Retinoic Acid Potentiates Orbital Tissues for Inflammation Through NF-κB and MCP-1

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PURPOSE. The orbit displays unique vulnerability to inflammatory conditions. The most prevalent of these conditions, thyroid eye disease (TED), occurs in up to 50% of patients with Graves' disease (GD). Whereas the pathology of both TED and GD is driven by autoantibodies, it is unclear why symptoms manifest specifically in the orbit.

METHODS. We performed retinoic acid treatment on both normal and TED patient–derived orbital fibroblasts (OFs) followed by mRNA and protein isolation, quantitative real-time polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay, RNA sequencing, and Western blot analyses.

RESULTS. Both normal and TED patient–derived OFs display robust induction of monocyte chemoattractant protein 1 (MCP-1) upon retinoid treatment; TED OFs secrete significantly more MCP-1 than normal OFs. In addition, pretreatment of OFs with thiopehenecarboxamide (TPCA-1) inhibits retinoid-induced MCP-1 induction, suggesting an NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)–dependent mechanism. We also found that treatment with cholecalciferol (vitamin D3) mitigates MCP-1 induction, likely because of competition between retinoic acid receptors (RARs) and vitamin D receptors (VDR) for their common binding partner retinoid nuclear receptors (RXRs).

CONCLUSIONS. Retinoids that naturally accumulate in orbital adipose tissue can act on orbital fibroblasts to induce the expression of inflammation-associated genes. These data suggest a potential role for retinoids in sensitizing the orbit to inflammation.

Keywords: inflammation, orbit, thyroid eye disease, Graves, pseudotumor

Although the eye displays immune privilege1 (reviewed in2), the eye socket (i.e., orbit) is highly vulnerable to inflammation.3 Orbital inflammatory conditions include thyroid eye disease (TED),1,5 lupus, polyangitis with granulomatosis, sarcoidosis, Sjögren, immunoglobulin G4–associated disease, orbital myositis, histiocytic disorders, and xanthogranuloma.3 Nonspecific orbital inflammation (NSOI) is also very common, representing 50% of orbital inflammatory conditions. Inflammatory masses, referred to as “orbital pseudotumor,” represent 8% to 10% of all orbital masses.6 Other systemic autoimmune or inflammatory conditions such as rheumatoid arthritis7,8 and inflammatory bowel disease9 can also affect the orbit. This represents a remarkable list of orbital inflammatory conditions essentially unmatched elsewhere in the body. Orbital inflammation causes a range of symptoms including redness, pain, swelling, and decreased vision; however, the mechanism underlying orbital sensitivity to inflammation is unclear.

TED is the most prevalent cause of orbital inflammation, occurring in up to 50% of patients with Graves' disease (GD).10 Although the biology underlying GD is understood,10–12 the mechanism of the orbitopathy remains ambiguous. The current prevailing hypothesis is that autoantibodies bind thyrotropin (TSHR) and insulin-related growth factor (IGF-1R) receptors expressed on orbital fibroblasts (OFs) to promote local inflammation.13 However, autoantibody animal models do not fully recapitulate human TED and do not address the mechanism of orbital specificity given that TSHR and IGR-1R are expressed throughout the body.

One unique attribute of orbital tissue is the presence of carotenoids stored in orbital fat,14 presumably a byproduct of retinoic acid's role in visual transduction and orbital biology.15–17 Here, we show that retinoids are present in orbital adipose tissues obtained from both normal and TED patients. In addition, OFs treated with all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9CRA) induce expression of a variety of inflammation-associated genes, including monocyte chemoattractant protein–1 (MCP-1), a previously identified key regulator of TED pathogenesis.18–20 This MCP-1 induction is dependent on NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), because treatment with thiopehenecarboxamide (TPCA-1), a potent inhibitor of IkB kinase (IKK) 2, mitigates MCP-1 expression. Interestingly, treatment of OFs with cholecalciferol (vitamin D3) also mitigates MCP-1 expression, consistent with the potential
link between vitamin D deficiency and TED pathogenesis. This is potentially due to competitive binding of vitamin D receptor (VDR) to retinoid nuclear receptors (RXRs), which are required for retinoic acid receptor (RAR) signaling. Taken together, these data suggest that retinoids stored in orbital adipose tissue may sensitize the orbit to inflammation and highlight retinoid signaling as a potential therapeutic target for orbital inflammatory conditions.

