Final Report NKFIH K105733

Orbital Connective Tissue Related Factors in the Pathogenesis of Graves' Orbitopathy

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Period: 2012-12-01 – 2015-11-30

Graves' orbitopathy (GO) is an autoimmune inflammatory disease of the orbital tissues which accompanies Graves' disease in 10-30 % of the cases. Orbital fibroblasts (OFs) are the main target cells in GO due to their expression of autoantigens specific to Graves' disease. Leukocyte infiltration leads to cytokine-dependent activation of the OFs, which augments the inflammatory and autoimmune processes and accounts for orbital tissue remodelling. Stimulated OFs deposit extracellular matrix components, mainly hyaluronan (HA) and proliferate in an unregulated manner. Furthermore, extracellular matrix remodelling requires proteolytic enzyme activity, which is regulated by the plasminogen activator type 1 (PAI-1). The role of PAI-1 in the pathogenesis of GO is still unclear. The purpose of this study has been to set up an *in vitro* model for the examination of the effect of hormonal, immune and local orbital factors on OFs. Responses were evaluated by the measurement of proliferation, PAI-1 expression and HA production. HA is synthesized at the plasma membrane by three isoenzymes named hyaluronan synthase (HAS) 1, 2 and 3, which possess different biochemical properties. However changes in the transcriptional level of HAS do not always correlate with changes in the HA secretion. HA has a high turnover rate; hyaluronidase (HYAL) 1 and 2 are the major hyaluronidases expressed in human tissues. HA is retained as a pericellular coat after its synthesis, anchored to the cell surface via the synthase enzyme or through binding to a surface receptor, and certain amount cleaved by

hyaluronidase is released from the pericellular matrix and incorporated as an integral component of the extracellular matrix. HYAL2 initiates the degradation of HA at the cell surface to smaller chains, which are then further degraded by HYAL1. Alterations in this process could affect the physiological role of HA in surrounding tissue. mRNA expression of HAS1, 2, 3 and HYAL1, 2 were also measured in the experiments.

During the first year of the project, connective tissue samples were collected and primary cultures of fibroblasts were established. Orbital connective tissue samples were obtained from patients undergoing orbital decompression surgery for severe GO (n=5) or during surgery for non-orbital eye diseases from patients with no history of thyroid diseases (n=5), while control non-orbital connective tissues were obtained during abdominal hernia operations from patients without thyroid diseases (n=3). Briefly, tissue samples were minced and placed in culture dishes containing culture medium allowing fibroblasts to proliferate. After two passages, cells were cryopreserved for later use. Fibroblasts from several tissue samples were cultured and tested simultaneously. Regardless of the anatomic site of origin, fibroblasts synthesized and released PAI-1 and HA into the culture medium, which could be detected by ELISA; expressed PAI-1, HA synthase (HAS) 1, 2, 3 and hyaluronidase (HYAL) 1, 2 mRNA. In summary, we established morphologically stable cell lines from surgical tissue samples, and we demonstrated that this model was a useful tool to study the effects of several factors on PAI-1 and HA production of OFs; the latter were related to the pathogenesis of GO. Due to the infrequent nature of severe GO requiring orbital surgery, we were able to start experiments only close to the end of the first year.

PAI-1 expression has been shown to be cell density dependent in various cell types by others. Those studies revealed that both PAI-1 synthesis and proliferation rate decreased during the process of growth towards confluency. In the first series of experiments, fibroblasts were cultured at different cell densities to study how PAI-1 expression is altered by the increased fibroblast proliferation during the course of GO. Regardless of anatomic site of origin, the proliferation rate measured by thymidine analogue incorporation, as well as protein and mRNA levels of PAI-1 per cell significantly decreased with increasing cell densities. We found no difference in the cell density dependent PAI-1 expression between normal and GO OFs. These in vitro findings indicate that high cell density is a negative regulator of proliferation and PAI-1 expression in the orbital connective tissue; we are the first to show this phenomenon in orbital fibroblasts. We assume that the growth state of OFs at different cell densities is the main determinant of PAI-1 expression. We have hypothesized that

confluent cultures with contact inhibition correspond to the healthy orbit, while pre-confluent cultures represent the expanding orbital tissue with higher fibroblast proliferation rate. Proliferation rate dependent regulation of PAI-1 expression in the orbit, together with other local factors, might have a regulatory role in the turnover of the orbital extracellular matrix under both normal and disease conditions. These results were presented by us at the XXV. Congress of Hungarian Society of Endocrinology and Metabolism (Pécs, Hungary, 5-7 June 2014) and at the 38th Annual Meeting of European Thyroid Association (Santiago de Compostela, Spain, 6-10 September 2014).

