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Investigation of factors affecting hyaluronan production of orbital fibroblasts in endocrine orbitopathy

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Introduction

Endocrine orbitopathy (EOP) is an autoimmune inflammation disorder that affects the orbital components, particularly the connective tissue and the extraocular muscles. The primary targets of the autoimmune process within the orbit are the fibroblasts. The pathogenetic link between orbital and thyroid autoimmunity is attributed to the presence of thyroid-stimulating hormone receptor (TSHR) antibodies (TRAb), as TSHR is expressed on both thyroid follicular cells and orbital fibroblasts (OFs). Stimulation of this receptor by autoantibodies initiates signaling pathways that enhance fibroblast proliferation and the production of inflammatory cytokines. Moreover, factors produced by immune cells infiltrating the orbit can both sustain inflammation and stimulate fibroblast activation.

Activated OFs expressing thymocyte differentiation antigen 1 (Thy1, CD90) may undergo differentiation into myofibroblasts, becoming more contractile through cytoskeletal rearrangement. The extracellular matrix (ECM) production of myofibroblasts of orbital origin is also altered. Hyaluronan (HA), a major ECM component produced by fibroblasts, is known for its high water-binding capacity, which contributes to edema formation, adversely affecting disease course in EOP.

Transforming growth factor β1 (TGF-β1) is a key regulator of differentiation towards the myofibroblast phenotype and is also known to govern ECM remodeling. However, the impact of TGF-β1-induced myofibroblast differentiation on HA metabolism and its role in the course of EOP remain unclear.

To treat the underlying autoimmune inflammation of the disease, glucocorticoids (GCs) have been used as a first-line therapy for decades. Thus, we also aimed to elucidate, how these drugs affect the proliferation and HA metabolism of fibroblasts of different origins.

Review of the literature

Epidemiology of endocrine orbitopathy

EOP is an autoimmune disorder that affects orbital tissues. It develops in association with Graves' hyperthyroidism in about 90% of cases, though it can also manifest in euthyroid state or in autoimmune thyroiditis. Approximately 20-30% of patients with Graves' disease develop EOP as an extrathyroidal complication. EOP occurs more frequently in women; however, it may follow a more severe course in men. Its annual incidence is 16 per 100,000 in women and 2.9 per 100,000 in men, with a prevalence rate of 0.25%. Environmental factors, such as smoking, alterations in the gut microbiome, elevated serum cholesterol levels, and deficiencies in vitamin D and selenium, can all influence disease onset. Smoking, in particular, plays a prominent role, increasing the risk of EOP by up to eight times. Additionally, genetic factors, such as polymorphisms affecting the peroxisome proliferator-activated receptor-γ (PPARγ), plasminogen activator inhibitor type 1 (PAI-1) and TSHR genes, may contribute to disease development.

Clinical manifestation, staging and diagnosis of endocrine orbitopathy

One of the most common symptoms of EOP is retrobulbar pressure and pain, often accompanied by exophthalmos (proptosis), double vision, upper eyelid retraction, redness and swelling of the eyelids and conjunctiva, exposure keratopathy or corneal ulcers. In severe cases, dysthyroid optic neuropathy (DON) can also develop. Sight-threatening EOP occurs in only 3-5% of cases. However, even mild forms can significantly impact the patient's quality of life.

The European Group of Graves Orbitopathy (EUGOGO) classifies EOP patients according to disease severity. In mild EOP, quality of life is minimally affected, and immunosuppressive or surgical treatment is unnecessary. Mild EOP involves one or more of the following symptoms: upper eyelid retraction of less than 2 mm, mild involvement of the eyelids and conjunctiva, exophthalmos up to 2 mm above the population average, and little or no diplopia. Corneal involvement can generally be managed with lubricants. In moderate to severe EOP, quality of life is substantially impacted, warranting the consideration of immunosuppressive therapy or surgery. It is characterized by at least one of the following symptoms: upper eyelid retraction greater than 2 mm, moderate to severe eyelid and conjunctival involvement, exophthalmos of at least 3 mm above the population average, and

temporary or permanent double vision. In severe, sight-threatening EOP, optic neuropathy or corneal ulcers are present.

The status of the orbits can be assessed using the American Thyroid Association (ATA) classification, which reflects disease severity and aids in tracking progression. For evaluating the involvement of orbital components and extraocular muscles, at least one MRI scan is recommended. Disease activity can be assessed by MRI and ^{99m}Tc-labeled diethylene-triaminepentaacetic-acid (^{99m}Tc DTPA) single photon emission computed tomography SPECT, as well as through clinical activity scoring (CAS). Another important diagnostic method is the measurement of TRAb.

Treatment of endocrine orbitopathy

The treatment of EOP is currently based on the guidelines of the European Group on Graves' Orbitopathy (EUGOGO) and the joint statement of the European and American Thyroid societies. In mild cases, selenium supplementation may have a beneficial effect on EOP progression. EOP is associated most commonly with Graves' hyperthyroidism. Both thyrostatic drugs and surgery are options for treating hyperthyroidism. In moderate to severe EOP accompanied by elevated TRAb levels, surgical treatment of hyperthyroidism is preferred. The treatment of hyperthyroidism with radioactive iodine may lead to worsening of the pre-existing EOP, and may potentially induce endocrine orbitopathy in individuals with high risk. For moderate to severe, immunologically active EOP, immunosuppressive therapy is required. The first-line treatment is intravenous GCs, typically methylprednisolone with a cumulative dose of 4.5 g (500 mg weekly for six weeks, followed by 250 mg weekly for another six weeks). Immunosuppressive therapy is most effective when the immunological process is at its peak. Initially, GCs were mainly used for their immunosuppressive effects, but they have also been shown to inhibit HA production, a key factor in the pathogenesis of EOP, but it has not been examined in OFs. Orbital irradiation, a cumulative dose of 20 Gy per orbit, administered in 10 sessions of 2 Gy to both orbits, is an additional option, which may be combined with GCs.

