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The role of orbital fibroblasts in the pathogenesis of endocrine orbital fibroblasts

by Erika Galgóczi

Supervisor: Endre V. Nagy, MD, PhD, DSc

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By Erika Galgóczi MSc

Supervisor: Endre V. Nagy MD, PhD, DSc
Doctoral School of Health Sciences, University of Debrecen

Head of the **Examination Committee**:	Margit Balázs PhD, DSc
Members of the Examination Committee:	Péter Igaz, MD, PhD, DSc
	István Ilyés, MD, PhD, DSC

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Introduction

Endocrine orbitopathy (EOP) accompanies Graves-Basedow disease (GD) in 20–30% of patients and is characterized by autoimmune inflammation and rearrangement of orbital tissues. The clinical aspects of EOP are consequences of volume expansion of the soft tissues in the bony orbit: swelling of the external eye muscles and expansion of the connective tissue. Because of the excessive intraorbital volume, orbital content shifts toward the front opening of the orbit, resulting in proptosis of the eye. Two major sight-threatening conditions, keratitis and optic neuropathy, are the consequences of prolonged proptosis and optic nerve compression, respectively. The primary targets of the autoimmune process are the orbital fibroblasts, which are activated by cytokines and growth factors produced by infiltrating immune cells. The results of this process are increased fibroblast proliferation of and hyaluronan (HA) production, followed by rearrangement of the extracellular matrix (ECM). Later during the natural course of disease, this process leads to maturation of preadipocytes into adipocytes. The role of plasminogen activator inhibitor 1 (PAI-1) in the pathogenesis of EOP was unknown. By increasing matrix stability, PAI-1 reduces the degradation of ECM components which may contribute to decreased contact inhibition and increased cell proliferation. HA production is a major determinant of the pathogenesis of EOP; the water binding capacity of HA *in vitro* is up to a thousand times its own weight. Any intervention that reduces local HA production can reduce the volume increase of orbital tissues and thus interfere with the pathogenesis of EOP. HA production has been successfully reduced by administration of 4-methylumbelliferone (4-MU) in animal models of autoimmune, chronic inflammatory and cancerous diseases. 4-MU is a commercially available OTC spasmolytic in many European countries.

Literature review

EOP most often coincides with GD disease, but may also occur before or after the thyroid dysfunction is diagnosed. Hashimoto's thyroiditis is less frequently accompanied by EOP. EOP is typically characterized by the expansion of orbital connective tissue and thickening of the external eye muscles. An early, common symptom of the disease is pain in the orbit The cause of the pain is the increased intraorbital pressure. Double vision, increased tearing, photosensitivity, exophthalmus, upper eyelid retraction, periorbital edema, and conjunctival hyperaemia may be present; all these symptoms and signs severely impair the patients' quality of life. In addition to disease severity, disease activity has also to be estimated using the Clinical Activity Score (CAS); an immunologically active process requires therapeutic intervention.

Several studies show that orbital fibroblasts (OF) are the primary targets of the autoimmune processes in EOP. OF activation is resulting in increased proliferation rate and HA production. Further, OF themselves produce inflammatory factors, and later differentiate into adipocytes and myofibroblasts. Excessive accumulation of some components of the ECM leads to a rearrangement of the orbital tissue. A series of cytokines and growth factors have been detected in the orbital tissues of patients with EOP.

EOP orbital tissues contain elevated level of non-sulfated glycosaminoglycan (GAG), mainly HA and collagen, which are produced by OFs. HA is one of the ECM components that, via its high water-binding capacity, greatly contributes to the expansion of orbital tissues. In EOP, the balance of the synthesis and degradation of ECM components changes due to OF activation. HA plays an essential role in cell-ECM and cell-cell interactions. The HA molecule is a

polysaccharide, composed of repeating subunits. Its biosynthesis is mediated by three transmembrane glycosyltransferase isozymes, the hyaluronan synthases HAS1, HAS2 and HAS3, while hyaluronidases HYAL1 and HYAL2 are responsible for HA degradation.

PAI-1 reduces the degradation of ECM components and thereby increases matrix stability; its role was unknown in the pathogenesis of EOP. PAI-1 expression is cell density dependent in many human cell types, including preadipocytes and dermal fibroblasts. Elevated PAI-1 protein expression may result in ECM accumulation. Analysis of orbital tissues in EOP has shown increased transcription level of the *PAI-1* gene. The role of PAI-1 in the pathomechanism of EOP, including its potential role in HA regulation, was not known.