We found that HA secretion into the medium and pericellular HA levels did not show a cell-density dependent pattern, but were highly dependent on the origin of fibroblasts: dermal fibroblasts synthesized greater amount of HA than OFs. We found significant positive correlation between HA released into the medium and retained in the pericellular matrix. Results of the RT-PCR showed that HAS1 and HAS3 expression were in the same order of magnitude, while HAS2 expression was the predominant HAS enzyme in all cell lines studied. The expression patterns of HAS enzymes were different in fibroblasts with distinct types of origin. Dermal fibroblasts had higher expression levels of HAS1 and HAS2 mRNA than OFs, while OFs had higher HAS3 expression than dermal fibroblasts. GO OFs had higher HAS3 expression than normal OFs. The expression of HAS1 and HAS2 decreased with increasing cell density in dermal fibroblasts. The expression of HYAL1 was more diverse than the expression of HYAL2. Dermal fibroblasts had the lowest HYAL1 expression, and GO OFs had lower HYAL1 expression than normal OFs. There was a tendency for lower HYAL1 and HYAL2 expression in confluent cultures than in pre- and postconfluent cultures.

Strong TGF-β expression has been found by others in the orbital tissue of patients with GO which correlated positively with the clinical activity score. We tested the effect of TGF-β in our model. Dose-response experiments were performed in a range of 0.01-10 ng/ml; 1 ng/ml TGF-β had the maximal effect on PAI-1 secretion during the 24 hour-treatment and this concentration was selected for further experiments. At each density TGF-β stimulated PAI-1 secretion in all tested cell lines irrespective of the site of origin. In both GO and control OFs, but not in dermal fibroblasts, more marked stimulation of PAI-1 secretion was seen with increasing densities. The same stimulation pattern was detected when PAI-1 mRNA levels were examined under the effect of TGF-β. Thus, the PAI-1 lowering effect of high cell densities has been partially reversed by TGF-β. Significant stimulatory effect of TGF-β on HA secretion into the medium and pericellular HA levels was seen at the highest cell

densities, irrespective of the origin of fibroblasts. The positive correlation between HA in the medium and pericellular HA seen in unstimulated cultures remained significant in cell cultures after 24-hour TGF-β treatment. The expression of HAS2, HAS3, HYAL1 and HYAL2 did not changed or slightly decreased in TGF-β treated cells, while HAS1 mRNA expression increased markedly in a cell density dependent manner especially in OFs. In orbital fibroblast cultures, TGF- β induced increase of PAI-1 secretion was directly proportionate to the changes of HA production. There was only a minor effect of TGF-β on the proliferation rate, slight non-significant increase was only observed at the highest density of OFs. From the point of disease course the matrix overproducing responses of orbital fibroblasts to TGF-β at high cell densities may be detrimental in GO. HA overproduction can diminish contact inhibition and lead to elevated proliferation. In combination with previous findings, ie. HA increased PAI-1 expression in a concentration dependent manner in human vascular smooth muscle cells, our data suggest that the TGF-β induced HA secretion at high cell densities may facilitate the cell-density dependent PAI-1 stimulation in OFs. Results described above were published in the Journal of Endocrinology: **Galgoczi E, Jeney F, Gazdag A, Erdei A, Katko M, M Nagy D, Ujhelyi B, Steiber Z, Gyory F, Berta E, Nagy EV. Cell density dependent stimulation of PAI-1 and hyaluronan synthesis by TGF-β in orbital fibroblasts. J Endocrinol. 2016 Mar 15. pii: JOE-15-0524. Epub ahead of print.**

The active phase of GO is characterized by inflammation targeting orbital tissues. Immunohistochemical evidence of the presence of cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) has been reported by others in the orbital connective tissues, and their presence is associated with T cell infiltration. We aimed to evaluate the effects of stimulation with these recombinant human cytokines on PAI-1 expression and HA synthesis of OFs. TNF-α (1-10 ng/ml) stimulated PAI-1 production after 24, 48 and 72-hour treatment; IFN-γ treatment (1-10 ng/ml) had no effect on PAI-1 protein level. Neither TNF-α, nor IFN-γ stimulated HA production of OFs in this range of concentrations.

The recently approved extension of the project period allowed us to complete the experiments to study the effect of hypoxia and thyroid stimulating hormone (TSH) treatment on HA and PAI-1 production of primary cell cultures. Cells were cultured under hypoxic (1% O_2, 5% CO_2, 94% N_2 or 5% O_2, 5% CO_2, 90% N_2) and normoxic (21% O_2, 5% CO_2, 74% N_2) conditions for 24, 48 and 72 hours. No changes in the PAI-1 and HA production were detected in hypoxia compared to normoxia. The effect of human TSH (10-1000 mU/L) on

PAI-1 and HA synthesis was also studied. We did not found any significant changes in these parameters under the effect of human TSH.

In addition, we have studied the effectiveness of B cell depletion by anti-CD20 antibody (rituximab) therapy in severe conventional therapy resistant GO. In all patients treated with rituximab a definite clinical improvement of GO has been observed by three independent methods: clinical activity score, MRI and SPECT. These results were published within the scope of this project: **Erdei A, Paragh G, Kovacs P, Karanyi Z, Berenyi E, Galuska L, Lenkey A, Szabados L, Gyory F, Ujhelyi B, Berta A, Boda J, Berta E, Bodor M, Gazdag A, Nagy EV. Rapid response to and long-term effectiveness of anti-CD20 antibody in conventional therapy resistant Graves' orbitopathy: A five-year follow-up study. Autoimmunity. 2014 Dec;47(8):548-55.**