In severe, sight-threatening cases of EOP, high-dose GC treatment is necessary. If no improvement is seen within two weeks or if vision deteriorates despite treatment, urgent orbital decompression, involving the removal of the bony orbital wall, is recommended.

Pathogenesis of endocrine orbitopathy

The activation of orbital fibroblasts

In the pathogenesis of EOP, the primary targets of the autoimmune process are fibroblasts located within the orbital tissues. The autoantigen responsible for linking thyroid and orbital autoimmunity is the TSHR, as this receptor is expressed on both the follicular cells of the thyroid and on OFs. Antigen-presenting cells (APCs) internalize and degrade the TSHR, presenting its peptides in association with MHC class II molecules to helper T cells. These T cells subsequently activate autoreactive B cells through CD154–CD40 interactions, promoting the production of TSHR-specific antibodies. Alternatively, autoreactive T cells, influenced by chemotactic factors, migrate to the orbital tissues, where they interact with the CD40 proteins on the surface of fibroblasts through their CD154 ligand, initiating fibroblast activation.

Insulin-like growth factor-1 receptor (IGF1R) has been shown to form a physical and functional complex with TSHR, and the simultaneous activation of these two receptors enhances the proliferation of OFs. Stimulation of TSHR by autoantibodies activates signaling pathways —such as PKA/CREB, mTOR, AKT, and NF-κB — that increase orbital fibroblast proliferation and the production of inflammatory mediators like IL-6 and IL-8. IGF1R, in turn, activates PI3K/AKT, Ras/Raf/MEK, and IRS signaling pathways, which promote proliferation, inflammation, adipogenesis, and fibrosis. Furthermore, IGF1R/TSHR signaling was also found to stimulate glycosaminoglycan production.

Factors produced by immune cells infiltrating the orbit also contribute to fibroblast activation in addition to maintaining the inflammatory state. IL-6, produced by both immune cells and fibroblasts, activates the JAK/STAT3 and PI3K/AKT signaling pathways, thus promoting cell proliferation and inflammatory cytokine production. IL-1β from activated macrophages enhances fibroblast proliferation and HA production via the JNK, p38, and AP-1 pathways. IL-15, derived from dendritic cells, monocytes, and macrophages, supports B- and T-lymphocyte activation, while IL-17, an inflammatory cytokine secreted by Th17 cells, induces chemokine production, promoting immune cell infiltration into the orbit.

While 15d-PGJ2, PGE2, and PGD2 are produced by T-lymphocytes, macrophages, and activated mast cells contribute to tissue volume increase by enhancing adipogenesis, inflammatory cytokine production, and ECM production.

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine that induces the expression of cell adhesion molecules (intracellular adhesion molecule - ICAM, vascular cell

adhesion molecule - VCAM), and plays a role in the regulation of cell proliferation, differentiation, and apoptosis. TNFα can indirectly promote the production of prostaglandins through the activation of cyclooxygenase-2 (COX-2) and activate the nuclear factor kappa B (NF-κB) signaling pathway, that regulates the expression of inflammatory mediators such as interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and platelet-derived growth factor BB (PDGF-BB) via binding to cell surface receptors.

OFs also actively contribute to maintaining the autoimmune inflammatory state. Through their expression of CD40, they are able to interact with the CD154 ligand on CD4+ T cells, which results in increased proliferation and production of inflammatory cytokines (such as interleukin-1 - IL-1 and interleukin-6 - IL-6). Interferon-γ (IFN-γ) secreted by autoreactive T lymphocytes promote the synthesis of HA and prostaglandin E2, while IL-2 plays an important role in the cell-mediated immune response.

OFs can be divided into two subtypes, $Thy1^-$ ($CD90^-$) and $Thy1^+$, based on the expression of the CD90 glycoprotein. The $Thy1^-$ fibroblasts – also called preadipocytes – have the ability to differentiate into adipocytes. These cells also express high levels of PPARγ, which promotes adipogenesis and inhibits the persistence of autoimmune inflammation. On the other hand, $Thy1^+$ fibroblasts differentiate into myofibroblasts under the influence of TGF-β.

Fibroblast to myofibroblast transition

Based on their origin, myofibroblasts can be divided into bone marrow-derived and non-bone marrow-derived cells. Bone marrow-derived myofibroblasts originate from circulating mesenchymal stem cells or CD34+ fibrocytes, while non-bone marrow-derived myofibroblasts can arise from fibroblasts, epithelial, endothelial, myoepithelial, smooth muscle, adipocytes, pericytes, or epithelial tumor cells.

For fibroblast cells to undergo differentiation into myofibroblasts, three main criteria must be met: the presence of biologically active TGF-β1, extracellular stress (derived from changes in the mechanical properties of the ECM and EDA-FN/integrin interactions), and the production of certain phenotype modulators (e.g., EDA–FN, HA). The temporary state, where activated OFs exhibit increased proliferation, migration, ECM synthesis, and cytokine release, without yet showing α smooth muscle actin (αSMA) positivity, is referred to as the proto-myofibroblast stage.

Proto-myofibroblasts are characterized by the rearrangement of the actin cytoskeleton, where G-actin monomers polymerize into F-actin stress fibers. This enables connections with

other cells and ECM components through cadherin-based adhesion and integrin-containing membrane complexes. Cadherins are calcium-dependent adhesion molecules on the cell surface, that link to the actin cytoskeleton via proteins of the catenin family. Integrins are transmembrane receptors that can interact with ECM components; α5β1 integrin can bind to fibronectin, while α2β1 integrin binds to collagen.

The formation of αSMA-positive stress fibers signifies the presence of mature myofibroblasts. In addition, mature myofibroblasts exhibit higher contractility and elevated expression of fibronectin and collagen.

The development of the myofibroblast phenotype is influenced by three main signaling pathways: the HA/CD44/EGFR, Wnt/β-catenin and the TGF-β1/Smad pathways.