**METHODS**

**Primary Orbital Fibroblast Culture**

Orbital fat was collected from Graves’ and normal patients during orbital decompression or blepharoplasty. Informed consent was obtained and study was performed in compliance with the University of Michigan Institutional Review Board (HUM00142787). After collection, 1 to 2 mL of fat was washed with Dulbecco’s phosphate-buffered saline (PBS) and placed in a 15-cm dish containing OF growth media (Dulbecco’s modified Eagle medium [DMEM] culture media supplemented with 10% fetal bovine serum, 1% Hepes, 2.5% sodium pyruvate, and 0.2% primocin antibiotic). Media was changed every seven days until plates reached subconfluence (∼4 weeks), on which P1 and P2 cells were used for experiments.

**Retinoid, TPCA-1, and Vitamin D Treatments for Gene Expression Analysis**

Before treatment, OF growth media was removed, and cells were washed with “no serum” media (DMEM culture media supplemented with 1% Hepes, 2.5% sodium pyruvate, and 0.2% primocin antibiotic). For gene expression analysis after retinoid treatment, cells were treated with varying concentrations of all-trans retinoic acid (ATRA) or 9-cis retinoic acid (9CRA) in “no serum” media. After six hours, media was removed, cells were collected in 2 mL Trizol solution (Ambion, Austin, TX, USA), and RNA was isolated using the ReliaPrep RNA tissue Miniprep System (Promega, Madison, WI, USA). The cDNA was synthesized using iScript Reverse Transcriptase Supermix (Bio-rad, Hercules, CA, USA), and all qRT-PCR experiments were run in triplicate using 2x SYBR Green qPCR master mix (bimake.com). The qRT-PCR primer sequences are shown in Supplementary Table S1. Gene expression relative to RPS13 was performed, and fold-change in expression was determined by normalizing to the dimethylsulfoxide (DMSO)-treated samples. For vitamin D experiments, cells were treated with either DMSO alone, 100 nmol/L D3 (cholecalciferol), 1 μmol/L ATRA, or both 100 nmol/L D3 and 1 μmol/L ATRA for six hours before collection. For TPCA-1 experiments, cells were pretreated with either DMSO or 1μmol/L TPCA-1 for 30 to 45 minutes, followed by treatment with DMSO or 1 μmol/L ATRA for an additional 5.5 hours before collection. RNA isolation, cDNA synthesis, and qRT-PCR was performed as previously described.

**ELISA Assessment of MCP-1 Secretion**

On confluence, cells were washed once with “no-serum” media and then treated with “no-serum” media containing either DMSO or 1 μmol/L ATRA for 48 hours, after which media was collected and stored at −80°C. Enzyme-linked immunosorbent assay (ELISA) experiments were performed using the Invitrogen CCL2 Uncoated ELISA kit according to the kit protocol with an N of 6.

**Immunofluorescence Staining of Cultured Orbital Fibroblasts**

OFs were grown to confluence in OF growth media in 15-cm plates containing three or four glass slides. On confluence, cells were treated with DMSO or ATRA as previously described. After treatment, slides were immediately fixed in 4% PFA for 15 minutes followed by three washes in PBS-Tween 0.2, one wash with PBS-Triton 0.5%, followed by a final wash in PCR-Tween 0.2%. Blocking was performed using 20% normal goat serum for 30 minutes followed by overnight incubation in primary antibody diluted in 5% NGS/PBS-Tween 0.2% (NF-κB p65 [D14E12] rabbit mAb, 1:1000; Cell Signaling Technology, Danvers, MA, USA). The following day, slides were washed three times with PBS-Tween 0.2% followed by a one-hour incubation in secondary antibody (Goat Anti-Rabbit Alexa Fluor 555, 1:300, Invitrogen, Carlsbad, CA, USA) in 5% NGS/PBS-Tween 0.2%. Slides were washed an additional three times in PBS-Tween 0.2% before mounting with Prolong Gold containing DAPI (Invitrogen).