Transforming growth factor β (TGF-β) plays an important role in cell growth and differentiation, as well as in tissue regeneration and in the regulation of fibrosis through ECM production. It is involved in many biological processes, including cell proliferation and ECM production. Increased TGF-β expression in the orbital tissues has been described in moderate to severe EOP, and its amount positively correlated with CAS. PAI-1 expression is known to be regulated by several cytokines and growth factors, including TGF-β.

4-MU has a beneficial effect on the pathogenesis of certain experimental autoimmune and chronic inflammatory and cancerous diseases in animal models and inhibits HA production *in vitro*. It can interfere in the HA production in two different ways: by binding to glucuronic acid, 4-MU acts as a competitive inhibitor, and reduces mRNA expression hyaluronan synthases.

According to current recommendations, patients with EOP are to be referred to specialized centres where endocrinologists, ophthalmologists, ophthalmic surgeons with experience in this

field, and several imaging modalities are available. Our unit at the University of Debrecen is well suited for the diagnosis and treatment of EOP patients.

Aims

EOP is a serious, sight-threatening disease that cannot fully resolve using the available treatment modalities. Better understanding the pathomechanism of the disease may result in new therapeutic interventions. We designed experiments on primary fibroblast cell lines generated from orbital tissue of patients with EOP to explore the roles of PAI-1 and HA in the pathogenesis of EOP. The major aims were:

1. To establish an *in vitro* model from surgical tissue samples, to test the behaviour of orbital fibroblasts exposed to different conditions

2. To model the changing fibroblast density during volume growth in EOP, and to investigate the effect of increasing cell density on PAI-1 and HA production in control orbital, EOP orbital, and dermal fibroblast cultures.

3. To investigate the effect of TGF-β on HA and PAI-1 production and proliferation of fibroblast cultures of different origin.

4. Identification of potential new treatment modalities which could inhibit the HA production of fibroblasts.

Methods

Tissue samples of patients treated and followed at the Division of Endocrinology, Department of Medicine, University of Debrecen, were used. Patients were selected based on predefined criteria. The study protocol was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen, and experiments were performed in accordance with the Declaration of Helsinki. All participating patients gave written informed consent. Five primary orbital fibroblast cell lines were generated from orbital connective tissue removed during orbital decompression surgery performed in EOP patients. Control orbital tissues were obtained during eye surgery (enucleation) in four patients with non-thyroid related eye disease, and without any thyroid disease. Control dermal connective tissues were obtained during four abdominal hernia surgeries.

Orbital fibroblast cell lines were generated according to the method previously described by Bahn et al. The connective tissue pieces were cut into 2x2 mm pieces and then washed in antibiotic-containing PBS and were placed in a Petri dishes. Depending on the proliferation potential of the cells, tissue pieces were removed after 2 to 4 weeks. After propagation, cells were suspended in freezing solution and stored in liquid nitrogen until used. Primary cell lines at low passage number (2-8) were used in the experiments. Cells were plated in 24-well plates at six different densities: $6.24x10^4$, $3.12x10^4$, $1.56x10^4$, $7.8x10^3$, $3.9x10^3$, and $1.95x10^3$ cells/cm^2. After 24 hours, cells were synchronized by 24-hour serum deprivation. Then cells were incubated for an additional 24 h with media or media containing the various treatments. Supernatants were collected and stored at -20°C until measurements were performed. Each experiment was

performed at least three times and in triplicate in each case. TGF-β used for the treatments was dissolved in M199. 4-MU and 4-methylumbelliferyl glucuronide (4-MUG) were dissolved in dimethyl sulfoxide (DMSO).

Cell Proliferation ELISA BrdU Colorimetric Kit for cell proliferation rate, *EZ4U* for metabolic activity, *Vybrant Cytotoxicity Assay Kit* for cytotoxicity, and *Caspase-Glo 3/7* kit were used to determine caspase activity according to the manufacturer's instructions. Commercially available *DuoSet Human Serpin E1 / PAI-1* and *DuoSet Hyaluronan* kits were used to determine PAI-1 protein and HA levels in the supernatant according to the manufacturer's instructions. For determination of pericellular HA, cells were treated with trypsin-EDTA solution. The cell suspension was centrifuged at 2200 G for 5 minutes. The supernatant was used for pericellular HA measurement. The HA content of FBS was taken into account in all measurements. In each case, the HA production was normalized to 10^5 cells. Absorbance and fluorescence were measured with a *Beckman Coulter, DTX 880 Multimode Detector*. In each case, the absorbance values of the treated cultures were compared to the measured values of the untreated cultures.

