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Research paper

Cyclosporin A inhibits PDGF-BB induced hyaluronan synthesis in orbital fibroblasts

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ABSTRACT

Orbital connective tissue changes are contributors to the pathogenesis in thyroid eye disease (TED). Activated fibroblasts respond to immune stimuli with proliferation and increased hyaluronan (HA) production. Cyclosporin A (CsA) was reported to be beneficial in the treatment of TED. PDGF isoforms are increased in orbital tissue of TED patients and enhance HA production. We aimed to study the effect of CsA on HA production and hyaluronan synthase (HAS1, 2 and 3) and hyaluronidase (HYAL1 and 2) mRNA expressions in orbital fibroblasts (OFs). Measurements were performed in the presence or absence of CsA (10 μM) in unstimulated or PDGF-BB (10 ng/ml) stimulated OFs. The HA production of TED OFs ($n = 7$) and NON-TED OFs ($n = 6$) were measured by ELISA. The levels of mRNA expressions were examined using RT-PCR. The proliferation rate and metabolic activity were measured by BrdU incorporation and MTT assays, respectively. Treatment with CsA resulted in an average 42% decrease in HA production of OFs ($p < 0.0001$). CsA decreased the expression levels of *HAS2*, *HAS3* and *HYAL2* ($p = 0.005$, $p = 0.005$ and $p = 0.002$, respectively.) PDGF-BB increased HA production ($p < 0.001$) and *HAS2* expression ($p = 0.004$). CsA could reduce the PDGF-BB-stimulated HA production ($p < 0.001$) and *HAS2* expression ($p = 0.005$) below the untreated level. In addition, CsA treatment caused a decrease in proliferation potential ($p = 0.002$) and metabolic activity ($p < 0.0001$). These findings point to the fact that CsA affects HA metabolism via *HAS2*, *HAS3* and *HYAL2* inhibition in OFs. In addition to its well characterized immunosuppressant properties, CsA's beneficial effect in TED may be related to its direct inhibitory effect on basal and growth factor stimulated HA production.

1. Introduction

Thyroid eye disease (TED) is an autoimmune process directed against orbital components and a common extrathyroidal manifestation of Graves' disease (GD). The most common symptoms are discomfort or pain behind the eye, conjunctival injection, diplopia and proptosis, the latter resulting from swelling of the extraocular muscles and expansion of the orbital connective tissue [1]. The presence of autoantibodies produced against the thyroid stimulating hormone receptor (TSHR) are responsible for the hyperthyroidism, as well as cell activation and tissue remodeling in the orbit [2]. Immune cells infiltrate the orbit and produce many factors such as cytokines, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), transforming growth beta (TGF-β), platelet derived growth factor isoforms (AA, AB and BB, with BB being the dominant), among others; these activate orbital fibroblasts (OFs) [3–5]. Fibroblasts respond to activation with increased proliferation and overproduction of extracellular matrix (ECM) components, including hyaluronan (HA) [6]. HA can hold a large amount of water, thereby resulting in tissue oedema [7]. Three isoforms of hyaluronan synthase (HAS1, HAS2 and HAS3) and two main degrading enzymes (hyaluronidase - HYAL1 and HYAL2) maintain the balance of ECM homeostasis in the tissues [8].

Cyclosporine A (CsA) is a potent non-steroidal immunosuppressive drug [9] and inhibitor of calcineurin [10]. In recent guidelines, addition of oral CsA to glucocorticoids (GCs) is a valid second-line treatment option for moderate-to-severe TED [11,12]. In active TED prednisone

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with CsA was more effective than prednisone monotherapy and a lower relapse rate could be achieved [13].

Earlier we have demonstrated that GCs have a direct beneficial pleiotropic effect in orbital fibroblasts [14]. Currently, there are few studies exploring the effect of CsA on HA synthesis. Its effect on orbital fibroblasts has not been investigated.

2. Materials and methods

2.1. Materials

Medium 199, stable glutamine (S/G), Dulbecco's phosphate-buffered saline (DPBS) and Fetal bovine serum (FBS) were purchased from Biosera (Nuaille, France). TrypLE Express were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Calcineurin inhibitor Cyclosporin A was purchased from Tocris (Bio-Techne, Minneapolis, MN, USA), dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (St. Louis, MO, USA) and Recombinant human PDGF-BB were purchased from R&D Systems (Bio-Techne, Minneapolis, MN, USA), TRI reagent solution was purchased from Molecular Research Center Inc. (Cincinnati, OH, USA). Cell proliferation ELISA BrdU (colorimetric) kits were purchased from Roche (F. Hoffmann-La Roche Ltd., Basel, Switzerland). A High-Capacity cDNA Reverse Transcription Kit and TaqMan Gene Expression Assays were purchased from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Preparing CsA solutions