**RA Extraction and Mass Spectrometry Analysis**

The all-trans retinol and all-trans retinoic acids were obtained from Sigma (St. Louis, MO, USA). The d6-all-trans retinol and d6- all-trans retinoic acids were purchased from Cambridge Isotopes Laboratories (Andover, MA, USA). All compounds were stored in amber vials in ethanol as 1 mg/mL stocks at −20°C. Acetonitrile, methanol, water, and formic acid used in the UHPLC-MS/MS method were from Fischer Scientific (Pittsburg, PA, USA), and all were Optima LC/MS grade.

Retinoids were extracted by homogenization in solvent using a probe sonicator (Branson 450, duty cycle 40%, output power level 4). Biological samples were homogenized in 4:1:7 MeOH: H2O: Acetonitrile (v:v:v). The homogenate was centrifuged at 15,000 g at 4°C for 20 minutes, and the supernatant was transferred to a new tube. One hundred microliters of acetonitrile, 200 microliters of water, and 10 microliters of deuterated internal standards d6-all-trans retinol and d6-all-trans retinoic acid in 1:1:1 ratio were added to each sample. The final mixture was vortexed and centrifuged at 15,000 g at 4°C for 20 minutes. The supernatant was transferred to freshly cleaned polypropylene vials, dried under N2, and reconstituted with 100 μL of 50:50 ACN:water. The dried extracts were injected onto an Acquity CSH C18 1.7 μm 2.1 × 100 mm column (Waters, Milford, MA, USA) that was heated to 40°C. A binary gradient system was used; eluent A was water with 0.1% formic acid and eluent B was acetonitrile with 0.1% formic acid. The gradient profile consisted of the following: linear ramp from 100% A to 95% B from zero to seven minutes, hold 95% B between seven to 28 minutes, return to 100% A from 28 to 28.1 minutes, and hold 100% A from 28.1 to 35 minutes. The flow rate was 0.250 mL/min and the sample injection volume was 10 μL. Pooled human plasma and master pools were used as quality controls. Master pools made from a small aliquot from all samples were injected at the beginning, end, and at regular intervals throughout each analysis batch to provide a measurement of the system’s stability and performance.

ESI-MS/MS data acquisition was performed in positive ion mode using an Agilent 6490 QQQ-LC-MS with multiple reaction monitoring (MRM) transitions programmed for both labeled and unlabeled internal standards. MRM...
transitions for target retinoids were: 269 > 91 (native all trans retinol) and 275 > 96 (labeled all trans retinol), 301 > 91 (native ATRA) and 307 > 96 (d6-ATRA). Quantitation was performed by isotope dilution mass spectrometry using native/labeled peak area ratios. LC-MS data were processed using MassHunter Quantitative Analysis software version B.08.00. For tissues the quantitative data were normalized to tissue weight and were reported as picomoles per milligram tissue. For cell lysates samples the quantitative data are reported as nanomoles per liter.

Transcriptome Analysis
Sequencing was performed by the UM Advanced Genomics Core (AGC), with Quant-seq libraries constructed and subsequently subjected sequencing. Raw data were then processed by the AGC to generate a raw count table of all genes for each sample, using UCSC hg19. Quality control and sequence alignment At the UM Bioinformatics Core, we downloaded the reads and count summary files from the Advanced Genomics Core's storage. FastQC results were reviewed to ensure that only high-quality data were used for expression quantitation and differential expression. Differential Expression Data were prefiltered to remove genes with less than 10 counts in all samples. Differential gene expression analysis was performed using DESeq2, using a negative binomial generalized linear model with a term to account for “PatientOrigin” (thresholds: linear fold change >1.5 or <−1.5, Benjamini-Hochberg FDR [Padj] <0.05). Plots were generated using variations of DESeq2 plotting functions and other packages with R version 3.3.3. Genes were annotated with NCBI Entrez GeneIDs and text descriptions. Functional analysis, including candidate pathways activated or inhibited in comparison(s) and GO-term enrichments, was performed using iPathway Guide (Advaita).