After the supernatant was removed from the cells grown in 24-well plate, they were washed twice with PBS and lysed in TRI reagent. Samples were stored at -20 ° C until RNA isolation. The thawed samples were homogenized and 100 µl of chloroform was added, then centrifuged at 16,000 G for 15 minutes. The upper aqueous phase was pipetted into a new DNase- and RNase-free Eppendorf tube. 375 µl of 100% isopropyl alcohol was added to precipitate RNA found in the aqueous phase and centrifuged at 16,000 G for 10 minutes. In the next step, after removing the supernatant, the pellet was washed with 1 ml of cold 75% ethanol. After centrifugation at

12,000 G for 10 min, the entire supernatant was removed and the isolated RNA was dissolved in nuclease-free water. Concentration was measured using a *NanoDrop* and stored at −20 ° C under pressure until reverse transcription was performed. Purified RNA samples were transcribed into cDNA using the *High Capacity cDNA Reverse Transcription Kit* as described by the manufacturer. *PAI-1, HAS1, HAS2, HAS3, HYAL1* and *HYAL2* expressions were detected relative to the expression of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene. The RT-PCR reaction was performed on a *BioRad CFX96 system*. Results were normalized to GAPDH mRNA expression levels using the ΔCT method. Statistical analyzes were performed using STATISTICA 7 software. Data were plotted as mean ± SD in each case. Repeated analysis of variance (ANOVA) used treatment as an internal factor (1 ng/mL TGF-β and 1 mmol/L 4-MU) and fibroblast origin as an intergroup factor (dermal, control, and EOP), and Fisher LSD post hoc analysis was used to estimate the differences. The significance level was set at $p < 0.05$.

Results

Investigation of the effect of cell density on PAI-1 and HA production

Dermal fibroblasts, control orbital fibroblasts, and EOP orbital fibroblasts produced different amounts of PAI-1 protein at all cell densities. The amount of PAI-1 protein per cell decreased with increasing cell density, and control OF, EOP OF, and dermal fibroblasts behaved similarly in this regard. The secreted amount of PAI-1 decreased continuously until cell confluence had been reached. We found that increasing cell density, regardless of origin, was a negative regulator of PAI-1 production in the cell lines studied. The *PAI-1* mRNA expression decreased in parallel with the concentration of PAI-1 protein. PAI-1 mRNA expression differed in cell lines of various

origin ($p < 0.01$); in descending order, dermal fibroblasts > control OF > EOP OF. The amount of HA in the supernatant and pericellular space did not show cell density-dependent behavior ($p = 0.6$ and $p = 0.2$, respectively), but the origin of the fibroblasts determined its quantity ($p < 0.001$). Post hoc analysis showed that untreated dermal fibroblasts produced higher amounts of HA than untreated OF (dermal vs. control $p < 0.01$; dermal vs. EOP $p < 0.001$). Similar results were obtained in pericellular HA levels (dermal vs. control $p = 0.013$, dermal vs. EOP $p < 0.001$). We found positive correlation between the amount of HA in the supernatant and in the pericellular space (dermal: $r = 0.72$, $p < 0.01$, control: $r = 0.77$, $p < 0.00001$, EOP: $r = 0.97$, $p < 0.000001$). *Hyaluronan synthase 1* (*HAS1*) and *hyaluronan synthase 3* (*HAS3*) expressions were of the same order of magnitude, while *hyaluronan synthase 2* (*HAS2*) was the most highly expressed enzyme in all cell lines studied. The expression pattern of HAS enzymes varied by cell type. Untreated dermal fibroblasts showed higher *HAS1* and *HAS2* mRNA expression than control OF, while untreated OFs showed higher *HAS3* expression than dermal fibroblasts. EOP OFs showed higher *HAS3* expression than control OFs. The *HAS1* and *HAS2* expression of dermal fibroblasts decreased with increasing cell density. The expression of *hyaluronidase 1* (*HYAL1*) mRNA showed a more diverse picture than the expression of *hyaluronidase 2* (*HYAL2*): untreated dermal fibroblasts had the lowest basal *HYAL1* expression levels, and the EOP OF's *HYAL1* expression was lower than that of control OF. We saw a lower but not significant trend in the production of *HYAL1* and *HYAL2* in confluent cultures compared to post or pre-confluent cultures. The proliferation rate of fibroblasts decreased with increasing cell density in all cell lines tested, regardless of the origin of the cells. We found a positive correlation between proliferation rate and PAI-1 secretion in all fibroblast lines examined (dermal $r = 0.81$ $p < 0.01$,