100 mM CsA stock solution was made in DMSO according to the manufacturer's instruction. Working solutions in DMSO were made from stock solution in a concentration range of 0.01–20 mM. To reach the final concentration (0.01–20 μM) dilutions with culture medium were made. The final concentration of DMSO in the medium was adjusted to 0.1% in all experiments, which is considerably lower than the concentration at which DMSO can inhibit cell proliferation and influence the effect of PDGF [15]. Since CsA precipitated in the medium at 20 μM concentration despite the presence of 0.1% DMSO, the results of this concentration were not evaluated, and the dose-response relationship was examined in the range of 0.01–10 μM.

2.3. Tissue samples and cell cultures

Orbital connective tissues were obtained from decompression surgeries of seven patients with TED. Before surgery, two patients underwent thyroidectomy, two patients received radioiodine treatment, one patient was on thyreostatic medication. All patients had received GC therapy and six patients received orbital irradiation. Decompression surgeries were carried out 12 or more months after the completion of GC treatment and irradiation. Patients had low-normal or suppressed TSH and normal thyroid hormone levels.

Control tissue samples were obtained from six patients who underwent enucleation surgery, with no disease of the tyroid and the orbit.

The primary human orbital fibroblast culture model described in our previous studies was used [14,16,17]. Briefly, orbital fibroblasts were seeded in 24-well or 96-well plates at confluent density. Incubation for 24 h in culture medium, followed by treatment with medium containing 10% (v/v) FBS with or without CsA (10 μM), PDGF-BB (10 ng/ml), or CsA combined with PDGF-BB for an additional 24 h. The supernatant and cells for mRNA extraction were harvested at the end of the 24-h treatment. Samples were stored at −20 °C until use. Not all cell cultures were included in all measurements.

This study was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen (ID: 5913/2012/EKU (84/13)). All patients gave written informed consent.

2.4. Proliferation assay

The proliferation assays were performed in 96-well plates according to the manufacturer's instructions. Briefly, the 5-bromo-2′-deoxyuridine (BrdU) solution was added to the cells at 10 μM final concentration in 100 μl culture medium and incubated for 2 h in a CO_2 incubator at 37 °C. Then the cells were fixed using FixDenat solution for 30 min. The peroxidase-conjugated anti-BrdU antibody was added for 90 min. Finally, 3,3′,5,5′-tetramethylbenzidine substrate was added for 10 min. The reaction was stopped by addition of 2 N H_2SO_4, and the absorbance was detected at 450 nm against a reference wavelength of 620 nm using a DTX 880 Multimode Detector (Beckman Coulter Inc., Brea, CA, USA).

2.5. Metabolic activity assay

The cells were seeded in 96-well plates, and medium with or without CsA or PDGF-BB was added to them for 24 h. The concentration of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was 0.5 mg/ml in the culture medium. The cells were incubated for 3 h in a CO_2 incubator at 37 °C. The blue formazan crystals were dissolved in (DMSO). The absorbance was measured at 550 nm (reference wavelength: 660 nm) using a Synergy H1 Microplate Reader (Agilent Technologies Inc. Santa Clara, CA, USA).

2.6. Quantitation of HA

Secreted HA levels in cell culture supernatants were measured using the DuoSet Hyaluronan Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The absorbance at 450 nm (reference wavelength: 620 nm) was detected using DTX 880 Multimode Detector (Beckman Coulter Inc., Brea, CA, USA).

2.7. Real-time polymerase chain reaction (RT-PCR)

TRI reagent was used for the isolation of RNA. High-Capacity cDNA Reverse Transcription Kit was used to reverse transcribe the mRNA to cDNA library. The expression of *HAS1, HAS2, HAS3, HYAL1* and *HYAL2* (HAS1–Hs00987418_m1, HAS2– Hs00193435_m1, HAS3– Hs00193436, HYAL1– Hs00201046_m1, HYAL2– Hs01117343_g1) was detected using the TaqMan Gene Expression Assay. The results were normalized to *GAPDH* (Hs02758991_g1) mRNA levels by the ΔCT method. The reactions were performed by the CFX Opus 96 Real-time PCR System (BioRad, Hercules, CA, USA).

2.8. Statistical analysis

Statistical analysis was performed using the STATISTICA 14.0 software (TIBCO Software Inc., Palo Alto, CA, USA). Data are presented as mean ± standard error of mean (SEM). Differences among groups were evaluated by repeated measures ANOVA followed by Fisher LSD post hoc test. Statistical significance was set at the 5% level ($p < 0.05$).