In the HA/CD44/EGFR signaling pathway, CD44 acts as a co-receptor by binding HA and recruiting epidermal growth factor receptor (EGFR) into the lipid rafts, initiating biphasic signaling processes via the extracellular signal-regulated kinase 1/2 (ERK1/2) and calmodulin-dependent protein kinase II CaMKII pathways. Early signaling events enhance the expression of genes related to differentiation, while later events stimulate the expression of genes associated with proliferation. TGF-β1 enhances the synthesis of HA, thereby further influencing the HA/CD44/EGFR signaling pathway.

In the Wnt/β-catenin pathway, In the absence of Dickkopf-related protein 1 (DKK1), the Wnt ligand simultaneously binds to the extracellular domain of the Frizzled (FZD) receptor and the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) coreceptors. When the Wnt ligand binds, the FZD receptor and the LRP5/6 coreceptor form a complex. Then the Wnt ligand indirectly phosphorylates LRP5/6 by initiating a signaling process that activates the Disheveled (DVL) protein, as well as recruits and activates casein kinase (CK1γ) and glycogen synthase kinase-3β (GSK-3β) kinases. The Wnt/FZD/LRP signaling pathway, mediated by DVL, inhibits the β-catenin inhibitory complex, thus promoting β-catenin accumulation and its translocation into the nucleus, where it induces the expression of pro-fibrotic genes. TGF-β1 also activates the Wnt signaling pathway by the inhibition of DKK1, further supporting myofibroblast differentiation.

The role of TGF-β signaling in myofibroblast differentiation

TGF-β is a 25 kDa polypeptide homodimer that plays a crucial role in cell proliferation, differentiation, motility, apoptosis, and ECM production. It has three main isoforms: TGF-β1, TGF-β2, and TGF-β3. Although fibroblasts and macrophages can produce all three, TGF-β1 is the dominant isoform. TGF-β is synthesized in its precursor form, which contains a homodimer and a pro-peptide. After synthesis, the TGF-β homodimer interacts with the Latency-Associated Peptide (LAP) to form the small latent complex (SLC), which remains intracellular, until the large latent complex (LLC) is formed. During this process, the Latent TGF-β-Binding Protein (LTBP) binds to the complex, and is then translocated into the extracellular space. TGF-β activation occurs through enzymatic and mechanical effects, such as by proteases or acidic pH conditions that lead to the denaturation of LAP. Additionally, thrombospondin-1 (TSP-1) and αV integrins are also capable of directly activating latent TGF-β. The TGF-β ligand released from the complex then binds to TGF-β RII, which then associates with TGF-β RI to form the functional receptor. While TGF-β RIII, also known as betaglycan, is capable of presenting TGF-β to TGF-βRII, thus promoting TGF-β signaling.

In the differentiation of fibroblasts into myofibroblasts, the TGF-β SMAD-dependent pathway plays a key role. Upon receptor activation, SMAD2 and SMAD3 are phosphorylated and form an oligomer with SMAD4. The SMAD complex translocates to the nucleus, where it regulates the transcription of genes, such as collagen (COL1A1), fibronectin (FN), and α-SMA, which serve as markers for myofibroblast differentiation. I-SMADs, such as SMAD7, inhibit the interaction between R-SMADs and the active receptor, reducing the number of receptors on the cell surface and acting as transcriptional repressors in the nucleus.

The role of hyaluronan in the pathogenesis of endocrine orbitopathy

The differentiation of OFs into myofibroblasts and the subsequent change in their ECM production is a significant factor in the pathogenesis of EOP. The overproduction of HA, a non-sulphated glycosaminoglycan polymer, that is a major component of the ECM, significantly contributes to tissue edema, due to its high water retention capacity.

The HA biosynthesis process is mediated by three transmembrane glycosyl-transferase isoenzymes, HA synthases (HAS) 1, 2, and 3, which construct the HA chain from UDP-glucuronic acid and UDP-N-acetylglucosamine as substrates. HAS1 synthesizes HA chains of a wide range ($2x10^5$-$2x10^6$ Da), HAS2 synthesizes larger polymers (>$2x10^6$ Da), and HAS3 creates shorter chains (<$3x10^5$ Da). After synthesis, the HA chains, anchored via the synthases to the cell surface or bound to cell surface receptors, remain in the pericellular space until released from the binding.

The hyaluronidases responsible for degradation can be classified into six subfamilies. In mammalian tissues, the enzymes HYAL1, found in lysosomes, and HYAL2, associated with the plasma membrane, are responsible for HA degradation. HYAL2 breaks long-chain HA into smaller oligosaccharides of 10-20 kDa. These HA units bind to cell surface HA receptors and enter the cell via endocytosis, where they are further degraded by HYAL1.

Recently, two new molecules have been identified that play a role in HA degradation: the cell migration-inducing protein (CEMIP/KIAA1199) and transmembrane protein-2 (TMEM2). CEMIP facilitates HA depolymerization through clathrin-mediated endocytosis, while TMEM2 regulates HA production through inhibition of CEMIP.

HA not only functions as a structural element but also plays a role in cell-ECM and cell-cell interactions. Among the cell surface receptors that bind HA, the cluster determinant 44 (CD44) is the most significant. Through its interaction with HA, it plays a role in numerous physiological and pathological processes, such as the retention of the pericellular matrix, cell-matrix signaling, cell migration, and the formation of cell-cell adhesion.

Other receptors, such as RHAMM (receptor for hyaluronan mediated motility), LYVE-1 (lymphatic vessel endothelial HA receptor), HARE (hyaluronan receptor for endocytosis), and toll-like receptor-4 (TLR-4), are also capable of interacting with HA. Meanwhile, the interactions between HA and HA-binding proteins, such as aggrecan and versican, play a role in cell-cell interactions and the regulation of the mechanical properties of tissues.