control OF r = 0.71, p < 0.0001, EOP OF r = 0, 70, p < 0.0001). The proportion of proliferating cells was higher at all densities of all but one of OF lines than of dermal fibroblasts (control OF vs. dermal p = 0.03, EOP OF vs. dermal p = 0.02). By post hoc analysis, we did not find any difference in the proliferation rate of control and EOP OF.

Effect of TGF-β treatment on PAI-1 and HA production by fibroblasts

TGF-β in the concentration range of 0.01–10.0 ng/mL was tested in confluent cultures. The effect on PAI-1 production did not differ at concentrations of 1 ng/mL or higher. TGF-β stimulated PAI-1 production at all densities in all cell lines examined, regardless of their origin (p < 0.000001). For both control and EOP OF, the increase in PAI-1 production was dependent on cell density (p <0.00001); the higher the density, the greater the increase in PAI-1 production. The cell density dependence of the stimulatory effect of TGF-β treatment on PAI-1 expression was typical of OF, but not of dermal fibroblasts. At the highest orbital fibroblast densities TGF-β treatment resulted in a 5-fold increase in PAI-1 protein and an average 10-fold increase in PAI-1 mRNA. We concluded that the PAI-1 inhibitory effect of high cell density under basic conditions can be partially reversed by TGF-β treatment.

Regarding the stimulatory effect of TGF-β on HA production, we found a difference at the highest cell density studied (p < 0.0001), and this effect did not depend on the origin of the fibroblasts. The same is true for the pericellular HA synthesis of OF, whereas dermal fibroblasts responded to TGF-β treatment at all densities with increased HA synthesis. A positive correlation was found between the supernatant HA content and the amount of pericellular HA of untreated cultures after 24 hours of TGF-β treatment (dermal r = 0.58 p = 0.02, control r = 0.95,

$p < 0.000001$, EOP $r = 0.89$, $p < 0.000001$). There was no difference between the HA production of preconfluent and confluent cultures under the influence of TGF-β, but the response of postconfluent cell density cultures differs from these ($p < 0.0001$). *HAS1* expression increased in a cell density-dependent way ($p = 0.03$), more pronounced in OF, whereas *HAS2*, *HAS3*, *HYAL1*, and *HYAL2* expression levels did not change after TGF-β treatment. With increasing cell density, the enhancing effect of TGF-β was more pronounced in all cell lines. In orbital fibroblasts, a 500 to 600-fold increase of *HAS1* mRNA was observed at the highest density, while in dermal fibroblasts it was only approximately 10 times. TGF-β treatment affected proliferation potential only at the highest cell density ($p < 0.01$). A weak inhibitory effect was observed in the other densities with 30% decrease ($p < 0.01$) in the lowest density of dermal fibroblasts and 18% decrease in EOP OF ($p = 0.04$).

Effect of 4-methylumbelliferone (4-MU) treatment on HA production by fibroblasts

The concentration of 4-MU was tested in the range of 0.125 to 6.0 mmol/L. In the concentration range studied, maximal reduction of HA synthesis at 24 hours of treatment was achieved at 1.0 mmol/L 4-MU concentration. The efficacy of 4-MU treatment was independent of cell density ($p = 0.931$).

A strong inhibitory effect of 4-MU on HA synthesis was observed: 85.3 ± 2.9% in control OF, 85.4 ± 2.8% in EOP OF, and 91.3 ± 1, 2% in dermal fibroblasts ($p < 0.001$). The efficacy of 4-MU treatment was independent of the fibroblast origin ($p = 0.352$). We found a comparable change in pericellular HA production of orbital fibroblasts after treatment. The metabolite of 4-MU, 4-methylumbelliferyl glucuronide (4-MUG), did not inhibit HA production.