3. Results

Based on data by others [13,18,19], dose-dependent influence of CsA treatment on hyaluronan production of OFs was tested in the range of 0.01–10 μM concentration (Fig. 1.). CsA decreased HA production in all studied concentrations ($p < 0.001$). The maximal inhibitory effect of CsA on HA production in all cell cultures was present at 10 μM concentration; as this was close to the one ever achieved in humans [19], for further experiments this concentration was selected.

CsA treatment (10 μM) caused a remarkable decrease in HA production ($p < 0.0001$) after 24 h (Fig. 2), average 42.5 % ± 5.6 % ($p < 0.001$) and 42.2 % ± 3.9 % ($p < 0.001$) in TED OFs and NON-TED OFs, respectively. The effect was not affected by the origin of orbital fibroblasts ($p = 0.362$). We did not find any differences in the basal HA

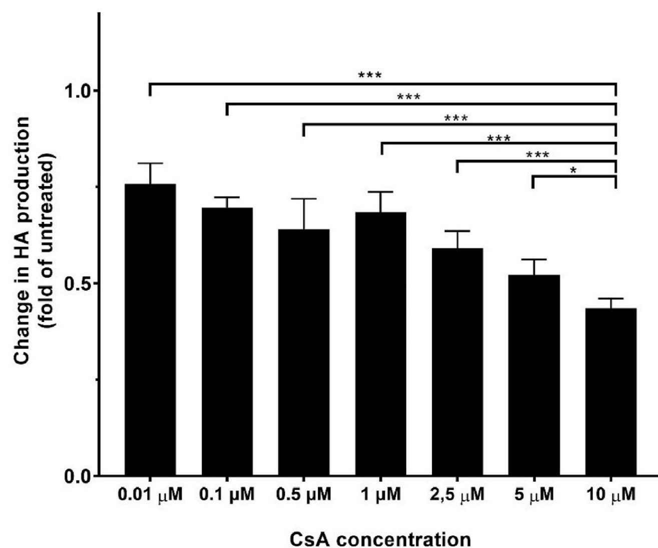

Fig. 1. The dose-dependent effect of CsA on hyaluronan production of orbital fibroblasts (n = 4). Data were analyzed using repeated measures ANOVA followed by Fisher LSD post hoc test. Results are shown as mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

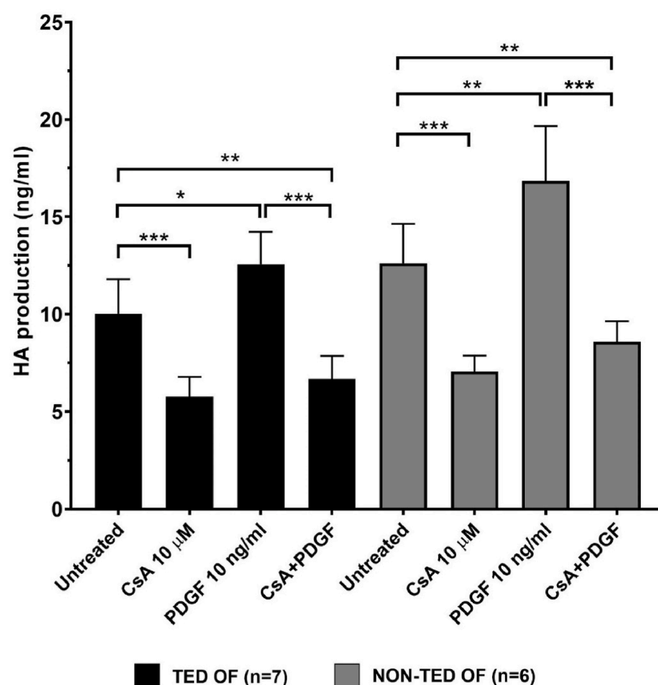

Fig. 2. Effect of 10 μM CsA on hyaluronan production of TED OFs and NON-TED OFs. Data were analyzed using repeated measures ANOVA followed by Fisher LSD post hoc test. Results are shown as mean ± SEM. *$p < 0.05$ **$p < 0.01$, ***$p < 0.001$.

production of TED and NON-TED fibroblasts ($p = 0.389$). The HA production increased after PDGF-BB treatment ($p < 0.001$), which is consistent with our previous findings [14]. CsA and PDGF-BB together resulted in the suppression of PDGF-BB stimulated HA production ($p < 0.0001$); CsA had such a strong inhibitory effect on HA production that it was able to reduce the PDGF-BB stimulated HA production below the untreated level ($p < 0.001$).