Objectives

- Investigating the effect of TGF-β1-induced myofibroblast differentiation on HA metabolism, the significance of which in the pathogenesis of EOP is not yet fully understood.
- To investigate the impact of GCs, as first-line therapies, on the proliferation and HA production of OFs, and mRNA expression of HA synthases and hyaluronidases under basal and PDGF-BB stimulated conditions.

Methods

Tissue samples and cell cultures

After informed consent was obtained from the patients according to the study protocol approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen (ID: 5913/2012/EKU (84/13)), orbital connective tissue explants were acquired during the decompression surgery of patients with EOP (n=7) and from connective tissue explants obtained from eye surgeries performed on non-EOP patients (n=8) for indications unrelated to thyroid disease. Cultures of dermal fibroblasts (DFs) (n=4) were established from skin tissue removed during abdominal hernia surgery.

During the course of the disease, all patients received thyreostatic medication and all patients received at least one 12-week course of i.v. GC. At least one year has passed between orbital surgery and the completion of GC treatment. Median time since the onset of EOP was 4 (min: 1, max: 9) years. In the two months prior to surgery, the patients with EOP were only receiving β-blockers, diuretics, and thyroid hormone replacement. At the time of surgery, the patients had low-normal or suppressed TSH levels, as well as high-normal thyroid hormone levels within the reference range. All patients were in the inactive phase of EOP at the time of surgery (CAS $\leq$ 3 on the 10 item scale).

OFs were cultured in accordance with the description by Bahn et al. The tissues were sliced into pieces measuring approximately 2x2 mm and seeded into culture dishes containing Medium 199 with Earle's salts with 20% (v/v) FBS, stable glutamine, and penicillin-streptomycin solution (Biosera, Nuaille, France). The cells were then cultured at 37°C, 5% CO2, in a humidified incubator. Every 3 to 4 days, the medium was changed. Resulting monolayer outgrows of adherent fibroblasts were serially passaged with TrypLE Express (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) dissociating reagent and cultured in Medium 199 with 10% (v/v) FBS under the same conditions. After reaching confluence, the fibroblasts were treated with TrypLE Express and passaged into T75 flasks. They were treated again with TrypLE Express, and after centrifugation (1000 g), the pellet was suspended in freezing medium and stored in liquid nitrogen until the experiments were performed. Since our experiments were conducted on primary cell cultures, we used cultures with a low passage number, between 2 and 6.

Treatments

For the experiments, fibroblast cells were seeded at a density of 1.56×10^4 cells/cm² in 24- or 96-well plates. To examine the effects of myofibroblast differentiation on HA metabolism, treatments were performed using DMEM High Glucose medium supplemented with 10% (V/V) FBS and stable glutamine and containing TGF-β1 5 ng/ml (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The cells were then incubated for an additional 24 or 72 hours.

To investigate the effects of GCs (Sigma Aldrich, St. Louis, MO, USA), treatments were performed using hydrocortisone (HC), prednisolone (P), methylprednisolone (MP), and dexamethasone (DEX) in a concentration range of 0.0001–1.0 μM, then in 1 μM concentration for 24 hours, later on, with or without PDGF-BB 10 ng/ml (R&D Systems, Bio-Techne, Minneapolis, MN, USA), 24 hours stimulation, on non-EOP and EOP OFs as well as on DFs.

Cell proliferation assay

The assay was carried out in 96-well plates per the manufacturer's (Roche, F. Hoffmann-La Roche Ltd, Basel, Switzerland) instructions. Cell cultures were treated for 2 hours with a 5-bromo-2′-deoxyuridine (BrdU) solution. BrdU is a synthetic analog of thymidine, which gets incorporated into DNA in place of thymidine during DNA synthesis. The cells were then fixed for 30 minutes using FixDenat solution, followed by addition of peroxidase-conjugated anti-BrdU antibody for 90 minutes. This was followed by an enzymatic color reaction by the addition of 3,3′,5,5′-tetramethylbenzidine substrate for 10 minutes; the intensity of the color change is proportionate to the amount of incorporated BrdU. Finally, a Beckman Coulter, DTX 880 Multimode Detector (Beckman Coulter Inc., Brea, CA, USA) was used to detect absorbance at 450nm (reference wavelength: 620 nm). The optical density (OD) values are reported as OD/well, with cells plated at a density of 5000 cells per well.

Metabolic activity assay

To evaluate the effect of applied treatments on metabolic activity, OFs were seeded into 96-well culture plates (5×10^3 cells/well). After treatment, cells were incubated with 0.5 mg/mL 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution for 4

hours at 37°C. Supernatants were carefully removed, and the adherent cells were dissolved in 100 µl/well of DMSO (Merck KGaA, Darmstadt, Germany). Cells are able to convert the MTT reagent, a yellow-colored tetrazolium salt, into a formazan derivative in proportion to their metabolic activity. Hence, the more metabolically active cells present in the sample, the more formazan is produced. Absorbance of the dye was measured at 595 nm, with background subtraction at 660 nm, using a Synergy H1 microplate reader (Agilent Technologies Inc. Santa Clara, CA, USA). In the MTT assay, the OD reflects the metabolic activity of the cells and is indirectly proportionate to the number of viable cells. The OD values are reported as OD/well, with cells plated at a density of 5000 cells per well.

Quantitation of hyaluronan

OFs were plated in 96-well plates. After each experiment, in order to quantify the amount of HA produced and released into the media, we collected conditioned media from the treated cells directly and assayed using a DuoSet Hyaluronan Kit (R&D Systems, Bio-Techne, Minneapolis, MN, USA).

To selectively measure the amount of HA retained in the pericellular coat, the cells were washed twice with DPBS to remove residual media and any unbound extracellular components, then treated with 0.05% (w/v) trypsin–EDTA solution at 37°C for 20 minutes to degrade extracellular matrix proteins and release cell-associated HA. To inactivate trypsin, FBS was added to a final concentration of 10% V/V. After centrifugation at 1000g for 5 minutes, HA was measured in the supernatants.

