Aspirin-Triggered Resolvin D1 Versus Dexamethasone in the Treatment of Sjögren’s Syndrome-Like NOD/ShiLtJ Mice - A Pilot Study

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Abstract

Resolvin D1 (RvD1) and its aspirin-triggered epimeric form (AT-RvD1) are endogenous lipid mediators (derived from docosahexaenoic acid, DHA) that control the duration and magnitude of inflammation in models of complex diseases. Our previous studies demonstrated that RvD1-mediated signaling pathways are expressed and active in salivary glands from rodents and humans. Furthermore, treatment of salivary cells with RvD1 blocked TNF-α-mediated inflammatory signals and improved epithelial integrity. The purpose of this pilot study was to determine the feasibility of treatment with AT-RvD1 versus dexamethasone (DEX) on inflammation (i.e., lymphocytic infiltration, cytokine expression and apoptosis) observed in submandibular glands (SMG) from the NOD/ShiLtJ Sjögren’s syndrome (SS) mouse model before experimenting with a larger population. NOD/ShiLtJ mice were treated intravenously with NaCl (0.9%, negative control), AT-RvD1 (0.01-0.1 mg/kg) or DEX (4.125-8.25 mg/kg) twice a week for 14 weeks beginning at 4 weeks of age. At 18 weeks of age, SMG were collected for pathological analysis and detection of SS-associated inflammatory genes. The AT-RvD1 treatment alone did not affect lymphocytic infiltration seen in NOD/ShiLtJ mice while DEX partially prevented lymphocytic infiltration. Interestingly, both AT-RvD1 and DEX caused downregulation of SS-associated inflammatory genes and reduction of apoptosis. Results from this pilot study suggest that a systemic treatment with AT-RvD1 and DEX alone attenuated inflammatory responses observed in the NOD/ShiLtJ mice; therefore, they may be considered as potential therapeutic tools in treating SS patients when used alone or in combination.

Keywords

Resolvins, Salivary Glands, AT-RvD1, RvD1, Sjögren’s syndrome, Submandibular Gland, Cytokine

Introduction

Sjögren’s Syndrome (SS) is an autoimmune disorder characterized by chronic inflammation of the salivary and lacrimal glands, resulting in secretory dysfunction [1]. Previous studies have found elevated levels of pro-inflammatory cytokines in salivary glands from SS patients [2] and SS mouse models [3]. Specifically, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18), interleukin-12 (IL-12), BAFF, interleukin-17 (IL-17) and interleukin-23 (IL-23) are significantly elevated [2-5]. Interestingly, the anti-inflammatory cytokines such as transforming growth factor beta 1 (TGFβ1) and interleukin-4 (IL-4) were expressed at low levels but interleukin-10 (IL-10) was overexpressed (for review, please see reference [3]). The etiology of SS remains unknown and treatment options are limited to either secretory agonists such as pilocarpine or saliva substitutes [6].

Previous studies demonstrated that human and animal cells convert ω-3 polysaturated fatty acids (PUFAs) into resolvins (Rv), which are anti-inflammatory and pro-resolving agents that control the duration and magnitude of inflammation and promote tissue repair [7]. Rv subtypes include the E-series (RvE1-2, derived from eicosapentaenoic acid [EPA]), the D-series (RvD1 and RvD2, derived from docosahexaenoic acid [DHA]), and aspirin-triggered forms (AT-RvD1) [8]. Rvs naturally inhibit key immuno-inflammatory processes in response to infection, injury or environmental challenges [9]. Interestingly, they are able to preserve tissue integrity and promote tissue repair and regeneration following an environmental insult [10-12]. Our previous studies indicate that ALX/FPR2, the receptor for RvD1, is expressed and active in the rat parotid cell line, Par-C10 [13]. Specifically, activation of ALX/FPR2 with AT-RvD1 blocked inflammatory signals caused by TNF-α and enhanced salivary epithelial integrity [13]. ALX/FPR2 is also expressed in mouse submandibular gland (SMG), and its activation with AT-RvD1 increases a diverse set of intracellular signaling pathways (e.g., intracellular calcium [Ca2+]i, Erk1/2, and Akt) to block TNF-α-mediated caspase-3 activation [14]. We also demonstrated that the machinery for RvD1 biosynthesis and its receptor are expressed and functional in human minor salivary glands (hMSG) with and without SS [15]. The expression levels of ALX/FPR2 seem to be unaltered in hMSG with and without SS [15], indicating that RvD1 could be used as a therapeutic option to treat salivary gland inflammation in SS patients.