In order to monitor the change in the mRNA expression pattern of the enzymes required for the synthesis of HA RT-PCR was performed. We found an average 2.39 ± 1.1-fold ($p = 0.023$) and 1.77 ± 0.58-fold ($p = 0.007$) increase in the *HAS1* expression of TED and NON-TED OFs, respectively, after CsA treatment (Fig. 3A). PDGF-BB did not change the mRNA levels of *HAS1* ($p = 0.579$). Changes observed in *HAS2* expressions reflect the changes in HA production. The basal levels did not depend on the origin of the cells ($p = 0.965$). The 10 μM CsA reduced the mRNA levels of *HAS2* in both cell types ($p = 0.005$) (Fig. 3B) causing an average 75 ± 16 % ($p = 0.019$) and 60.5 ± 16 % ($p = 0.026$) decrease in TED and NON-TED OFs, respectively. PDGF-BB is a known stimulator of *HAS2* which is supported by our results ($p = 0.004$). CsA treatment reduced the *HAS2* expression even under PDGF-BB stimulated conditions ($p = 0.003$). The *HAS2* expression pattern resembled that of HA production (Fig. 2). We also found a marked reduction in *HAS3* (Fig. 3C) expression after the CsA treatment; the decrease was 35.5 ± 26.4% ($p = 0.043$) in TED OFs and 43.1 ± 23.2 % in NON-TED OFs ($p = 0.019$). PDGF-BB alone did not cause alteration in *HAS3* mRNA levels of the OFs ($p = 0.166$).

Among the enzymes required for the degradation of HA molecules, CsA did not affect the expression of *HYAL1* (Fig. 4A). In contrast, the treatment caused an average 39.7 ± 18.3 % ($p = 0.022$) and 33.3 ± 13.4 % ($p = 0.007$) reduction of *HYAL2* (Fig. 4B) expression in TED and NON-TED OFs, respectively. PDGF-BB alone had no effect on mRNA expression levels of HYALs.

The effect of CsA on the proliferation of OFs was assessed by BrdU incorporation assay (Fig. 5). The NON-TED OFs had higher proliferation rate ($p < 0.008$). The proliferation rate was decreased by 23.5 ± 15.4% in TED OFs ($p = 0.026$), 31.3 ± 11.5% in NON-TED OFs ($p = 0.004$) after incubation with 10 μM CsA compared with untreated cultures. PDGF-BB increased the proliferation rate of the cell cultures ($p = 0.006$). The PDGF-BB stimulated proliferation decreased to the level of untreated cells after CsA treatment. In PDGF-BB stimulated cells CsA was able to decrease proliferation, but this did not reach the degree of reduction seen in the cultures treated with CsA alone (Fig. 5).

CsA treatment (Fig. 6) caused a decrease in metabolic activity ($p < 0.0001$). This reduction was average 15.4 ± 3.7 % in TED OFs ($p < 0.0001$) and 11.6 ± 7.5 % in NON-TED OFs ($p = 0.002$). The PDGF-BB treatment did not alter the metabolic activity of the cells ($p = 0.188$).

4. Discussion

During the course of TED several factors are present in the orbit that may interfere with the HA synthesis of fibroblasts: TSH-R/IGF-1R activation, TNF-α, TGF-β, IL-1β, PDGF-BB [20,21]. In addition to the ECM remodeling resulting from elevated HA synthesis, the proliferation potential of cells found in orbital connective tissue is increased in vivo [1]. CsA is a well-known immunosuppressive agent used for the treatment of autoimmune diseases and after organ transplantation [22]. In the treatment of TED CsA may have clinical benefit when used in combination with glucocorticoids in patients who did not respond to steroids alone [11,12]. The immunosuppressive effect of CsA is mediated by the formation of complex with cyclophilin, which can inhibit the activity of protein phosphatases including calcineurin. Calcineurin inactivation inhibits nuclear factor of activated T cells (NFAT)-1 dephosphorylation and its transfer into the nucleus [23]. NFAT-1 is responsible for transcription of many genes, including the transcription and production of IL-2, which is required for the growth and proliferation of T and B cells [22].