The absorbance was measured at 450 nm (reference wavelength: 620 nm) using a Beckman Coulter DTX 880 Multimode Detector. The amount of HA was expressed in ng/ml of culture medium or ng/well.

Hyaluronan isolation

Supernatants were collected and stored at -20°C until HA isolation was performed. For protein digestion 110 µL 1 mg/mL Proteinase K (Merck KGaA, Darmstadt, Germany) in 20 mM Tris buffer (Merck KGaA, Darmstadt, Germany) with 1% SDS was added to 1 mL supernatant and incubated at 60°C for 4 hours. For HA precipitation 4 ml of pre-chilled 96% ethanol (VWR International, Avantor, Radnor, PA, USA) was added and incubated at -20°C overnight. Next day the samples centrifuged at 14000 g for 10 minutes. The pellet was washed with pre-chilled 75% ethanol and centrifuged again at 14000 g for 10 minutes. Air

dried pellets were resuspended and collected in TBE buffer (Merck KGaA, Darmstadt, Germany) and placed to 100°C for 3 minutes, then stored in refrigerator overnight.

Hyaluronan gel-electrophoresis

We used a modified agarose gel electrophoresis method based on Cowman's technique. Briefly, the gel was pre-run for 10 hours at 40 V, then isolated HA samples and standards - HA solutions with different molecular weight (R&D Systems, Bio-Techne, Minneapolis, MN, USA)- were loaded in Bromophenol Blue loading buffer (0.02% bromophenol with 2 M sucrose in TBE (Merck KGaA, Darmstadt, Germany) and run in 1.5 % agarose gel in TBE buffer (Tris/boric acid/EDTA) for 0.5 h at constant 20 V and then for 3.5 h at constant 40 V. Immediately after the run, the gel was placed into 30 % ethanol for 1 hour. The gel was stained overnight in Stains All (0.00125 % in ethanol). Destaining the gel was performed in dark for 30 minutes in 10 % ethanol and then in water. To remove the purple background, the gel was left on the benchtop. Afterwards, the gel was scanned, and the densitometric analysis of the obtained images were performed using Image Studio Digits Ver. 5.2 (LI-COR Biotechnology – GmbH, Bad Homburg, Germany). Summarized results were obtained using values of consecutive regions from the densitometric profiles of the relevant samples of each OF culture.

Real-time polymerase chain reactions (RT-PCR)

After the 24- and 72-hour treatments, the supernatant was removed from the cells, and the cells were lysed in TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). The samples were stored at -20°C until RNA isolation. The thawed samples were homogenized, and 100 µl of chloroform (Merck KGaA, Darmstadt, Germany) was added. The total RNA in the suspension was extracted with chloroform. Then samples were centrifuged for 15 minutes at 16,000 g and the upper phase was pipetted into a new DNase- and RNase-free tube, avoiding contact with the interphase. To precipitate the RNA from the aqueous phase, 375 µl of room temperature 100% isopropanol (VWR International, Avantor, Radnor, PA, USA) was added, followed by centrifugation for 10 minutes at 16,000 g. The supernatant was removed, and the pellet was washed with 1 ml of cold 75% ethanol followed by a final centrifugation step at 12,000 g for 10 minutes. After removing the supernatant, the isolated RNA was dissolved in nuclease-free water (Applied Biosystems, Thermo Fisher Scientific,

Waltham, MA, USA), and its concentration was measured using the Synergy H1 Microplate Reader. RNA samples were stored at -20°C until reverse transcription. The purified RNA samples were converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

Finally, the mRNA expressions of the genes of interest (ACTA2, CEMIP, FN, GAPDH, HAS1, HAS2, HAS3, HYAL1, HYAL2, TMEM2, COL1A1) were detected using TaqMan Gene Expression Assay (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR reaction was performed on the CFX Opus 96 Real-Time PCR System. To quantify gene expression levels, the ΔCT method was used, and the expressions of target genes were normalized to the mRNA level of the housekeeping gene, GAPDH.

Statistical analysis

The statistical analysis of the data obtained during the experiments was performed using GraphPad Prism 10 (GraphPad, Boston, MA, USA) or STATISTICA 14.0 software (TIBCO Software Inc., Palo Alto, CA, USA). Repeated measures analysis of variance (ANOVA) was performed, followed by Tukey or Dunnett's post hoc tests, where the applied treatment was considered as the within subject factor and origin and the duration of treatment as between subject factors.

In all experiments level of statistical significance was set at $p<0.05$. Data were expressed as mean ± standard error of the mean (SEM).

Results

The effect of myofibroblast differentiation on hyaluronan metabolism

Alterations in the expressions of genes serving as markers of myofibroblast differentiation indicate that fibroblast cells underwent a transdifferentiation process in response to stimulation with TGF-β. The mRNA expression of αSMA and fibronectin was similar in both type of untreated OFs (EOP and non-EOP) at both 24 and 72 hours. TGF-β increased the αSMA expression at 24 and 72 hours in both groups ($p < 0.0001$). COL1A1 expression increased over time in untreated EOP OFs ($p<0.01$) and increased further after 24- and 72-hour TGF-β treatment in non-EOP and EOP OFs as well (EOP-OFs 24 hours: $p < 0.001$; EOP-OFs 72 hours: $p < 0.001$, non- EOP OFs 24 hours: $p < 0.01$, non-EOP OFs 72 hours: $p < 0.01$). In EOP OFs fibronectin expression was increased during TGF-β treatment at

both time points (EOP-OFs 24 and 72 hours: $p < 0.01$), while only a tendency to increase was observed in non-EOP OFs. The effect of TGF-β on the expression of these markers was even more pronounced over time.

Treatment with TGF-β did not affect the proliferation rate of OFs at 24 hours. However, after 72 hours despite a decrease in the baseline proliferation rates of both EOP ($p<0.0001$) and non-EOP OFs ($p<0.0001$), the proliferation rates observed in TGF-β-treated cultures remained higher compared to untreated cultures.

