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Anti-cancer and analgesic effects of resolvin D2 in oral squamous cell carcinoma

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ABSTRACT

Oral cancer is often painful and lethal. Oral cancer progression and pain may result from shared pathways that involve unresolved inflammation and elevated levels of pro-inflammatory cytokines. Resolvin D-series (RvDs) are endogenous lipid mediators derived from omega-3 fatty acids that exhibit pro-resolution and anti-inflammatory actions. These mediators have recently emerged as a novel class of therapeutics for diseases that involve inflammation; the specific roles of RvDs in oral cancer and associated pain are not defined. The present study investigated the potential of RvDs (RvD1 and RvD2) to treat oral cancer and alleviate oral cancer pain. We found down-regulated mRNA levels of *GPR18* and *GPR32* (which code for receptors RvD1 and RvD2) in oral cancer cells. Both RvD1 and RvD2 inhibited oral cancer proliferation *in vitro*. Using two validated mouse oral squamous cell carcinoma xenograft models, we found that RvD2, the more potent anti-inflammatory lipid mediator, significantly reduced tumor size. The mechanism of this action might involve suppression of IL-6, C-X-C motif chemokine 10 (CXCL10), and reduction of tumor necrosis. RvD2 generated short-lasting analgesia in xenograft cancer models, which coincided with decreased neutrophil infiltration and myeloperoxidase activity. Using a cancer supernatant model, we demonstrated that RvD2 reduced cancer-derived cytokines/chemokines (TNF-α, IL-6, CXCL10, and MCP-1), cancer mediator-induced $CD11b^{+}Ly6G^{-}$ myeloid cells, and nociception. We infer from our results that manipulation of the endogenous pro-resolution pathway might provide a novel approach to improve oral cancer and cancer pain treatment.

1. Introduction

Oral squamous cell carcinoma (SCC) is the most common oral malignancy (Wu et al., 2011). Survival of oral SCC patients has modestly improved in the past few decades; recurrence is common (Hunter et al., 2005; Ran and Yang, 2017). Approximately half of all oral SCC patients treated with surgery, chemotherapy, or radiation therapy will be cured. Outside of survival, pain is the primary concern of oral cancer patients (Benoliel et al., 2007; Epstein et al., 2007; Viet and Schmidt, 2012). Oral cancer pain usually becomes more severe with disease progression (Benoliel et al., 2007). Terminal oral SCC patients often experience excruciating pain during the final months of life (Benoliel et al., 2007). Ineffective pain control correlates positively with poor outcome and diminished quality of life in oral SCC patients (Reyes-Gibby et al., 2014; Viet and Schmidt, 2012).

Chronic inflammation is a hallmark of oral SCC (Choi and Myers, 2008; Feller et al., 2013a; Patel et al., 2016; Wu et al., 2011). Acute inflammation is typically self-limited, initiated by pro-inflammatory cytokines and actively resolved by specialized lipid mediators (i.e., pro-resolution, anti-inflammatory mediators) that return inflamed tissues to homeostasis (Ji et al., 2011; Prevete et al., 2017a; Serhan et al., 2008). These specialized pro-resolving mediators are derived from ω-3 or ω-6 essential polyunsaturated fatty

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acids (PUFA), through the activity of lipoxygenases (Serhan et al., 2002). A potentiated pro-inflammatory response or a suppressed anti-inflammatory response can impair resolution and lead to chronic inflammation; inflammation increases cancer proliferation, progression, and metastasis (Feller et al., 2013a; Prevete et al., 2017a). The well-known pro-inflammatory pathway associated with oral SCC involves cyclooxygenase-2 (COX-2) (Feller et al., 2013b; Hunter et al., 2005; Pandey et al., 2008), the inducible enzyme that converts arachidonic acid to prostaglandins. COX-2 inhibitors, however, generate slight or no improvement in oral premalignant lesions (Feller et al., 2013a; Hunter et al., 2005; Papadimitrakopoulou et al., 2008; Wirth et al., 2008). COX-2 inhibitors slightly reduce oral cancer pain in animal models (Harano et al., 2010). Moreover, COX-2 inhibitors might prolong inflammation by blocking synthesis of endogenous pro-resolution mediators (Ji et al., 2011; Serhan et al., 2002, 2008). The role of counter-regulatory mechanisms (i.e., the pro-resolution pathway) has never been studied in the context of oral SCC progression and pain.

Resolvins, such as resolvin D1 (RvD1) and resolvin D2 (RvD2), represent a family of pro-resolution mediators naturally synthesized from docosahexaenoic acid (DHA) (Serhan et al., 2002, 2008). They exert potent pro-resolving and anti-inflammatory actions by regulating immune cell migration and infiltration, removing cell debris and apoptotic cells, and through inhibition of pro-inflammatory cytokines synthesis (Ji et al., 2011; Serhan, 2014; Sulciner et al., 2017). The benefits of RvDs have been reported in many disease models associated with inflammation including cancer of the lung, liver, pancreas, stomach, and colon, as well as in models of inflammatory pain and postoperative pain (Halder et al., 2015; Huang et al., 2011; Ji et al., 2011; Kuang et al., 2016; Lee et al., 2013a; Prevete et al., 2017b; Serhan, 2014; Wang and Strichartz, 2017; Xu et al., 2010). RvD1 and RvD2 attenuate or alleviate pain through inhibition of transient receptor potential (TRP) ion channels and reduction of inflammatory mediators including tumor necrosis factor alpha (TNF-α) and IL-6 (Claria et al., 2012; Ji et al., 2011; Xu et al., 2010). In addition, these resolvins inhibit glial activation and spinal cord synaptic transmission (Ji et al., 2011). Low doses of RvD1 and RvD2 are sufficient for treatment in animal models of inflammation and are not immunosuppressive; no side effects have been observed (Ji et al., 2011; Prevete et al., 2017a; Sulciner et al., 2017). Accordingly, RvD1 and RvD2 are attractive therapeutic candidates for cancer and cancer symptom treatment.