In the present study, we focused on another aspect of CsA treatment. We aimed to clarify how CsA influences hyaluronan production of orbital fibroblasts, since hyaluronan is a key matrix component during the course of TED, plays a role in fluid accumulation (oedema), as well as in the immune and fibrotic processes. Studies by other groups resulted in controversial data in this respect: CsA treatment increased intra- and extracellular HA in osteoblasts [24] and in gingival fibroblasts [25], especially low molecular weight HA, and the expression of *HAS3* and *HYAL1-2* increased [26], while did not affect the production of HA in

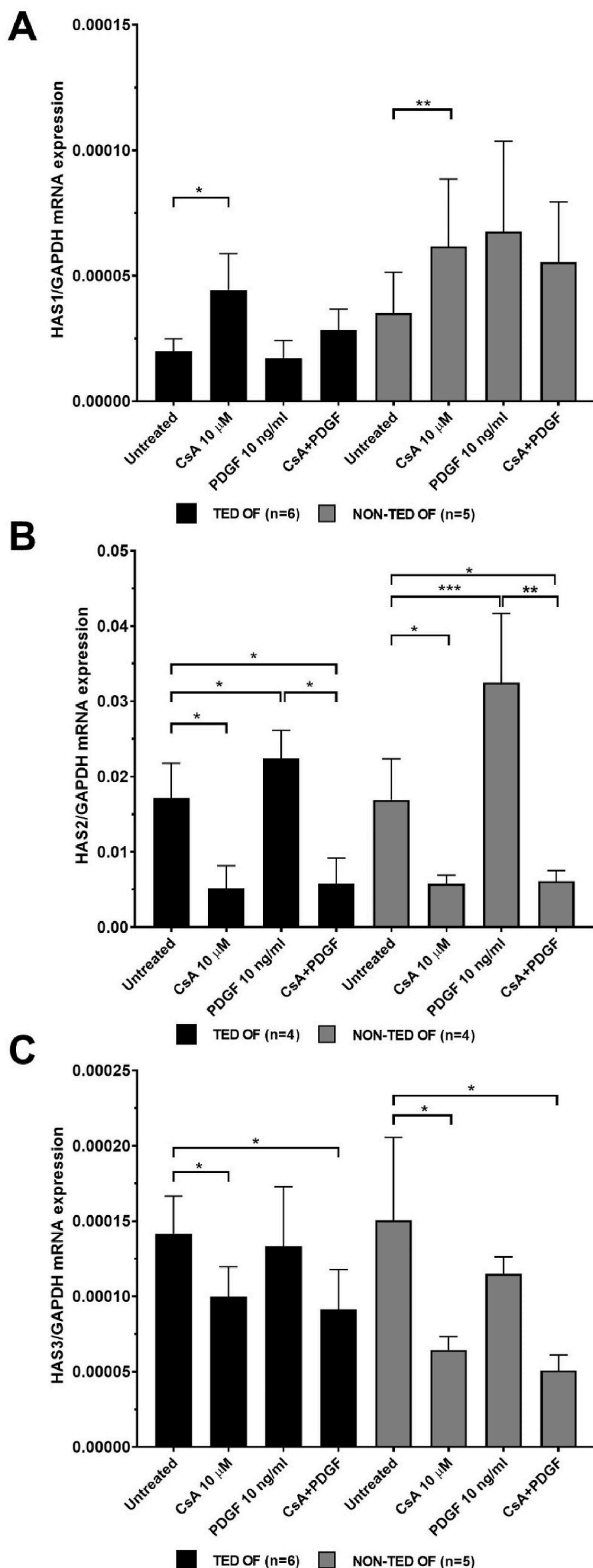

Fig. 3. The expressions of (**A**) *HAS1* (**B**) *HAS2* and (**C**) *HAS3* of TED OFs and NON-TED OFs after CsA or/and PDGF-BB treatment. Data were analyzed using repeated measures ANOVA followed by Fisher LSD post hoc test. Results are shown as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