One of the current treatments for SS includes the use of oral corticosteroids (see reviews [6,16]) and parotid irrigation with corticosteroids [17]. Additionally, dexamethasone (DEX) has been previously shown to control acute inflammation in rat SMG during retrodental viral delivery [18]. Furthermore, DEX serves as a potent...
anti-inflammatory drug that inhibits polymorphonuclear neutrophil function and tissue invasion during acute inflammatory responses [19]. Additionally, DEX causes a reduction of systemic cytokine expression in several conditions [20-22]. Corticosteroids, however, often result in debilitating side effects involving multiple organ systems [23], increasing the appeal of alternative anti-inflammatory therapies, such as resolvins.

In the present study, we compared the effects of AT-RvD1 and DEX on the inflammatory features (i.e., lymphocytic infiltration, cytokine expression and apoptosis) observed in SMG from the well-established NOD/ShiLtJ SS-like mouse model that exhibits many features of the human disease lymphocytic infiltration and cytokine upregulation [24].

Materials and Methods

Experimental animals

Female NOD/ShiLtJ mice were treated via tail vein injection twice a week with NaCl (0.9%, used as negative control), different doses of the stable RvD1 analog, AT-RvD1 (0.01-0.1 mg/kg) and dexamethasone (DEX, 4.125-8.25 mg/kg) from 4 weeks of age until 18 weeks of age (Figure 1). At 18 weeks of age the mice were anesthetized with 80-100 mg/kg Ketamine + 5 mg/kg Xylazine and subsequently euthanized by abdominal exsanguination. All animal usage, anesthesia, and surgeries were conducted under the strict guidelines and approval of the University of Utah Institutional Animal Care and Use Committee.

Histopathological evaluation

At 18 weeks of age, NOD/ShiLtJ mice were sacrificed and SMG were removed, frozen, sectioned, and stained with hematoxylin & eosin. Sections from the top, middle, and bottom of the gland were visualized using an Olympus BX53 upright research microscope (Olympus, Tokyo, Japan). Grading of SMG histological sections was performed as described by Chisholm & Mason [25]. Briefly, numerical scores were assigned as follows, 1: Slight infiltrate, 2: Moderate infiltrate of less than one focus per 4 mm², 3: One focus per 4 mm², 4: More than one focus per 4 mm². Focus represents an aggregate of 50 or more lymphocytes.

RNA extraction and preparation of cDNA

Total RNA (from nine different frozen SMG, three from each of the following treatments: NaCl 0.9%, AT-RvD1 0.1 mg/kg and DEX 8.25 mg/kg) was extracted using the Bio-Rad Aurum Total RNA Kit (Olympus, Tokyo, Japan). Grading of SMG histological sections was performed as described by Chisholm & Mason [25]. Briefly, numerical scores were assigned as follows, 1: Slight infiltrate, 2: Moderate infiltrate of less than one focus per 4 mm², 3: One focus per 4 mm², 4: More than one focus per 4 mm². Focus represents an aggregate of 50 or more lymphocytes.

cDNA using the Bio-Rad iScript Advanced cDNA Synthesis Kit (Bio-Rad).