The basal expression of the *HAS1* was extremely low in all cell lines tested. There was no difference in basal *HAS2* expressions of different fibroblasts ($p = 0.654$). 4-MU treatment reduced *HAS2* expression in all cell lines ($p < 0.001$), and there was no difference in the efficacy (ANOVA $p = 0.268$). The mean reductions were $72 \pm 12\%$, $76 \pm 18\%$, and $91 \pm 3\%$ for control OF, EOP OF, and dermal fibroblasts, respectively. Based on post hoc analysis, this decrease was significant in all cases (control OF $p = 0.008$, EOP OF $p = 0.0234$, dermal $p = 0.0012$). 4-MU treatment caused an increase in *HAS3* mRNA ($p = 0.030$) regardless of the origin of the fibroblasts ($p = 0.163$). The increase from baseline expression were 36% for control OF, 99% for EOP OF, and 34% for dermal fibroblasts. For *HYAL1*, basal expressions did not differ by site of origin ($p = 0.203$) and 4-MU treatment caused a decrease in all cell lines ($p = 0.017$) The degree of inhibition depended on cell origin ($p = 0.042$); post hoc analysis showed that the reduction was significant only in control OF with 4-MU treatment ($45 \pm 13\%$). There was no difference in *HYAL2* expression in fibroblasts of different origin ($p = 0.353$). 4-MU treatment caused an increase in *HYAL2* mRNA levels ($p < 0.0001$), and this effect was independent of the origin of the cells ($p = 0.368$). The post hoc test showed that the effect of 4-MU was significant in all cell lines. The increase from baseline was $157 \pm 72\%$ in control OF, $160 \pm 24\%$ in EOP OF, and $135 \pm 24\%$ in dermal fibroblasts.

The reduction in proliferation rate after 4-MU treatment was $78 \pm 8\%$ for control OF, $61 \pm 12\%$ for EOP OF, and $80 \pm 3\%$ for dermal fibroblasts compared to untreated cultures. Both the basal and the reduced proliferation rates after treatment were independent of the origin of the cells ($p = 0.387$); 4-MU treatment caused a uniform decrease ($p < 0.001$) regardless of whether dermal or orbital fibroblasts were treated ($p = 0.259$).

Metabolic activity was decreased by 4-MU treatment: 29 ± 18% in control OF, 34 ± 11% in EOP OF, and 30 ± 10% in dermal fibroblasts. The effect did not depend on the origin of the cells. In the cytotoxicity assay, which is based on the reaction of the enzyme glucose-6-phosphate (G6PD) released from cells during apoptosis or necrosis, the rate of necrosis/apoptosis in untreated cultures was 3.8 ± 3.5%, while after 4-MU treatment it was 5.7 ± 5.5%, i.e. the proportion of damaged or dead cells in treated cells did not differ from the untreated cell lines ($p = 0.316$). To demonstrate that 4-MU treatment did not show pro-apoptotic activity, caspase-3 and caspase-7 activity measurements were also performed; no difference was found in activities after 24 hours of treatment.

Combined effect of 4-MU and TGF-β treatment

The increase in the TGF-β-induced HA production was significant in all cell lines studied, and TGF-β-stimulated HA growth was reduced by 4-MU treatment. During 4-MU and TGF-β co-treatment, 4-MU prevented the stimulatory effect of TGF-β and reduced the HA synthesis below baseline levels. In this respect, there was no difference between fibroblasts of different origin ($p = 0.242$). The increase in *HAS1* mRNA expression induced by TGF-β was decreased by 4-MU treatment. However, for *HAS2*, *HAS3*, *HYAL1* and *HYAL2*, no difference was found after TGF-β stimulation between 4-MU treated and untreated cells.

Discussion

Orbital fibroblasts are considered the primary target of EOP autoimmune processes because they can to be activated by cytokines produced by immune cells infiltrating orbital connective tissue. Activated orbital fibroblasts produce large amounts of ECM components and proliferate increasingly. The accumulation of HA in the orbit, more specifically due to its unique high water-binding capacity, causes tissue swelling and promotes the infiltration of inflammatory immune cells. PAI-1 maintains the balance between the ECM synthesis and degradation. During the course of EOP, the overproduction of HA creates a looser matrix and high-water environment that affects cell-cell and cell-ECM relationships.