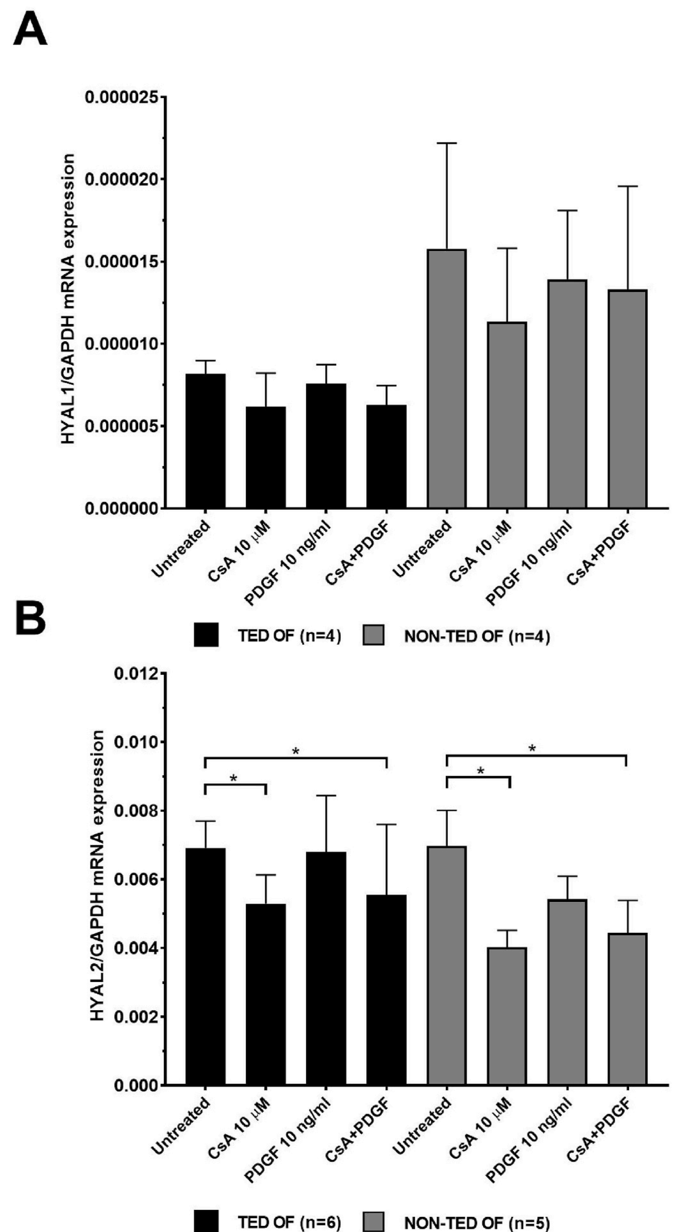

Fig. 4. The expressions of (**A**) *HYAL1* and (**B**) *HYAL2* of TED OFs and NON-TED OFs after CsA or/and PDGF-BB treatment. Data were analyzed using repeated measures ANOVA followed by Fisher LSD post hoc test. Results are shown as mean ± SEM * p < 0.05.

fibroblasts isolated from rat cardiac allograft and normal, non-transplanted tissue [27]. In addition to CsA, there is another commercial drug, tacrolimus (FK506), which has similar mechanism of action; in human dermal fibroblasts after tacrolimus treatment HA production was not affected, and no difference was found in the expressions of HA synthases and hyaluronidases [28]. We assumed that the *in vitro* effect of CsA on HA metabolism depended on the examined cell type.

The same primary human orbital fibroblast culture model and 24-h drug exposure was used as in our previous tests with other potential disease-modifying agents [14,16,17]. We found that in OFs 10 μM CsA reduced the production of HA, and inhibited *HAS2* and *HAS3* expressions, while increased *HAS1* expression. The divergent effects of CsA on the expression of the different HAS enzymes may be the result of a compensatory mechanism, as Zhang and colleagues, using *HAS2* siRNA in preadipocytes, found a marked reduction in HA production and *HAS2*

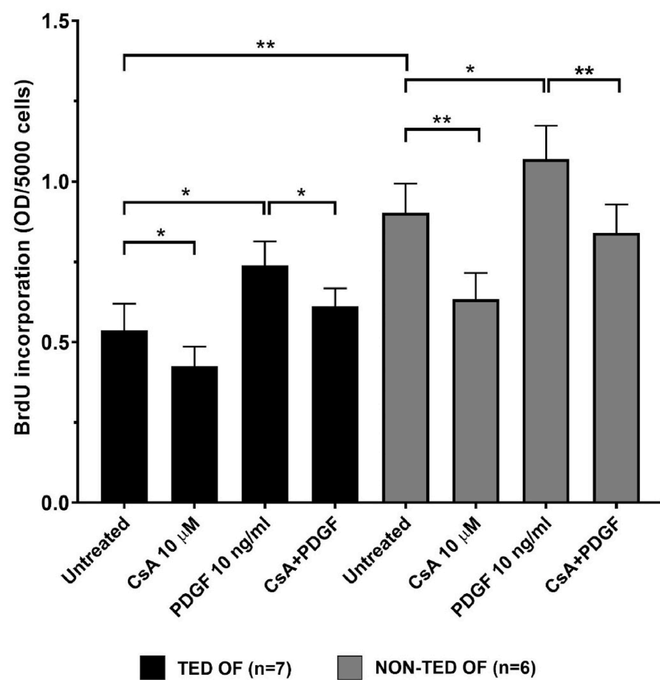

Fig. 5. The effect of CsA or/and PDGF-BB treatment on proliferation potential of TED and NON-TED OFs. Data were analyzed using repeated measures ANOVA followed by Fisher LSD post hoc test. Results are shown as mean ± SEM. *p < 0.05; **p < 0.01.