PCR array

Detection and quantification of mouse gene expression was performed with three different SMG per mouse treatment (i.e., NaCl 0.9%, AT-RvD1 0.1 mg/kg, DEX 8.25 mg/kg) using both the SS and the Apoptosis and Survival Tier 1 PrimePCR Pathway Plates, according to the manufacturer’s instructions (Bio-Rad). These kits profile the expression of genes encoding proteins that are important in apoptosis, survival and SS. Quantitative PCR was performed for each sample individually using a CFX Connect Thermocycler (Bio-Rad). All results were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All replicates were then pooled to determine the relative expression level of each gene between the control and treatment groups. A high sensitivity genomic DNA (gDNA) control primer was used to detect non-transcribed gDNA contamination. CT values of gDNA were greater than 35 for all replicate PCR samples run, indicating that the level of gDNA contamination was minimal and did not affect gene expression profiling results. Data were analyzed using the CFX Manager software, version 3.1 (Bio-Rad). Statistical significance was determined using a t-test.

Western blot analysis

One half of a SMG was lysed in 200 μl of 2× Laemml buffer (120 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 1 mM dithiothreitol and 0.002% (w/v) bromophenol blue), sonicated for 60 sec with a sonic dismembrator (model 120; microtip; amplification 45%; Fisher Scientific, Pittsburgh, PA), and boiled for 5 min. Twenty microliters of SMG lysates were subjected to electrophoresis with 4-15% (w/v) Mini-Protean TGX Precast Gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline [0.137M NaCl, 0.025M Tris (hydroxymethyl)aminomethane, pH 7.4] containing 0.1% (v/v) Tween-20 (TBST), then immunoblotted overnight at 4°C in TBST containing 3% (w/v) BSA with rabbit anti-phospho Akt (1:500 dilution; Cell Signaling Technology). After incubation, membranes were washed three times for 20 min each with TBST and incubated for 1 h with peroxidase-linked goat anti-rabbit IgG antibody (1:2,000 dilution; Cell Signaling Technology). The membranes were treated with Clarity Western ECL Substrate (Bio-Rad), and protein bands were visualized and quantitated using a ChemiDoc MP and Image Lab v4.1 software (Bio-Rad). Membranes were treated with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-probed with rabbit anti-pan Akt (1:500 dilution; Cell Signaling Technology) and with rabbit anti-pan-actin (1:1000 dilution; Cell Signaling Technology) for signal normalization followed by peroxidase-linked goat anti-rabbit IgG antibody (1:2,000 dilution; Cell Signaling Technology).

![Image](image_url)
**TUNEL analysis**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed with a Click-iT TUNEL Alexa Fluor 488 Imaging Assay (Life Technologies, Grand Island, NY) following the manufacturer’s instructions using TO-PRO-3 iodide (Life Technologies) as a nuclear marker. Briefly, frozen sections were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, permeabilized with Triton X-100 (1% in PBS) for 5 min and treated with 1% Sodium citrate for an additional 5 min. Then, sections were washed twice in PBS, and incubated with 100 μl of terminal deoxynucleotidyl transferase (TDT) reaction buffer (Component A) for 10 min at room temperature. The buffer was removed and the TUNEL reaction cocktail containing TDT was added, then sections were incubated in a humidified chamber overnight at room temperature. After treatment, sections were washed three times with 3% BSA in PBS for 5 min each and then incubated with 100 μl of Click-iT reaction mixture (containing Alexa Fluor 488 azide) for 1 h at room temperature, while being protected from light exposure. The sections were washed with 3% BSA in PBS. The staining was completed by incubation with TO-PRO-3 for 10 min at room temperature. The negative control was processed using a reaction mixture without terminal deoxynucleotidyl transferase (TDT) cocktail. The positive control section was incubated with DNase I (3 units/ml) for 1 h to induce DNA strand breaks. Positive and negative controls were performed in frozen mouse submandibular gland sections from C57BL/6 mice. TUNEL-positive nuclei images were taken using a Carl Zeiss LSM 710 confocal microscope, and visualized using the ZEN software (black edition; Carl Zeiss, Thornwood, NY). TUNEL-positive staining was calculated from a tile image of entire tissue sections, and quantified by mean intensity of images (see Supplemental Figure 1 and Supplementary Table 1).