Statistics
Repeat-measures one-way analysis of variance (ANOVA) and Student’s paired t-tests were performed in GraphPad Prism 7.00 to determine variance and statistical significance between treatment groups. All gene expression experiments were performed with an N of at least 5 (OFs from distinct patients).

RESULTS

Retinoids in Orbital Adipose Tissue
The distinct color difference between preaponeurotic orbital and nasal fat is attributed to increased presence of carotenoids (Fig. 1A). To assess which specific retinoid species are present in orbital fat, we performed mass spectrometry on orbital fat samples isolated from both normal and TED patients. The mean ATRA concentration was 0.0037 pmol/μg in TED samples and 0.0079 pmol/μg in normal samples; however, this difference was not statistically significant (P = 0.13, Fig. 1B). All-trans retinol was also present in higher concentrations in both samples (0.031 in TED, 0.023 in normal, P = 0.14, Fig. 1B).

Retinoids Promote Inflammatory Cytokine Expression in Orbital Fibroblasts
The presence of retinoids in orbital fat led us to question their effect on resident orbital fibroblasts. To test this, we treated primary OFs with 0.01, 0.1, 1, and 10 μmol/L ATRA and 9CRA. We found that both ATRA and 9CRA treatment resulted in a dose-dependent induction of the inflammatory cytokine monocyte chemoattractant protein-1 (MCP-1) (Figs. 2A, 2B). A 1-μmol/L dosage for both 9CRA and ATRA showed maximal induction, so that concentration was used for all future experiments. In addition to MCP-1, we also looked at the effect of ATRA treatment on other TED-associated genes and found significant induction of versican (P = 0.03) and interleukin-1 beta (IL-1β) (P = 0.02), and significant reduction in nuclear factor erythroid 2-related factor 2 (P = 0.0098) that regulates expression of antioxidant proteins (Supplemental Fig. S1). We then compared ATRA-induced MCP-1 induction in normal versus TED-derived OFs and found that whereas both showed a significant increase in mRNA expression (Fig. 2C), TED-derived OFs displayed significantly more MCP-1 protein secretion compared with normal OFs (Fig. 2D). In addition to MCP-1, ATRA treatment also induced expression of the retinoic acid receptors RARβ and RARγ, but not RARα (Fig. 2D) and inhibited expression of thyroid hormone receptors TRα and TRβ (Supplemental Fig. S2).
Retinoic Acid Potentiates Orbital Inflammation

MCP-1 Induction Is NF-κb Dependent

MCP-1 is a known modulator of orbital inflammation. Many factors induce MCP-1 expression in vitro, including tissue plasminogen activator, lipopolysaccharide, IL-1β, tumor necrosis factor–alpha, platelet-derived growth factor, interferon gamma, and interleukin-17. All of these factors promote MCP-1 expression primarily via activation of the transcription factor complex NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells).

Functional interaction between NF-κB and the retinoic acid (RA) pathway has been established in several different cell types. In endothelial cells, adjacent NF-κB and RA response elements are required for synergistic transcriptional regulation of ICAM-1. In keratinocytes, RA promotes expression of interleukin-8 (IL-8) in an NF-κB dependent manner. ATRA has also been shown to induce NF-κB activation via phosphorylation and subsequent degradation of IκB. In OFs, ATRA treatment promotes phosphorylation and nuclear translocation of the NF-κB p65 subunit.

To test whether ATRA-induced MCP-1 expression is NF-κB dependent, we used TPCA-1, a potent IKK inhibitor that prevents phosphorylation and subsequent degradation of the inhibitory protein IκB. We treated OFs with 1 μmol/L TPCA-1 for 30 minutes before ATRA treatment and found that TPCA-1 significantly inhibited MCP-1 induction.

Dermal Fibroblasts Display NF-κb Dependent MCP-1 Induction

To assess whether the observed ATRA response is unique to OFs, we performed ATRA treatment on primary human dermal fibroblasts (DF). We found that upon ATRA treatment, DFs significantly induce expression of MCP-1 (Supplemental Fig. S3A, P = 0.0001). This induction is also NF-κB-dependent, because pretreatment with TPCA-1 inhibits MCP-1 induction (Supplemental Fig. S3B, P = 0.0036).