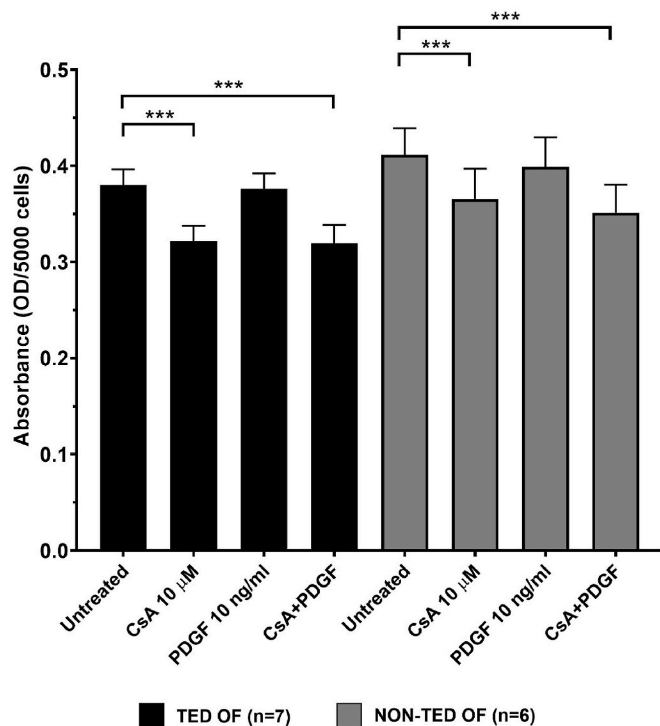

Fig. 6. The effect of CsA and/or PDGF-BB treatment on metabolic activity of TED and NON-TED OFs. Data were analyzed using repeated measures ANOVA followed by Fisher LSD post hoc test. Results are shown as mean ± SEM. ***p < 0.001.

expression in parallel with a 60% increase in *HAS1* mRNA expression [29]. Similar results were obtained in human breast cancer cell lines after HAS2 siRNA treatment; the *HAS1* expression increased [30]. The inhibition of *HAS2* and *HAS3* by CsA in OFs may lead to a compensatory increase in HAS1 transcripts. We assume that, considering the marked decrease in HA production, the observed increase in *HAS1* expression has a smaller effect in restoring the balance of HA production. *HAS2* is the dominant synthase in OFs [17] and *HAS3* has a critical role in inflammation and tumorigenesis [31], thus, reduction of these two synthases may have a beneficial effect in TED. After CsA treatment, *HAS2* mRNA expression levels changed in parallel with HA production, and *HAS3* expression also decreased. There is a little data available about the mRNA expressions of enzymes responsible for the degradation of HA under CsA treatment. Our results show that *HYAL1* expression is not affected by CsA treatment, while *HYAL2* expression is inhibited; this reveals that CsA not only inhibits HA synthesis, but also reduces the turnover rate of HA.

PDGF isoforms were found increased in orbital tissue from TED patients [5]; in addition, PDGF-BB increases HA production in OFs [3,14]. We found that CsA decreases HA production in OFs, even under stimulation by PDGF-BB. *HAS2* expression increased as a result of PDGF-BB treatment, and CsA was able to completely abolish this effect. Taken together, we assume that under PDGF-BB stimulated conditions, the reduction of HA production achieved by CsA treatment is most probably due to the inhibition of *HAS2* expression. CsA inhibits proliferation of epithelial cells [32], neural stem cells [33] and regulatory T cells [34]; we have found the same effect in orbital fibroblasts. CsA was also able to reduce the PDGF-BB-stimulated proliferation of OFs to the baseline level. Both TED and NON-TED OFs were exposed to the same conditions during experiments, thus their basal behaviours did not necessarily reflect the disease-specific differences, such as the higher proliferation rate of TED OFs. In our previous study we did not find any difference between the proliferation rates of TED and NON-TED OFs [17]. However, in the current experiment, in accordance with Li and colleagues [35], the NON-TED OFs had higher proliferation potential. We assumed that this controversial result depended on the current state of the fibroblasts. Moreover, individual differences may have had greater impact on proliferation of the cells than those effects that previously had affected the tissue from which they originated.