**Statistical analysis**

Data presented are means ± S.E.M. of results from three or more determinations. Graphpad Prism software (Graphpad Software, Inc., La Jolla, CA) was used to perform one-way ANOVA, followed by pairwise post hoc multiple comparisons using Dunnett’s test to generate P-values and 95% confidence intervals (CI) of the differences of the means. P-values equal to or less than 0.05 represent significant differences between experimental groups. A 4-fold regulation threshold was used to determine significant differences in gene microarray studies.

**Results**

**Effects of AT-RvD1 and DEX on lymphocytic infiltration in NOD/ShiLtJ mouse SMG**

The degree of lymphocytic infiltration was estimated by counting the number of lymphocytes in SMG from NOD/ShiLtJ mice as described in MATERIALS AND METHODS. As shown in table 1 and Supplemental figure 2, SMG from NOD/ShiLtJ mice treated with NaCl (0.9%, used as negative controls) displayed a focus score of 4 in at least two out of three sections from each gland. Similarly, SMG from NOD/ShiLtJ mice treated with AT-RvD1 (0.1 mg/kg, used as target group) displayed focus scores comparable to SMG from mice treated with saline. In contrast, sections of SMG from mice treated with DEX (8.25 mg/kg) displayed a decrease in focus scores with the majority of the sections observed being 1 or 2. One gland displayed a focus score of 4 in two of the three sections. These results indicated that DEX treatment is effective in decreasing lymphocytic infiltration in these mice.

**Effects of AT-RvD1 and DEX on inflammatory genes in mouse SMG**

To determine whether AT-RvD1 and DEX treatment affected the expression of SS-associated inflammatory genes, we performed a microarray analysis. As shown in figure 2A, systemic AT-RvD1 (0.1 mg/kg) treatment caused a significant downregulation of the SS-associated inflammatory genes: CXCL10, IL-2, IL-4, IL-5, and IL-12β in SMG from NOD/ShiLtJ mice as compared to SMG from mice treated with saline. As shown in figure 2B, systemic DEX (8.25 mg/kg) treatment caused a significant downregulation of the SS-associated inflammatory genes: Foxp3 and IL-12β in SMG from NOD/ShiLtJ mice as compared to SMG from mice treated with saline.

To determine whether AT-RvD1 and DEX treatment affected the expression of apoptotic and survival genes, we performed a second microarray analysis. As shown in figure 2C, systemic AT-RvD1 (0.1 mg/kg) treatment caused a significant downregulation of the pro-apoptotic genes: TNF-α, Casp1, Hspa1a, and Hspa1b in SMG from NOD/ShiLtJ mice as compared to SMG from mice treated with saline. As shown in figure 2D, systemic DEX (8.25 mg/kg) treatment caused a significant downregulation of the pro-apoptotic genes: Foxp3, IL-12β, TNF-α and NF-xB in SMG from NOD/ShiLtJ mice as compared to SMG from mice treated with saline.

**Effects of AT-RvD1 and DEX on cell death in mouse SMG**

To assess the effect of AT-RvD1 and DEX treatment on cell death in SMG of NOD/ShiLtJ mice, we quantified the mean intensity of TUNEL positive cells. As shown in figure 3, SMG from NOD/ShiLtJ mice systemically treated with AT-RvD1 (0.1 mg/kg) showed a significant decrease in mean intensity of TUNEL positive cells as compared to SMG from mice treated with saline. Similarly, SMG from mice systemically treated with DEX (8.25 mg/kg) showed a significant decrease in mean intensity of TUNEL positive cells as compared to SMG from mice treated with saline. These results indicate that both AT-RvD1 and DEX were effective in decreasing the overall apoptosis levels in SMG from NOD/ShiLtJ mice.

**Effects of AT-RvD1 and DEX on phosphorylation of Akt in mouse SMG**

Our previous studies showed that RvD1 is able to cause phosphorylation of the survival Akt pathway, blocking apoptosis in salivary cells [13]. Therefore, to determine whether decreased apoptosis correlated with Akt phosphorylation, we decided to test whether SMG from NOD/ShiLtJ mice systemically treated with AT-RvD1 displayed a sustained activation of this pathway. As shown in figure 4, systemic treatment with AT-RvD1 (0.1 mg/kg) did not cause a sustained phosphorylation of Akt in SMG from NOD/ShiLtJ mice as compared to SMG from mice treated with saline.