Metabolic activity of OFs did not change in the first 24 hours of TGF-β treatment, however a modest increase was observed after 72 hours (non-EOP OFs: $p < 0.001$, EOP-OFs $p < 0.01$).

The quantity of HA released into the media was not affected by TGF-β. However, a 6,1-fold and a 4,7-fold increase were detected in pericellular HA levels after 72 hours of TGF-β treatment of OFs and EOP-OFs, respectively ($p < 0.0001$).

TGF-β treatment markedly increased HAS1 mRNA expression in both EOP and OFs ($p < 0.0001$), while no changes were observed in HAS2 and HAS3 mRNA expression.

HYAL1 expression increased with time; this increase in HYAL1 expression has been completely abolished by TGF-β induced transdifferentiation towards myofibroblast phenotype ($p < 0.0001$). TGF-β treatment did not affect HYAL2 mRNA expression.

TGF-β increased the mRNA expression of TMEM2 (non-EOP OFs 24 hours: $p < 0.001$; EOP-OFs 24 hours: $p < 0.01$, non-EOP OFs 72 hours: $p < 0.0001$, EOP-OFs 72 hours: $p < 0.001$) whereas that of CEMIP decreased at both time points in OFs.

The molecular weight of HA fractions appearing in the media was examined using gel-electrophoresis. We have found that the proportion of high molecular weight HA (HMW-HA) had a tendency to increase as a result of TGF-β induced transdifferentiation.

The effect of glucocorticoids on cell proliferation and hyaluronan metabolism

Our other objective was to investigate how GCs used in the treatment of EOP affect the proliferation rate, HA production, and the mRNA expression of HA synthases and hyaluronidases in fibroblasts of different origins, both independently and following PDGF-BB stimulation.

The lowest concentrations of the different GCs with maximal inhibitory effect on HA production of OFs were tested in the range of 0.0001–1.0 μM concentration. Both methylprednisolone and dexamethasone exerted a significant decreasing effect on the HA

secretion in 0.01 μM concentration (MP: $p < 0.01$, DEX: $p < 0.001$). In contrast, the inhibitory effects of prednisolone and hydrocortisone were observed at 0.1 μM (P: $p < 0.001$, HC: $p < 0.01$). Since GCs exerted maximal inhibitory effects at a concentration of 1.0 μM in all fibroblast cultures tested (MP: $p < 0.001$, DEX: $p < 0.001$, P: $p < 0.001$, HC: $p < 0.01$), this concentration was used in further experiments.

HA production of untreated OFs and DFs did not differ. GC treatment markedly decreased HA production ($p < 0.0001$) and the degree of inhibition did not depend on the origin of the cells. Regardless of the baseline HA concentration, all applied GCs were effective ($p < 0.0001$).

The HAS1 expression of fibroblasts was not affected by the origin of the cells. Although HAS1 expression of OFs had a tendency to be increased under the effect of GCs, the Dunnett's test showed that HC was the only GC that caused a significant increase in HAS1 expression of EOP-OFs.

The expression of HAS2 and the effect of GC treatment on that were not affected by the origin of the OFs. GC treatment significantly reduced HAS2 mRNA levels in OFs ($p = 0.009$). The baseline expression of HAS2 was higher in DFs compared to OFs ($p = 0.005$). HAS2 expression was reduced by all types of GC used ($p < 0.0001$), except for HC in EOP-OFs.

Expression of HAS3 did not depend on whether the cells originated from EOP or non-EOP orbital connective tissue. HAS3 expression of DFs and OFs did not differ. Each GC reduced HAS3 expression in all cases, regardless of origin (HC: $p < 0.0005$, P: $p < 0.0001$, MP: $p < 0.0005$, DEX: $p < 0.0001$).

HYAL1 mRNA expressions were too low in DF cultures to accurately measure its baseline and post-treatment expression. HYAL1 mRNA of OFs decreased in response to all types of GCs tested (HC: $p < 0.0007$, P: $p < 0.0062$, MP: $p < 0.05$, DEX: $p < 0.002$).

The baseline HYAL2 expression of EOP-OFs, OFs and DFs was not different. GCs affected neither OFs nor DFs. The lack of effect was independent from the origin of cells.

The origin of the cells did not affect the response to PDGF-BB treatment, and PDGF-BB increased HA production in all cultures ($p < 0.0001$). GC treatment reduced HA production even under PDGF-BB- stimulated conditions ($p < 0.0001$), and the effect was cell-origin-dependent ($p = 0.004$); it was more intense in DFs. All types of GCs were able to decrease the PDGF-BB-stimulated HA production below the baseline HA production of the cells ($p < 0.0001$).

To investigate if GCs may alter the mRNA expression of HA synthases in the presence of PDGF-BB, we examined the cultures in which the 24 h 10 ng/mL PDGF-BB treatment increased HA synthase expression. PDGF-BB increased HAS1 mRNA levels in three of four EOP-OFs, three of four K-OFs and three of four DFs ($p = 0.022$) to a comparable extent ($p = 0.381$). In OFs, GCs had no effect on PDGF-BB-stimulated HAS1 expression. In contrast, in DFs GCs significantly reduced the stimulated HAS1 expression ($p = 0.048$).

PDGF-BB treatment increased HAS2 expression in OFs ($p = 0.003$) regardless of the origin of the cells. The Dunnett's test showed that only MP and DEX were able to reduce ($p < 0.001$, $p < 0.002$, respectively) PDGF-BB-induced HAS2 expression, while HC and P were not ($p = 0.997$, $p = 0.151$, respectively). In DFs, the effect of PDGF-BB on HAS2 expression was negligible.

