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Insulin-like growth factor 1 promotes the proliferation and adipogenesis of orbital adipose-derived stromal cells in thyroid-associated ophthalmopathy

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ABSTRACT

Thyroid-associated ophthalmopathy (TAO) is characterised by increased volume of the orbital contents involving adipose tissue, but the factors responsible for stimulation of orbital adipogenesis remain uncertain. Previous studies have shown that insulin-like growth factor 1 (IGF-1) is increased in the orbital fatty connective tissues of patients with TAO. The present study was conducted to investigate the effects of IGF-1 on orbital adipose-derived stromal cells (OADSCs) derived from TAO patients and to identify the signalling mechanisms involved. Our results showed that IGF-1 significantly promoted the cell proliferation and lipid accumulation of TAO OADSCs. The mRNA expression of adipogenic markers (adiponectin, leptin, adipocyte fatty acid binding protein [*AP2*] and fatty acid synthase [*FAS*]) was increased in TAO cultures treated with IGF-1. Further research demonstrated that the protein levels of peroxisome proliferator-activated receptor-γ (PPARγ) were up-regulated when OADSCs were treated with IGF-1. We also found that the inhibition of either IGF-1 receptor (IGF-1R) or phosphoinositide 3-kinase (PI3K) activity decreased the levels of IGF-1-stimulated mRNA encoding adiponectin, leptin, *AP2*, and *FAS*, as well as PPARγ protein levels. Moreover, the expression of phosphorylated Akt (p-Akt) protein in TAO cells was up-regulated by IGF-1, while a specific PI3K inhibitor (LY294002) or an antibody of IGF-1R blocked this effect. These results indicate that IGF-1 is a pro-proliferative and pro-adipogenic factor in TAO OADSCs. IGF-1 enhances the adipogenesis of TAO OADSCs by up-regulation of PPARγ via the activation of the IGF-1R and PI3K pathways, suggesting that the blocking of IGF-1R or inhibition of PI3K signalling might be a potential novel therapeutic approach to TAO.

1. Introduction

Thyroid-associated ophthalmopathy (TAO), also known as Graves' ophthalmopathy (GO), is an autoimmune inflammatory disease, that is characterised by expansion of the orbital adipose tissue and enlargement of the extraocular muscles (Garrity and Bahn, 2006; Naik et al., 2010). These changes lead to most of the signs and symptoms of TAO, including exophthalmos, eyelid retraction, conjunctival injection, chemosis, corneal ulceration, diplopia, and even loss of vision in extreme cases (Bahn, 2010; Bartalena and Tanda, 2009). While most patients with TAO have enlargement of both the orbital adipose compartment and the extraocular muscles, the severity of proptosis appears to be more closely related to orbital adipose expansion than to extraocular muscle enlargement (Nishida et al., 2002; Peyster et al., 1986). Thus expansion of the orbital fat compartment represents a major component of the disease process (Kumar et al., 2004).

This expanded orbital adipose tissue volume is thought to be due to both hyaluronic acid-related oedema and the emergence of a population of newly differentiated fat cells within these tissues (Bahn, 2003). These newly differentiated fat cells within the orbit suggest that resident orbital adipose-derived stromal cells (OADSCs) are stimulated by some factors to undergo adipocyte differentiation (adipogenesis). Several groups have confirmed that *de novo* adipogenesis within the orbital tissues is enhanced in TAO (Crisp et al., 2000; Kumar et al., 2004). TAO is most likely initiated by autoreactive T lymphocytes, which trigger a cascade of events, including the secretion of cytokines. These cytokines stimulate the proliferation of orbital fibroblasts, the expansion of adipose tissue, and the secretion of hydrophilic glycosaminoglycans from fibroblasts (Bartalena and Tanda, 2009). Thus, orbital adipose tissue expansion is thought to be driven by mediators present within the orbital

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tissue, but the factors responsible for stimulation of orbital adipogenesis remain uncertain.

Insulin-like growth factor 1 (IGF-1) is a polypeptide, the expression of which in most tissue is known to regulate adipose tissue mass through its regulation of adipogenesis (Grohmann et al., 2005; Smith et al., 1988). A number of studies have demonstrated that IGF-1 levels were increased in samples of eye fat and muscle, which were obtained after decompression surgery from patients with TAO (Hansson et al., 1986; Matos et al., 2008). Local production of IGF-1 by orbital fibroblasts was elevated in TAO patients (Song et al., 2012). These finding suggest that IGF-1 might be actively involved in the evolution of the orbital adipose tissue expansion in TAO. However, adipose-derived stromal cells (ADSCs) exhibit depot-specific differences in the expression of adipocyte-specific genes, and they vary in their adipogenic potential. Activators of peroxisome proliferator-activated receptor-γ (PPARγ) enhance differentiation of ADSCs from subcutaneous sites, but the cells from omental sites are refractory to these agents (Adams et al., 1997). IGF-1 activation of the Akt pathway was impaired in visceral, but not subcutaneous, preadipocytes from obese subjects (Cleveland-Donovan et al., 2010). Much remains unknown about the effects of IGF-1 on OADSCs in TAO.

In the present study, we investigated the role of IGF-1 on the proliferation and adipogenesis of OADSCs in TAO patients. The phosphoinositide 3-kinase (PI3K) signalling cascades in OADSCs were also investigated to understand better the signalling mechanisms that might be engaged and utilised to develop new therapeutic approaches for patients with TAO.

