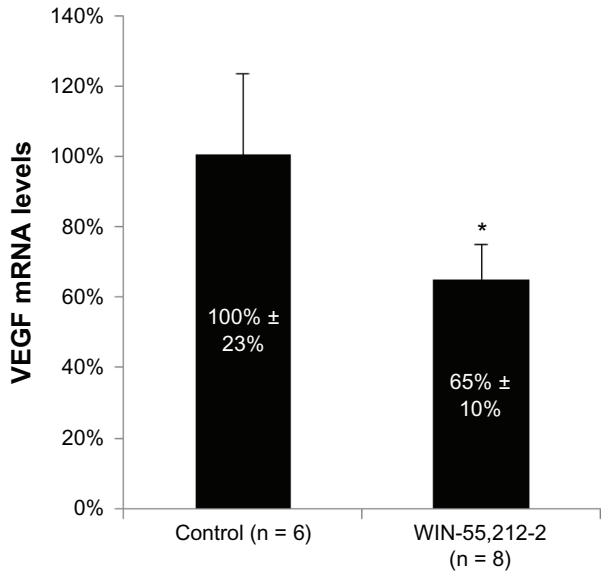

Figure 1 Comparison between vascular endothelial growth factor (VEGF) messenger RNA (mRNA) levels in control OSA-8 cells cultured for 48 hours with dimethylsulfoxide (0.1%, v/v) or WIN-55,212-2 (1 μM).
Notes: Data are reported as the mean ± standard deviation of three independent experiments. *Statistically significant at $P < 0.05$.

used successfully in patients with human cancer for the treatment of symptoms resulting from both cancer and antineoplastic therapies, including nausea and vomiting, weight loss, lack of appetite, and pain. Although these benefits have been known for years, it is only recently that the clinical anticancer effects of cannabinoids have been investigated.[4]

Our findings give rise to at least two further lines of research. First is the need to address the role of VEGF in the establishment of canine osteosarcoma and development of metastasis. Second is the need to elucidate the exact signaling mechanisms governing the inhibitory effects of cannabinoids on VEGF expression, although the involvement of cannabinoid receptors has been suggested by the fact that CB_1 and CB_2 antagonists block the beneficial action of WIN-55,212-2 in skin carcinoma.[12] Further research is currently underway in our laboratory with WIN-55,212-2 and the OSA-8 cell line to assess its precise mechanism of action.

Canine osteosarcoma accounts for approximately 85% of all canine skeletal tumors and, although advances in disease management have been achieved, 40%–50% of dogs treated by amputation followed by adjuvant therapy survive about one year and only 10%–20% survive more than 2 years.[27] In the United States, the number of new cases is estimated to exceed 10,000 per year, representing 10 times more than that in people.[18,19] These, in addition to the finding that VEGF levels in human osteosarcoma are indicative of a poor prognosis[22] and given the similarities between canine and human osteosarcoma,[18] make the canine OSA-8 cell line an attractive in vitro model for assessing the antiangiogenic effects of cannabinoid treatment, and future work will benefit both comparative angiogenesis research and canine patients with cancer.

Conclusion

In conclusion, the cannabinoid agonist, WIN-55,212-2, was shown to reduce the expression of VEGF-A in a canine osteosarcoma cell line. On the basis of the results reported here, we suggest that cannabinoid receptor agonists may have the potential for use as clinical angiogenesis inhibitors. Our results suggest a first step toward the use of cannabinoids as potential adjuvants to chemotherapeutics in the treatment of canine cancers.

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Disclosure

The authors report no conflicts of interest in this work.

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