PDGF-BB enhanced ($p = 0.049$) HAS3 mRNA expression in OFs (three out of four in EOP OFs, three out of four in K-OFs). Only MP and DEX treatment could decrease the PDGF-BB-induced increase in HAS3 expression ($p = 0.035$ and $p = 0.029$, respectively). The post hoc test showed that MP ($p = 0.044$) and DEX ($p = 0.045$) were able to reduce the HAS3 expression even below the baseline. PDGF-BB had no effect on HAS3 expression in DFs.

HYAL1 mRNA expression decreased after PDGF-BB treatment ($p < 0.003$), in both EOP and non-EOP OFs. GCs had no effect on the reduced HYAL1 expression caused by PDGF-BB. Neither PDGF-BB treatment alone nor in combination with GC influenced HYAL2 expression.

PDGF-BB increased the proliferation rate of every cell line ($p < 0.0001$). None of the GCs studied was able to prevent the PDGF-BB-stimulated proliferation of OFs in 1 μM concentration.

Discussion

In the active phase of endocrine orbitopathy, fibroblasts are the primary targets of the autoimmune process. Activated, $Thy1^+$ OFs may undergo myofibroblast differentiation. TGF-β, which we used in our studies for inducing myofibroblast differentiation, is a multifunctional cytokine that plays a key role not only in cell growth, motility, and matrix remodeling, but also in the induction and maintenance of the myofibroblast phenotype. This is supported by the observation that in severe cases of endocrine orbitopathy, increased TGF-β expression correlates with clinical activity scores. Although myofibroblast transdifferentiation contributes significantly to the remodeling of orbital tissues, its effect on the HA synthesis of OFs is still not fully understood.

In our studies, the mRNA expression of myofibroblast markers (αSMA, COL1A1, fibronectin) increased following TGF-β stimulation, and transdifferentiation towards the myofibroblast phenotype occurred as a result of TGF-β stimulation.

The increased expression of αSMA is responsible for the contractile phenotype and activates the Hippo signaling pathway through YAP/TAZ mechanotransduction proteins. These proteins can interact with the Smad transcription factors involved in canonical TGF-β signaling, which support the nuclear translocation of YAP/TAZ and the expression of target genes in fibroblasts. Inhibition of YAP reduces Smad activity, indirectly modulating the expression of αSMA. Although αSMA is often regarded as a key marker of myofibroblasts, mesenchymal stromal cells from various origins cultured on plastic surfaces also exhibit basal αSMA expression, which can be further enhanced by TGF-β. We also observed this phenomenon in both K-OF and EOP-OF cultures.

COL1A1, the main chain of type I collagen, is abundant in the extracellular matrix. Its expression increased during TGF-β-induced myofibroblast differentiation. A direct interaction can be suggested between COL1A1 expression and YAP/TAZ activity, as silencing YAP inhibits the expression of profibrotic genes such as COL1A1.

Fibronectin is a multifunctional ECM protein, that promotes cell proliferation and migration, contributes to the stabilization of the ECM structure, and facilitates the incorporation of other proteins, such as collagens. Additionally, during orbital tissue remodeling, fibronectin may function as a co-stimulator for infiltrating immune cells, by mediating the release of inflammatory cytokines through activation of toll-like receptors (TLRs). In our experiments, fibronectin expression in EOP-OF cultures significantly

increased after 72 hours of TGF-β treatment, while no changes were observed in the non-EOP cultures.

We observed that the baseline proliferation rate of OFs decreased with time in confluent cultures, which may suggest contact inhibition as a negative regulator. However, after TGF-β1-treatment, this decrease was significantly lower. The MTT conversion rate did not change within 24 hours but increased moderately after 72 hours. Although TGF-β inhibited proliferation in most cell types, under certain conditions, such as in endocrine orbitopathy, where TGF-β had a profibrotic effect, the proliferation of OFs increased. Furthermore, based on the results of Meran's group, we hypothesize that the increase in pericellular HA concentration contributed to maintaining a higher proliferation rate of TGF-β treated cells compared to untreated cells. We demonstrated that the pericellular HA increased, while, in the supernatant, a shift towards high molecular weight HA was found without a change in the total HA production. Along the same line, and in concordance with our previous studies, HAS2 was the predominant synthase in OFs. TGF-β treatment was a potent stimulator of HAS1 expression in OFs, while HAS2 and HAS3 remained at the unstimulated level. The contribution of HAS1 to HA synthesis substantially increased in OFs with myofibroblast phenotype. Albeit HAS1 has lower enzyme activity than other HA synthases, the HAS1 dependent pericellular HA coat can rapidly expand in response to cytokines, which suggest that orbital myofibroblasts expressing HAS1 may achieve higher HA content in the orbital connective tissue during inflammation.

We found that during TGF-β induced transdifferentiation towards the myofibroblast phenotype, HYAL1 and CEMIP expression levels decreased. The increased TMEM2 expression seen in our *in vitro* model may contribute to the decreasing CEMIP expression in response to TGF-β; based on recent studies, which verified that TMEM2 facilitates the accumulation of HA by suppressing CEMIP expression. We conclude that CEMIP and its regulator TMEM2 may have a role in HA accumulation during myofibroblast differentiation in OFs.

Fibroblasts derived from the orbit, whether from TED or non-TED sources, respond similarly to TGF-β treatment regarding their HA metabolism and proliferation rate. As for the myofibroblast markers, αSMA and COL1A1 increased at a similar rate regardless of the origin of the cells, while the increase in fibronectin expression following TGF-β treatment was significant only in TED OFs.

According to the results obtained, we can conclude that during myofibroblast differentiation, the increased expression of HAS1 and TMEM2, along with the decreased

mRNA levels of the hyaluronidases HYAL1 and CEMIP, may be responsible for the accumulation of pericellular HA and the increase in the HMW-HA fraction. While the HA accumulating in the pericellular space, due to its high water-binding capacity, contributes to the volume increase and edema of orbital tissues, it may also enhance fibroblast proliferation and leukocyte infiltration, thereby worsening the progression of EOP. Our observations suggest that hyaluronidases could be potential targets in the treatment of EOP.