PDGF-BB can increase HA production in dermal fibroblasts via stimulating the NF-κB pathway [36], as there is an NF-κB binding site in the *HAS2* and *HAS3* promoter region [31]. Since CsA can inhibit NF-κB [37], we assume that CsA can hinder the activation of PDGF-BB-stimulated NF-κB signaling, thus diminishing its effect. Further, by inhibiting the NFAT pathway, CsA can reduce *HAS3* and increase *HAS1* expression [38]. Pham and his colleagues demonstrated that the NFAT element of the calcineurin pathway and the NF-κB transcription factors directly interact with each other [39], and suggested a possibility of new therapeutic targets by inhibiting the two factors or the relationship between them. Although the above interactions were not directly studied here, we think that CsA contributes to the improvement of TED by inhibiting both HA synthesis and OF proliferation of via calcineurin-NFAT and NF-κB pathways (Fig. 7). We found a slight decrease in the metabolic activity of OFs after CsA treatment; in orbital fibroblasts, we have already shown a similar phenomenon, when the decrease in metabolic activity was not caused by the cytotoxic effect of a substance, but rather its significant reducing effect on HA synthesis [16].

Recently it has been proven that CsA has had a positive effect in several disorders associated with HA deposition. As a positive result, the skin tightness in scleroderma was reduced by CsA treatment [40]. CsA may have antifibrotic and anticirrhotic effects in primary biliary cirrhosis and in some patients with chronic active hepatitis [41], as well as it can improve the pulmonary physiology in idiopathic chronic fibrosing interstitial pneumonia without idiopathic pulmonary fibrosis [42], and extend the patients' chances for long-term survival with idiopathic pulmonary fibrosis [43]. These data in accordance with our results in OFs, suggest the applicability of CsA in the treatment of diseases accompanied by increased HA production and fibrosis.

The main limitation of the present study is its *in vitro* nature. We used

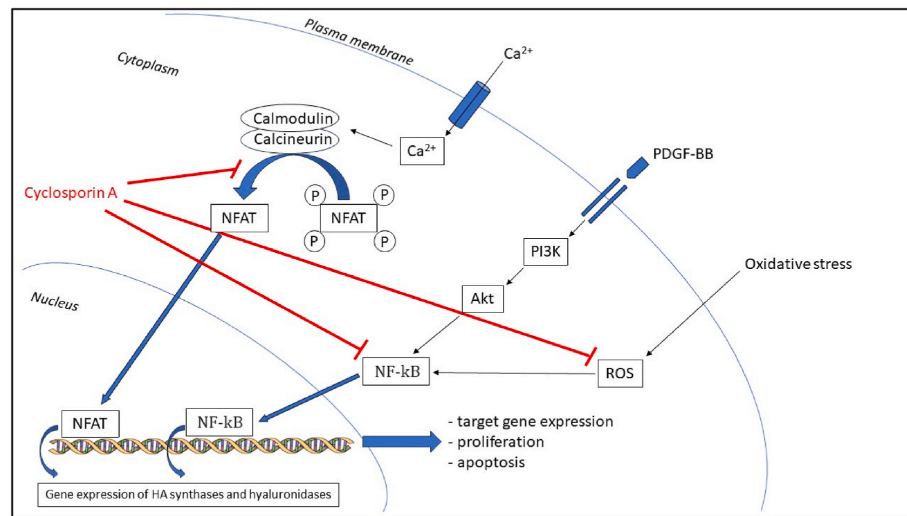

Fig. 7. Schematic figure of the hypothetic mechanism of action of CsA on HAS and HYAL gene expression. Increased intracellular Ca^{2+} binds to calmodulin and forming complex with calcineurin, that activates NFAT. PDGF-BB binds to receptors and stimulates NF-κB signaling. Both factors translocate to the nucleus and upregulate target gene expression. Cyclosporin A could inhibit the NFAT and NF-κB and suppress the expression of the key enzymes of HA synthesis.NFAT-nuclear factor of activated T cells, NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells, Akt - Protein kinase B, PDGF-BB – Platelet derived growth factor BB, ROS – reactive oxygen.

primary orbital fibroblast cultures established from tissue explants. The cells were removed and deprived from the milieu that is present in the orbit during the pathogenesis of TED, including endocrine, immune and local factors. Further, TED OFs were originated from connective tissues of patients with inactive disease.

5. Conclusion

Our results show for the first time the effect of CsA on the expression pattern of enzymes involved in HA metabolism in orbital fibroblasts. The decrease in HA synthesis may contribute to the beneficial effect of CsA in TED. Further studies are required to elucidate the precise mechanisms how the effect of CsA on connective tissue may contribute to its immunomodulatory effect in TED.

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CRediT authorship contribution statement

Erika Galgoczi: Writing – original draft, Software, Methodology, Data curation, Conceptualization. **Zsanett Molnar:** Writing – review & editing, Methodology. **Monika Katko:** Writing – review & editing. **Bernadett Ujhelyi:** Writing – review & editing, Resources. **Zita Steiber:** Writing – review & editing, Resources. **Endre V. Nagy:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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