**Discussion**

Salivary gland inflammation and dysfunction are hallmarks of SS [26]. Current anti-inflammatory treatments do not fully relieve salivary gland chronic inflammation without causing adverse side effects [6,16]. The complex interaction between multiple inflammatory pathways requires future studies to achieve a deeper mechanistic understanding of SS etiology and pathogenesis. Such complex interactions suggest that a combinatorial treatment may be a better therapeutic approach than individual treatments. Due to its lack of adverse side effects, AT-RvD1 offers an attractive alternative option to the use of corticosteroids. RvD1 has shown to be useful in treating a variety of conditions such as experimental colitis, alveolar
epithelial cell injury, smoke-induced lung inflammation, colon cancer, rheumatoid arthritis, chronic obstructive pulmonary disease, ischemic injuries and uveitis [27-34]. Moreover, our previous in vitro studies indicate that RvD1 and AT-RvD1 block inflammatory signals in the rat parotid Par-C10 cell line and in freshly isolated submandibular gland cells [13,14], suggesting a potential therapeutic benefit for SS in vivo.

Our results indicate that AT-RvD1 treatment was not effective in decreasing lymphocytic infiltration in NOD/ShiLtJ mice (Table 1). In contrast, systemic treatment with DEX prevented lymphocytic infiltration in two out of three NOD/ShiLtJ mice (Table 1). Reduction of lymphocytic infiltration has been observed previously with DEX treatment for patients suffering from autoimmune diseases, such as rheumatoid arthritis [35] and systemic lupus erythematosus [36]. While DEX appeared to have a more directly observable effect on lymphocytic infiltration than AT-RvD1 as evidenced by histopathological evaluation, we wanted to determine the effects of both treatments on other indicators of inflammation. To this end, we performed gene expression analyses using SS- and apoptosis-associated gene expression panels. We found that AT-RvD1 and DEX affected the expression of mostly non-overlapping groups of genes (Figure 2A, Figure 2B, Figure 2C and Figure 2D). AT-RvD1 significantly reduced the expression of SS-related pro-inflammatory cytokines as well as apoptotic and survival genes (e.g., IL-2, -4, -5, and -12β, TNF-α, Casp1, Foxp3, and Cxcl10, Figure 2A) in SMG from NOD/ShiLtJ mice as opposed to mice treated with saline (used as negative control). Interestingly, DEX was able to downregulate IL-12β (similar to AT-RvD1); however, it significantly downregulated different pro-inflammatory genes compared to those downregulated by AT-RvD1 (Figure 2B). Our results are consistent with previous findings showing that RvD1 reduces cytokine expression in alveolar epithelium [29], primary human lung fibroblasts [30], colonic epithelium [31], and in rat eyes [33]. In the case of DEX, our results are also consistent with previous studies showing reduction of cytokine expression in human corneal epithelium [37], airway epithelium

Figure 2: Detection and quantification of mouse apoptosis and survival gene expression and SS gene expression. Expression levels of genes important to apoptosis and survival as well as Sjögren's syndrome (SS) were detected and quantified by PCR array as described in MATERIALS AND METHODS and represented on a scatter plot. Black dots indicate no change in gene expression from control, red dots indicate upregulation, and green dots indicate downregulation. Regression lines for upregulation (red) and downregulation (green) are also shown. (A) Expression levels after treatment of AT-RvD1 (0.1 mg/kg) as compared to control treatment of NaCl (0.9%) using a SS gene array; (B) Expression levels after dexamethasone (DEX) treatment (8.25 mg/kg) as compared to control treatment of NaCl using an apoptotic and survival gene array; (C) Expression levels after treatment of At-RvD1 (0.1 mg/kg) as compared to control treatment of NaCl using an apoptotic and survival gene array; (D) Expression levels after dexamethasone (DEX) treatment (8.25 mg/kg) as compared to control treatment of NaCl using an apoptotic and survival gene array.
and in brain microvascular endothelial cells [39]. The different effects elicited by AT-RvD1 and DEX on anti-inflammatory cytokine gene expression suggest that using them in combination may be an effective treatment for SS.