We have shown that the increasing cell density is a negative regulator of both PAI-1 production of orbital fibroblasts and their proliferation potential. Contact inhibition is common in tissues and regulates cell proliferation. We hypothesize that in the system we have created simulates orbital conditions in health and in EOP. The confluent cultures model the healthy orbit, while preconfluent cultures resemble extensive, high ECM-producing fibroblasts with higher proliferation rates. Orbital fibroblasts were found to have higher proliferative capacity than dermal fibroblasts. We found intense, transcriptionally regulated PAI-1 synthesis that changes parallel with cell density in both dermal and orbital fibroblasts; PAI-1 production per cell is inversely proportional to cell density. To the best of our knowledge, our research team was the first to describe that orbital fibroblasts behave in this way. We hypothesize that the proliferative state of orbital fibroblasts at different densities is a major determinant of PAI-1 expression. Our results confirm the assumption that TGF-β may be one of the cytokines responsible for the

structural changes in orbital connective tissue in EOP. TGF-β was known to regulate cell growth, cell motility, ECM transformation, and pericellular proteolytic activity at the transcriptional level, to stimulate PAI-1 transcription, and to enhance transcription of genes encoding ECM components. In addition, TGF-β has been shown to be present in the orbit of EOP patients. Our results clearly show that TGF-β is an effective stimulator of PAI-1 synthesis in orbital fibroblasts at all cell densities studied, including high densities, where this effect is highly counterproductive and may well be coupled to disease progress. High concentration of PAI-1 in the EOP orbits is responsible for the increased matrix stability, thus contributing to the increase in the amount of ECM and the proliferation potential of fibroblasts.

HA production per cell does not show a cell density-dependent pattern but it is highly dependent on the origin of the fibroblasts. The expression pattern of HAS enzymes also differs in the three cell types. Changes in the transcriptional level of *HAS*s do not always correlate with HA secretion. Cells responded to TGF-β with increased HA production at higher densities, and their PAI-1 production increased in parallel. Because both EOP and control OF responded to TGF-β in the same manner, we hypothesized that it is the presence of immune cells and cytokines that contributes to the pathogenesis of the disease, rather than a possible difference between EOP and control orbital tissues. We confirmed that TGF-β treatment enhances *HAS1* mRNA expression. In terms of disease course, TGF-β-induced matrix overproduction may be especially harmful at higher fibroblast densities. Accumulation of HA can turn off contact inhibition and generate increased proliferation potential. Our data show that TGF-β-induced HA synthesis at higher cell densities promotes cell density-dependent PAI-1 stimulation in orbital fibroblasts. Based on our results, contact inhibition may be an important negative regulator of PAI-1 synthesis in orbital

fibroblasts. Further, high proliferation rates may be associated with PAI-1 synthesis. We observed that the usual decrease in PAI-1 synthesis caused by increasing cell density is partially reversed by TGF-β.

Our objectives included the identification of a drug, a potential suppressor inhibitor of HA synthesis. This effect would be especially useful if works under stimulated conditions. Consistent with previous work by others on other cell lines, we found that 4-MU inhibits HA production by orbital and dermal fibroblasts. 4-MU is known to be a competitive substrate of HA synthesis. We found that the presence of this competitive inhibitor affected both hyaluronan synthases and hyaluronidases in orbital fibroblasts at transcriptional level. This effect on HA synthesis was independent of the origin of the fibroblasts; 4-MU reduced the amount of HA in the supernatant in all cases. In addition, decreased HA synthesis was associated with a strongly decreasing proliferative potential. We conclude that 4-MU is a candidate for clinical studies in the treatment of EOP.

The hyaluronan synthase expression pattern of fibroblasts depends on their anatomical origin; dermal fibroblasts have higher *HAS1* expression than control or EOP orbital fibroblasts. The *HAS3* pattern was, in descending order, EOP OF > control OF > dermal fibroblasts. *HAS2* was the most prevalent isoform of the enzymes responsible for HA production in orbital fibroblasts, its concentration was two orders of magnitude higher than that of the other two isoforms, *HAS1* and *HAS3*. The increase in HA concentration is not due to reduced degradation, but due to overproduction of the HAS enzymes.