The inflammatory mediators and cytokines produced by immune cells infiltrating the orbit and activated fibroblasts, such as TNF-α, IFN-γ, IL-1β, IGF-1, as well as TGF-β and PDGF not only contribute to sustaining the inflammatory state but also enhance the ECM synthesis of fibroblasts, contributing to the remodeling of orbital tissues. We demonstrated that GCs, used as a first-line treatment, are effective in treating EOP not only due to their immunosuppressive effects but also because they can directly influence HA production by OFs, thereby positively impacting the course of the disease.

In our experiments, all 24-hour GC treatments (hydrocortisone, prednisolone, methylprednisolone, and dexamethasone) reduced HA production of fibroblast cells, regardless of origin, which finding is further supported by the decreased expression levels of HAS2 and HAS3.

Gebhardt et al. demonstrated that after dexamethasone treatment, HA production in DFs decreased, while the mRNA levels and enzymatic activity of HYAL1 and HYAL2 remained unchanged. In contrast, other studies have observed a decrease in HYAL1 expression in mouse macrophages following dexamethasone treatment, which aligns with our observations as well. We suggest that the reduction in HA production primarily results from changes in HAS2 and HAS3 expression, while the decrease in HYAL1 levels likely has a lesser impact.

PDGF-BB, known for its stimulatory effect on HA synthesis, consistently enhanced both HA production and cell proliferation. In our experiments, none of the GC treatments affected the proliferation rates or influenced the PDGF-BB-induced increase, confirming that GCs do not exert their effects through fibroblast proliferation. Additionally, we observed that all applied GC treatments — whether administered alone or in the presence of PDGF-BB — reduced the HA production of fibroblasts.

GCs could possibly influence the effects of PDGF-BB in multiple ways. Beyond their in vivo immunosuppressive effect, which inhibits T-cell activation and infiltration, the activated glucocorticoid receptor complex directly interacts with the NF-κB p65 subunit,

blocking the transcription of inflammatory genes and thereby reducing inflammation and PDGF-BB expression.

Furthermore, GCs affect cellular energy balance through phosphorylation of AMP-activated protein kinase (AMPK). AMPK activation indirectly inhibits HA synthesis by reducing the availability of essential precursors, such as UDP-N-acetylglucosamine and UDP-glucuronic acid.

In line with previous findings, we observed that orbital and DFs display distinct HAS mRNA expression patterns and respond differently to PDGF-BB treatment. PDGF-BB increased HAS2 and HAS3 expression in both EOP and K-OF cells, whereas this effect was not observed in DFs. GC treatments significantly reduced HAS2 mRNA levels in all fibroblast cultures examined, except for hydrocortisone, which had no effect on HAS2 expression in EOP OFs. Only methylprednisolone and dexamethasone were able to inhibit the PDGF-BB-induced increase in HAS2, while all GCs reduced HAS3 expression effectively.

Our results suggest GCs as a first-line treatment, are effective in treating EOP not only due to their immunosuppressive effects. The excessive accumulation of HA, primarily due to the activation of OFs and their differentiation toward a myofibroblast phenotype, significantly contributes to edema and the development of characteristic eye related symptoms. By reducing HA production through the inhibition of synthase enzymes, GCs can positively impact the course of EOP and improve patients' quality of life.

New Findings

1. During myofibroblast differentiation, the increased expression of HAS1 and TMEM2, along with the decreased mRNA levels of the hyaluronidases HYAL1 and CEMIP, are responsible for the accumulation of pericellular HA and the shift towards the HMW-HA fraction.

2. The increased HA production of OFs may play a role in the TGF-β-induced proliferation observed in this cell type.

3. GCs used in the treatment of EOP reduce HA production in both OFs and DFs even after PDGF-BB stimulation. This effect is attributed to the inhibition of HAS2 and HAS3 expressions.

4. GCs do not affect the proliferation of OFs and are unable to mitigate the proliferation-enhancing effects of PDGF-BB.

Summary

The differentiation of OFs into myofibroblasts, primarily driven by TGF-β1, is a key factor in the pathogenesis of EOP. Myofibroblasts are characterized by enhanced ECM production, with HA being one of its major components. Due to its high water-binding capacity, HA significantly contributes to tissue edema, thereby facilitating the progression of EOP. We investigated the impact of myofibroblast differentiation on HA metabolism of OFs, as well as the effect of GCs on fibroblast proliferation and HA metabolism.

Following TGF-β1 stimulation, OF cells underwent myofibroblast transdifferentiation. The proliferation rates of the cultures decreased with time, although this was less pronounced in TGF-β1-treated cells at 72 hours. We found increased amount of pericellular HA, while the HA released into the supernatant remained unchanged compared to untreated cells. TGF-β1 enhanced HAS1 expression at both time points examined, whereas HYAL1 and CEMIP expressions were reduced following differentiation. In contrast, TMEM2 expression was significantly elevated after 72 hours of TGF-β1 treatment. Additionally, we found that the proportion of HMW-HA increased following TGF-β-induced transdifferentiation.

None of the GCs affected fibroblast proliferation, nor did they decrease the PDGF-BB-induced increase in proliferation. Hydrocortisone was the only GC that increased HAS1 expression, while all GCs reduced HAS2 and HAS3 mRNA expression. DFs exhibited the highest baseline levels of HAS2. HYAL1 expression consistently decreased in response to GC treatment, while HYAL2 expression remained unaffected. GCs reduced HA production in all fibroblast cultures irrespective of origin, even in the presence of PDGF-BB, with a more pronounced effect in DFs. Only methylprednisolone and dexamethasone could significantly reduce the PDGF-BB-induced increase in HAS2 and HAS3 expressions, while none of the GCs affected PDGF-BB-stimulated HAS1 expression. Furthermore, none of the GCs was able to inhibit the reduction in HYAL1 expression induced by PDGF-BB treatment.

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