2. Materials and methods

2.1. Orbital tissue specimens

Orbital adipose tissue specimens were obtained from euthyroid patients who underwent orbital decompression surgery for severe TAO ($n = 12$). This research was conducted according to the principles of the Declaration of Helsinki; informed consent was obtained, and the Shanghai Ninth People's Hospital Ethics Committee approved the study. The orbital adipose tissue specimens were placed in sterile containers on saline-soaked gauze and were transported at room temperature to the laboratory.

2.2. Isolation and culture of orbital adipose-derived stromal cells

The procedure of OADSC isolation has been described previously (Lin et al., 2008). Briefly, the orbital adipose tissues samples were fragmented with surgical scissors and were suspended in 0.075% collagenase type 1 (Sigma–Aldrich, Saint Louis, MO) and phosphate buffered saline (PBS) at 37 °C. After 60 min of digestion, the fragmented tissues were filtered through a 70 μm filter. The remaining cells were centrifuged at 350 g for 10 min at room temperature. After resuspension of the pellet, the cells were counted and plated in 10 cm dishes with DMEM/F12 containing 10% foetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), penicillin (100 U/ml), and gentamicin (20 μg/ml) in a humidified 5% CO2 incubator at 37 °C. The media were replaced every 3–4 days.

2.3. Surface immuno-phenotyping

For cell surface antigen immuno-phenotyping, three- to five-passage OADSCs were detached and stained with fluorescein isothiocyanate (FITC)-conjugated antibodies and were analysed with FACSCalibur. Antibodies against the human antigens CD29, CD31, CD44, CD45 were purchased from eBioscience (San Diego, CA).

2.4. CCK-8 assay for cell proliferation

Logarithmic growth phase OADSCs were digested with trypsin in a single-cell suspension and were seeded into 96-well tissue culture plates at a density of 1.5×10^3 cells per well. After seeding and incubation at 37 °C for 12 h, the medium was changed from DMEM/F12 containing 10% FBS to serum-free DMEM/F12 for serum starvation, and the new medium was maintained for 24 h. IGF-1 alone (BioVision Inc., Milpitas, CA) at 1, 10, 20, 100, and 200 ng/ml, or in combination with the specific blocking antibody for IGF-1R, αIR3 (1 μg/ml), which was added separately to DMEM/F12 containing 5% FBS in the experimental or control groups. After 72 h of culture, the cells were treated with Cell Counting Kit-8 reagent (CCK-8; Dojindo Lab, Kumamoto, Japan) and were incubated at 37 °C for 4 h. The data of OD (optical density) values at 450 nm were read by a microplate reader (BioTek ELx 800, Winooski, VT).

To analyse the growth kinetics of IGF-1-treated OADSCs, cells were seeded into 96-well plates at a density of 2×10^3 cells/well. After serum starvation, the cells were treated with DMEM/F12 containing 5% FBS in the absence or presence of IGF-1 (10 ng/ml), or in both IGF-1 (10 ng/ml) and αIR3 (1 μg/ml) for the indicated lengths of time. CCK-8 was added at the time points of 0, 1, 2, 3, 4, 5, and 6 days after culture and incubation at 37 °C for 4 h. The data on OD values at 450 nm were read by a microplate reader (BioTek ELx 800, Winooski, VT).

2.5. Adipocyte differentiation

Several studies were performed to determine the impact on adipogenesis of IGF-1. For these studies, confluent OADSCs were treated for 10 days with IGF-1 (10 ng/ml), IGF-1 (10 ng/ml) plus αIR3 (1 μg/ml; Calbiochem, La Jolla, CA), IGF-1 (10 ng/ml) plus LY294002 (10 μM; Cell Signaling Technology, Danvers, MA, USA), LY294002 (10 μM), or no treatment, and were cultured in adipocyte differentiation medium (without insulin) consisting of serum-free DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with biotin (33 μM; Sigma), pantothenic acid (17 μM; Sigma), transferrin (10 μg/ml; Sigma), triiodothyronine (T_3) (0.2 nM; Sigma), carbaprostacyclin ($cPGI_2$; 0.2 μM) (Cayman Chemical, Ann Arbor, MI), and, for the first 4 days only, dexamethasone (1 μM; BioVision Inc., Milpitas, CA) and isobutylmethylxanthine (IBMX; 0.1 mM; Sigma) (Valyasevi et al., 2002; Kumar et al., 2011). The media were replaced every 2 days during the 10-day differentiation period.

2.6. Oil Red O staining

The cells induced to differentiate as adipocytes were washed gently with PBS twice, were fixed with 4% fresh formaldehyde in PBS for 15 min at room temperature and were stained with filtered Oil Red O (Sigma) solution (60% isopropanol and 40% water) for 30 min. The Oil Red O-stained cells were observed with an Olympus microscope after washing. Finally, the absorbed dye in the cells was extracted with 100% isopropanol and was quantified by measuring its optical absorbance at 492 nm.

2.7. RNA isolation, reverse transcription, and semi-quantitative real-time polymerase chain reaction analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration and purity of the total RNA were determined spectrophotometrically at OD260 nm and OD280 nm. DNase I was used to eliminate genomic DNA contamination. The cDNA was synthesised from 1 μg of total RNA using a PrimeScriptTM RT reagent