Vitamin D3 Treatment Mitigates MCP-1 Induction

Retinoic acid receptors (RAR) belong to a superfamily of nuclear receptors, which heterodimerize with retinoid nuclear receptors (RXR) and bind DNA to influence transcription. Vitamin D receptor (VDR) belongs to the same superfamily and also requires RXR heterodimerization. To test this hypothesis, we treated OFs with both 1 μmol/L ATRA and 100 nmol/L D3 and found that D3 treatment partially mitigates MCP-1 expression (Fig. 4B, P = 0.02). These data are consistent with our recent study, which linked low serum vitamin D levels to TED incidence in GD patients and suggest that achieving high-normal vitamin D levels via supplementation may be protective against orbital inflammation.

MCP-1 Induction Is NF-κb Dependent

FIGURE 2. Retinoic acid treatment promotes MCP-1 induction in orbital fibroblasts. (A) Dose response of MCP-1 expression upon 9-cis retinoic acid treatment in OFs (RM one-way ANOVA P = 0.0272, N = 5). (B) Dose response of MCP-1 expression upon ATRA treatment in OFs (RM one-way ANOVA P = 0.0389, N = 6). (C) ATRA treatment promotes significant induction of MCP-1 expression in both normal (N = 6) and TED OFs (N = 7). (D) ATRA treatment promotes MCP-1 secretion in both normal and TED OFs, TED OFs show increased secretion compared with normal OFs (N = 3). (E) ATRA treatment promotes expression of retinoic acid receptors (RAR) α and β (N = 8). (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
**FIGURE 3.** MCP-1 induction is NFκB dependent. (A) Increased nuclear (left panel - yellow asterisk, right panel—white outline) expression of p65 (red) in ATRA (bottom panels) versus DMSO (top panels) treated TED OFs (scale bars: 100 μm). (B) TPCA-1 treatment mitigates ATRA-dependent p65 phosphorylation. (C) 1 μmol/L TPCA-1 treatment mitigates ATRA response in OFs (N = 9 (** **P < 0.0001, error bars: SEM).

**FIGURE 4.** Vitamin D partially inhibits MCP-1 induction. (A) Schematic of vitamin D and retinoic acid receptor heterodimerization with retinoid x receptors. (B) Vitamin D treatment inhibits MCP-1 induction in ATRA-treated OFs (N = 5). (*) P < 0.05, error bars: SEM.

**ATRA Treatment Promotes Similar Gene Expression Changes in Normal and TED OFs**

After observing gene expression changes in TED-associated genes upon ATRA treatment, we next sought to uncover additional pathways affected by ATRA. To do this, we performed 3′ RNA sequencing (Quantseq) on both TED and normal OFs treated with DMSO (control) or 1 μmol/L ATRA. We first compared TED and normal DMSO-treated OFs to assess the baseline differences between the two patient populations and found 29 significantly differentially expressed genes (Fig. 5A). We then looked at DMSO versus ATRA treatment in each of our two patient populations.

We found that ATRA treatment resulted in significant differential expression of 221 genes in TED OFs and 199 genes in normal OFs, 130 of which were differentially expressed in both populations (Fig. 5A). Top differentially expressed genes displayed similar expression patterns for both populations, suggesting despite differences at baseline, the two populations respond similarly to ATRA treatment (Fig. 5C). In addition, genes that are differentially expressed at baseline (DMSO) maintain their expression pattern upon ATRA treatment (Fig. 5B), suggesting these genes are not affected by ATRA treatment. Interestingly, although MCP-1 expression was induced in 16 of 19 patients, these changes were not significant. This is potentially due to
limited sensitivity of the Quantseq assay, which only maps 3' reads and has been shown to be less sensitive than whole transcript RNAseq.\textsuperscript{41} The quantitative real-time polymerase chain reaction (qRT-PCR) assay used in previous figures to highlight MCP-1 expression changes provides a more sensitive and accurate picture of MCP-1 gene expression.