Of particular interest is gene expression for the pro-inflammatory cytokine, TNF-α, which was downregulated in both AT-RvD1 and DEX treatments. TNF-α is a secreted by macrophages, mature T helper cells and epithelial cells, and has been identified as a key regulator of inflammatory responses [40]. The role of TNF-α in the pathogenesis of primary SS has not been fully evaluated; however, there are a few studies indicating that TNF-α may play a role [2,4,40]. Specifically, TNF-α has been shown to be upregulated along with its receptors in serum [4] and salivary glands [2] from SS patients. Neutralization of TNF-α in NOD mice by transgenic expression of soluble TNF-receptor significantly reduced lymphocytic infiltration in SMG and lacrimal glands associated with decreased expression of the cell adhesion molecules, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) [41]. These results suggest a role for TNF-α in the initiation and progression of salivary and lacrimal gland destruction in NOD mice. Our previous studies indicate that TNF-α upregulates cell adhesion molecules and binding of lymphocytes in the human submandibular gland cell line (HSG) [42]. Additionally, TNF-α causes salivary gland dysfunction of Par-C10 cells and primary cells [13,14,43,44]. Interestingly, blocking TNF-α alone did not show improvement in patients using anti-TNF-α therapy [45], suggesting that other cytokines may also contribute to SS pathogenesis. This pilot study will serve as a foundation for our future pre-clinical study in which we will increase the population size and study whether other cytokines (such as IFN-γ and IL-10) are downregulated after AT-RvD1 treatment.

Our findings suggest that AT-RvD1 and DEX individually affect apoptosis independently from lymphocytic infiltration (Figure 3). These results are consistent with previous in vivo studies showing that RvD1 reduces apoptosis induced by lipopolysaccharide (LPS) in the liver [46]. Additionally, RvD1 reduced LPS-induced pulmonary cellular apoptosis in mice [47]. Furthermore, AT-RvD1 treatment significantly decreased apoptosis and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in acute lung injury [48].

Similarly, the use of DEX has been associated with decreased apoptosis but with deleterious effects including a reduction in insulin-secreting cells [49], a decrease of neural progenitor cells in the hippocampus [50] and diminishing oligodendrocyte progenitor cells resulting in hypomyelination. More recently, a study showed that DEX increases apoptosis in osteoblast and osteocytes, leading to bone loss [51]. These results highlight the positive impact of AT-RvD1 and the secondary effects that could be caused by DEX and indicate the need for further studies combining these two treatments to resolve inflammation associated with SS.

Our studies showed that neither AT-RvD1 nor DEX caused a sustained phosphorylation of Akt in mouse SMG (Figure 4). Furthermore, Akt phosphorylation did not correlate with the decreased apoptosis observed in mice treated under these conditions (Figure 4). Our previous studies showed that activation of ALX/FPR2 with AT-RvD1 induces transient Akt phosphorylation in rat parotid Par-C10 cells [13] as well as in mouse SMG [14]. These results indicate that AT-RvD1 initiates rapid signaling mechanisms to block stress generated by tissue culture conditions. Therefore, it is possible that Akt displayed a transient unmeasurable phosphorylation at the time point studied (at least in the case of AT-RvD1). In contrast, DEX has been shown to inhibit Akt phosphorylation in several tissues caused by dexamethasone-induced reductions in PI3 kinase activity. Future studies to measure Akt phosphorylation over time will be necessary to better understand the AT-RvD1 and DEX mechanisms to prevent cell apoptosis [52].

We were able to test the feasibility of using AT-RvD1 and DEX before using these treatments in a large scale population. In this pilot study, we were able to examine effects and associations that warrant further studies (see review, [53]). Given our findings, future studies will determine whether AT-RvD1 and DEX alone and in combination prove to be useful in the treatment of experimental SS.

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References