In the present study we hypothesized that RvD1 and RvD2 counter-regulate the pro-inflammatory microenvironment driven by oral SCC. Using cell culture and mouse models of oral cancer we examined the effect of RvD1 and RvD2 on cytokine production, immune cell infiltration, tumor growth, and nociceptive behaviors. We provided evidence that RvD2 significantly inhibited tumor growth. We proposed a mechanism of action that includes attenuated tumor-promoting cytokines and hypoxia-induced necrosis. RvD2 alleviated acute pain caused by cancer mediators and transiently reduced chronic cancer pain.

2. Materials and methods

2.1. Cell culture of human oral SCC, normal oral keratinocytes, and dysplastic oral keratinocytes

HSC-3 cells (Japanese Collection of Research Bioresources, passage 4–10) were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing antibiotic (penicillin/streptomycin, 10 U/mL) and 10% fetal bovine serum (FBS). Premalignant dysplastic oral keratinocytes (DOKs, Sigma Aldrich, passage 4–8) were cultured in DMEM with 10% FBS and 5 μg/ml hydrocortisone. Normal oral keratinocytes (NOKs, Lifeline Cell Tech, passage 4–8) were grown in Lifeline® DermaLife K Medium. All cells were incubated with 5% CO_2 at 37 °C. Separate new batches of HSC-3 cells were purchased and used in 2012, 2014, and 2015. DOKs and NOKs were purchased in 2015.

2.2. Animals

Six to 8 week-old female athymic NU/J (*Foxnl*^{nu}) nude mice and BALB/cJ mice (The Jackson Laboratory) were used in accordance with National Institutes of Health guide for the care and use of laboratory animals and the New York University Institutional Animal Care and Use Committee. Investigators blinded to drug treatment performed all behavioral testing. Immunocompromised athymic nude (NU/J) mice were used to create the paw and tongue SCC xenograft model. To study the acute effect of cancer mediators on nociceptive behaviors and immune infiltration we used BABL/cJ mice, which have an intact immune system.

2.3. Real-time PCR

Total RNA from cultured cells was extracted with Qiagen AllPrep DNA/RNA Micro Kit (Qiagen Inc.). Reverse transcription was carried out with Quantitect Reverse Transcription Kit (Qiagen Inc.). All qPCR assays were performed with Taqman gene expression assay kits (Applied Biosystems Inc.). Primers were purchased from Life Technologies (human *GPR18*, Hs01921463; human ALX/*FPR2*, Hs027591275_s1; human *GPR32*, Hs00265986_s1). The housekeeping gene *GUS-β* was used as the internal control (human *GUS-β*: Hs00939627_m1). Relative quantification analysis of gene expression data was conducted according to the $2^{-\Delta\Delta CT}$ methods. Samples were run in triplicate.

2.4. Real-time cell growth profiling

Real time growth kinetics of HSC-3 cells with various RvD treatment concentrations were examined using the Real-Time Cellular Analyzer (RTCA) (xCELLigence System) (Kho et al., 2015). Electrode impedance was represented as the Cell Index calculated with the manufacturer-developed algorithm. For each experiment, a cell suspension (100 μL) with a density of 1.0×10^4 cells/well was added to each well. Cell growth was monitored for 18 h to reach the middle of the logarithmic growth phase. The plate was then removed from the RTCA machine, and 100 μL of freshly prepared media with the various concentrations of RvDs or vehicle (0.01% Ethanol) were added to each well. The plate was reinserted into the RTCA machine and cell growth was further assessed for up to 86 h. Six wells were used for each treatment. Normalization of the growth curves and slope calculations (based on the most linear phase of the entire growth curve) were performed using the RTCA Software 1.2.

2.5. Paw xenograft model

Nude (NU/J) mice were inoculated with 10^6 HSC-3 cells in 50 μL of DMEM and Matrigel into the plantar surface of the right hind paw (Ye et al., 2011, 2014). This paw xenograft model allows better monitoring of tumor development, as well as the measurement of both mechanical and heat hypersensitivity of the paw. By post inoculation day (PID) 14, a visible tumor had developed in the paw of each mouse and nociceptive behaviors were evident. 100 μL vehicle (0.01% ethanol in PBS), RvD1 (100 ng or 200 ng in PBS) or RvD2 (100 ng or 200 ng in PBS) was administered daily through intraperitoneal (IP) injection beginning on PID15. Five to 8 animals were used in each treatment group. RvD1 and RvD2 were obtained from Santa Cruz Biotechnology.