**DISCUSSION**

Inflammatory conditions represent a major disease burden worldwide. Autoimmune conditions are common, and tissue damage may be caused by either auto-antibodies specific to an antigen unique to the diseased tissue, such as Graves' disease, or more commonly as “collateral damage” without a clear pathogenic signal, such as thyroid-associated orbitopathy or rheumatoid arthritis. The ocular orbit and the tissues contained therein, for example, muscles, nerves, connective and adipose tissues, are particularly vulnerable to systemic autoimmune inflammatory conditions that seemingly have nothing in common with one another or with orbital tissues. A key question is: Why is the orbit is uniquely vulnerable to inflammation?
Orbital tissues contain retinoids. This is likely due to their proximity to the eye, which synthesizes, uses, and recycles retinoids. We hypothesize that the retinoids stored in orbital fat are produced in the eye and diffuse through the sclera. Given the unique predisposition of orbital tissues to inflammation and the finding that orbital tissues are a depot for retinoids, we hypothesized that retinoids may sensitize the orbit to inflammation by promoting expression of inflammatory cytokines. Expression of inflammatory cytokines may prime orbital tissues for invasion by circulating T cells and macrophages associated with a pre-existing systemic inflammatory condition. The most common orbital inflammatory disease is thyroid eye disease (TED), also known as thyroid-associated orbitopathy (TAO). TED patients with a more severe disease course may require orbital decompression surgery, which often includes removal of orbital fibrous and adipose tissues. This provided an opportunity to study fibroblasts and adipocytes that originate from the orbits of patients with TED. As a control, we used fibroadipose tissue from the superior medial orbital fat pad—a source of fibroblasts that has been shown to be otherwise similar to fibroblasts from the deep orbit.

In this article, we describe our finding that RA treatment of orbital fibroblasts leads to expression and secretion of MCP-1, a cytokine implicated in the pathogenesis of TED. Whereas RA treatment of both control and TED fibroblasts resulted in MCP-1 induction, fibroblasts from TED patients demonstrated increased MCP-1 secretion in response to RA. MCP-1 induction occurred through the NF-κB pathway, as blocking NF-κB inhibited MCP-1 induction. This provides a new biological framework for understanding the susceptibility of orbital tissues to inflammation, which might lead to novel therapies that target the proinflammatory role of the retinoid pathway.

It is important to note that RA-induced cytokine expression occurred in fibroblasts irrespective of their origin, orbital or dermal. Interestingly, both the orbit and the skin are targeted by autoimmune thyroid disease, in the form of the orbitopathy and dermopathy. Severe form of the dermopathy is known as pretribial myxedema. In the skin, RA also plays a critical physiologic role in maintaining dermal appendages and regulating epithelial turnover. Hence, our findings suggest that RA may be a common mechanism through which orbital and dermal tissues are primed de novo for involvement in autoimmune conditions. Indeed, dermatologic inflammatory conditions are extremely common.

Retinoic acid receptors belong to a family of nuclear receptors that include thyroid hormone receptor and vitamin D receptor. Both of these receptors compete with RA receptors for heterodimerization with RXR, a requirement for DNA binding. Interestingly, vitamin D deficiency is an independent risk factor for the development of TED. To test whether vitamin D can block the proinflammatory RA response, we treated OFs with both RA and vitamin D. Indeed, MCP-1 induction was partially inhibited in the RA response, we treated OFs with both RA and vitamin D. Interestingly, 130 genes were differentially expressed in both populations of cells, revealing that the response to RA is a fundamental property of orbital fibroblasts and not a byproduct of the disease state. Future studies will aim to dissect these pathways to identify opportunities at targeted therapy that would safely suppress the proinflammatory RA signals.

In summary, these data provide the first mechanistic explanation for the unique susceptibility of orbital tissues to inflammation. The RA pathway is highly amenable to pharmacologic manipulation, including the use of retinoid analogs. The finding that vitamin D can reduce the inflammatory response in OFs and that vitamin D deficiency is a clinical risk factor suggests that therapy focused on this pathway carries significant promise for preventing orbital inflammation in TED and other inflammatory conditions.

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