We found that 4-MU did reduce HA production in dermal and orbital fibroblasts even under TGF-β-stimulated conditions. The direct effect of 4-MU on HAS genes was unclear. In our study, 4-MU reduced *HAS1* and *HAS2* expression while *HAS3* levels remained unchanged. In addition to *HAS*s, hyaluronidases were also affected. Increased *HYAL2* expression was found upon 4-MU treatment. This might promote the first steps of HA degradation, which may have a beneficial effect on the course of EOP. Our results showed that 24 h of 4-MU treatment did not increase the level of apoptosis in either orbital or dermal fibroblast cultures, but further studies are needed to clarify this. The concentration of 4-MU required to the maximum inhibitory effect was not toxic to the cells. Our cytotoxicity study has shown that the decrease of metabolic activity based on formazan conversion generally overestimates the effect of 4-MU on cell viability.

The above-mentioned properties of 4-MU contribute to a new potential direction in the treatment of EOP. In our work, we demonstrated that 4-MU interferes with fibroblast activation in at least two ways: by reducing proliferation and inhibiting HA synthesis. Because the tissue microenvironment and its HA content contribute to the maintenance of local inflammatory processes, 4-MU may positively influence the course of EOP. 4-MU treatment may indirectly affect cell-cell interactions, which are important in antigen presentation. Further, 4-MU is known to inhibit adipogenesis, another important factor in disease progression in EOP.

One potential weakness of our study is its *in vitro* nature. In the human orbit, the environment is much more complex: systemic immune and endocrine, as well as local factors, increased tissue pressure, and changed blood supply are present together. However, any of these factors may be a potential point of intervention in the pathomechanism of the disease.

Summary

Endocrine orbitopathy (EOP) is an autoimmune disorder of retroocular soft tissues which may accompany Graves' disease (GD). In addition to leading to decrease in quality of life, in severe cases, it may result in loss of vision. Hyaluronan (HA) overproduction is a major factor in the pathogenesis of EOP, playing a key role in the autoimmune inflammation of the orbital connective tissue and external eye muscles.

We have shown that increasing cell density of orbital fibroblasts is a negative regulator of proliferation and plasminogen activator inhibitor-1 (PAI-1) secretion in both control and EOP-derived fibroblasts. With increasing cell density, the amount of PAI-1 protein and mRNA expression per cell decreased in all examined cell lines, while the amount of HA per cell produced by fibroblasts did not show density dependence. The negative regulatory effect of increasing cell density was partially inhibited by transforming growth factor β (TGF-β). TGF-β stimulated *PAI-1* expression, and this effect is more marked with increasing cell density in orbital fibroblasts, while was missing in dermal fibroblasts. Increased HA production by TGF-β may reduce contact inhibition, and lead to increased proliferation rate, which is further supported by elevated PAI-1 synthesis.

HA production of the fibroblasts was reduced in all cell lines after 4-methylumbelliferone (4-MU) treatment. 4-MU treatment decreased hyaluronan synthase 2 (HAS2) and increased hyaluronan synthase 3 (HAS3) and hyaluronidase 2 (HYAL2) expression, while hyaluronidase 1 (HYAL1) expression remained unchanged. EOP fibroblasts and fibroblasts from healthy orbits behaved similarly: 4-MU inhibited both basal and TGF-β-stimulated HA production and reduced the proliferative potential in all fibroblast lines examined.

Any intervention that reduces HA overproduction may be beneficial in EOP. As no suitable animal model of EOP is available, clinical trials should provide additional information on 4-MU's applicability as a treatment option.

Appendix:

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List of publications related to the dissertation

1. **Galgóczi, E.**, Jeney, F., Katkó, M., Erdei, A., Gazdag, A., Sira, L., Bodor, M., Berta, E., Ujhelyi, B., Steiber, Z., Győry, F., Nagy, E. V.: Characteristics of Hyaluronan Synthesis Inhibition by 4-Methylumbelliferone in Orbital Fibroblasts.
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2. **Galgóczi, E.**, Jeney, F., Gazdag, A., Erdei, A., Katkó, M., M. Nagy, D., Ujhelyi, B., Steiber, Z., Győry, F., Berta, E., Nagy, E. V.: Cell density dependent stimulation of PAI-1 and hyaluronan synthesis by TGF-[béta] in orbital fibroblasts.
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3. Sumanszki, C., Kiss, E., Simon, E., **Galgóczi, E.**, Soós, Á., Patócs, A., Kovács, B., Nagy, E. V., Reismann, P.: The Association of Therapy Adherence and Thyroid Function in Adult Patients with Phenylketonuria.
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Total IF of journals (all publications): 16,571
Total IF of journals (publications related to the dissertation): 8,518

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *