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Peroxisome Proliferator-Activated Receptor-γ Gene Expression in Orbital Adipose/Connective Tissues is Increased During the Active Stage of Graves' Ophthalmopathy

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The mechanisms involved in the increase of orbital retro-ocular adipose tissue that occurs in Graves' ophthalmopathy (GO) are still unclear. In this condition, the orbital tissue shows glycosaminoglycans deposition produced by activated fibroblasts capable of undergoing adipocytic differentiation. Many genes are involved in adipogenic mechanisms including the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ). We evaluated the level of expression of the PPAR-γ gene in normal and GO orbital adipose/connective tissue specimens using a quantitative and sensitive reverse transcription (RT) competitive polymerase chain reaction (PCR) assay. Our results show that the expression of PPAR-γ was significantly greater in adipose/connective tissue from patients in the active stage of GO than in controls (150.8 ± 103.9 and 24.0 ± 4.9 amol/micro g of total RNA respectively, $p < 0.05$), while there was no significant difference between patients with inactive GO (58.8 ± 40.6 aM/μg total RNA) and controls. These results suggest that increased PPAR-γ gene expression in the active stage of GO may be dependent on the inflammatory process in this disease. We speculate that the increased orbital fat tissue observed in GO may be a consequence of the anti-inflammatory PPAR-γ action.

Introduction

MANY OF THE CLINICAL SIGNS and symptoms of Graves' ophthalmopathy (GO) can be explained by an increase in the volume of orbital tissues as measured by computed tomography (1,2). The overall increase of orbital volume may result both from an accumulation of hydrated hyaluronan in the orbital muscles and connective tissues and from an expansion of the adipose tissues within the orbit (3).

Fatty orbital adipose tissue in GO has been the subject of much research in recent years (3–7). The development of adipose tissue requires adipocytic differentiation of fibroblasts. Adipocyte precursor cells have been isolated from the stromal-vascular fraction of neonatal and adult human adipose/connective tissues from several regions of the body (8). These cells, termed preadipocyte fibroblasts, are thought to be a subpopulation of fibroblasts, and are capable of undergoing adipocytic differentiation when cultured in an appropriate medium (9). In 1996, Sorisky and colleagues (5) showed that human orbital fibroblasts contain such a subpopulation capable of *in vitro* adipogenesis. Fibroblasts derived from the connective/adipose tissues of the orbit differ from those of other anatomic regions in many aspects including the capacity for adipocytic differentiation. Smith and colleagues (10) have reported that heterogeneous phenotypes exist even among different types of orbital fibroblasts. Fibroblasts derived from the perimysium of orbital muscles do not differentiate into adipocytes while those derived from connective/adipose orbital tissue are capable of adipocytic differentiation when incubated under identical culture conditions (10). Thus, it appears that functional changes in these types of orbital fibroblasts might account for the increase in orbital adipose tissue seen in patients with GO.

Adipocytic differentiation from preadipocytes is a complex process that appears to be controlled by several transcription factors (11–13). Peroxisome proliferator-activated receptor (PPAR)-γ is an important member of the nuclear hormone receptor superfamily of ligand-dependent transcription factors and has a dominant regulatory role in adipocytic differentiation (14,15). In the present study, we determined the PPAR-γ gene expression in adipose tissues from patients in the active and nonactive stage of GO, as well as in normal orbital fat/connective tissue specimens.

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Patients and Methods

Patients

Orbital adipose specimens were obtained and immediately frozen during orbital decompression surgery involving 13 patients with severe GO, ranging from 21 to 55 years of age (median, 35 years). Four patients (patients 1, 2, 3, and 11) were males and 9 were females. Information on the activity, duration, and therapy for ophthalmopathy is provided in Table 1. Thyroid status of the patients at the time of surgery was euthyroidism in 9, hyperthyroidism in 2 (patients 2 and 6), and hypothyroidism in 2 (patients 1 and 10).

Four patients (patients 1 to 4) were in the active stage of GO. The disease was defined as active because of the presence of progressive proptosis and severe orbital congestion, characterized mainly by inflammatory changes including chemosis, conjunctival congestion, periorbital pain, and swelling or optic neuropathy. All of them had Mouritz Clinical Activity Score (CAS index) equal to or higher than 5, compatible with an active orbital disease (16). Three of the patients had received previous treatment with corticosteroids and one was taking prednisone at the time of surgery. Two months prior, patient 1 had received 1000 mg of methylprednisolone per day for 3 days and was taking 60 mg of prednisone per day at the time decompression surgery was performed to alleviate optic neuropathy. His CAS index was 8 at the time of surgery and his body mass index (BMI) was 25.0 kg/m^2. Patients 2 (CAS index 9, BMI 22.0 kg/m^2) and 3 (CAS index 6, BMI 21.8 kg/m^2) required surgery because they developed corneal ulceration. Patient 2 had received 60 mg of prednisone per day for 2 weeks 1 month previously; patient 3 had never received corticosteroid treatment. Patient 4 (BMI 24.0 kg/m^2) had received prednisone and orbital irradiation 6 months previously but was not taking corticosteroids at the time orbital decompression was performed because of progressive proptosis and congestive ophthalmopathy with persistent swelling and hyperemia of the lids and conjunctiva, with a CAS index of 5.

The nine patients (patients 5 to 13) whose disease was defined as inactive had been clinically stable for at least 6 months before surgery. They had stable exophthalmometric and extraocular motility measurements. None presented with orbital congestion or chemosis although many complained of difficulties closing the eyes. Patients 5, 9, 10, and 11 had CAS index of 1 and the remaining had CAS index of 0. Surgery was indicated in the nonactive disease patients for rehabilitative reasons as well as to reduce symptoms related to incomplete lid closure. Patients 5 to 13 had BMI ranging from 18.3 to 34.0 kg/m^2 (median, 25.4).

Orbital tissue was obtained from 4 female controls without thyroid disease, ranging in age from 17 to 39 years (median, 35.5 years), who had undergone ocular surgery for conditions not affecting the soft tissues of the orbit (cosmetic blepharoplasty in two and strabismus surgery in two). Specimens were frozen in liquid nitrogen immediately after collection. Informed consent was obtained before surgery for all patients and controls, with the approval of the local ethics committee.

Methods

RNA extraction. Total RNA from adipose tissue was extracted by the trizol isolation method (Life Technologies, Rockville, Maryland). The RNA samples were quantified by spectrophotometry. The integrity of the total RNA samples was assessed by agarose gel electrophoresis and ethidium bromide staining. The RNA samples were stored at −70°C until use.

PPAR-γ RNA analysis. The absolute mRNA concentration of the PPAR-γ was measured by reverse transcription (RT) reaction followed by competitive polymerase chain reaction (PCR). After cDNA synthesis by specific RT from tissue RNA preparations, the method relies on the coamplification of known amounts of a competitor DNA with the target cDNA.

Competitor. The homologous competitor was provided by Dr. Hubert Vidal (Human Nutrition Research Center of

Table 1. Clinical Data and Treatment Modalities in Thirteen Patients with Graves' Ophthalmopathy

							ORB. Treatment		
					Thyroid treatment		Corticosteroids		Irradiation
Patient	Age	DTD (years)	DO (years)	ORB. status	At surgery	Previous	At surgery	Previous	Previous
1	38	6	0.5	Active	L-thyroxine	RAI	+	+	−
2	45	1	1	Active	—	—	−	+	−
3	42	0.3	0.3	Active	PTU	Methimazole	−	−	−
3	55	3	1.5	Active	L-thyroxine	L-thyroxine	−	+	+
5	35	11	11	Inactive	L-thyroxine	Thyroidectomy	−	−	−
6	40	2	2	Inactive	PTU	Methimazole	−	−	−
7	35	4	4	Inactive	—	RAI, methimazole	−	−	−
8	30	6	3	Inactive	PTU	Methimazole	−	−	−
9	29	2	2	Inactive	—	—	−	+	−
10	33	2	2	Inactive	PTU	RAI, PTU	−	−	+
11	42	4	4	Inactive	PTU	Methimazole	−	+	−
12	21	2	2	Inactive	L-thyroxine	Thyroidectomy	−	+	+
13	21	5	5	Inactive	PTU	Methimazole	−	−	−

M, male; F, female; DTD, duration of thyroid disease; DO, duration of orbitopathy; ORB, orbitopathy; RAI, radioactive iodine; PTU, propylthiuracil.

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Lyon, Lyon, France) who constructed the competitor by modifying the sequence of cDNA encoding PPAR-γ (17).

RT-competitive PCR. For each mRNA, first-strand cDNA synthesis was performed from 0.2 μg of total RNA in the presence of 100 mM of dNTPs, 0.1 M of dithiothreitol (DTT) and 37.5 pmol of the designed antisense primer, with 200 units of a thermostable reverse transcriptase (Superscript II Gibco BRL, Rockeville, MD) in a final volume of 20 μL. This RT medium was incubated for 60 minutes at 42°C, then for 15 minutes at 70°C in a thermocycler (GeneAmp PCR system 9600 Perkin Elmer, Norwalk, CT).

PCR was performed with a mix of: 10 mmol/L Tris-HCl, pH 8.3, 100 mmol/L KCl, 0.75 mol/L ethyleneglycoltetraacetic acid (EGTA), 5% glycerol, 0.2 mmol/L deoxynucleoside triphosphates, 45 pmol specific sense primer: TCTCTCCGTAATGGAAGACC 37.5 pmol antisense primer: GCATTATGAGACATCCCCAC and 5 units Taq polymerase, in a final volume of 112.5 μL. Six aliquots (18.75 μL) of the mixture were then transferred to microtubes containing a different but known amount of competitor and RT medium (1.25 μL). After 4 minutes at 95°C, the tubes were subjected to 40 cycles of amplification, including denaturation for 40 seconds at 95°C, hybridization for 50 seconds at 55°C, and elongation for 50 seconds at 72°C.

PCR product analysis. The PCR products were analyzed in 2% agarose (Gibco BRL) gel, stained with ethidium bromide, and visualized under UV light (Fig. 1). The density of the competitor and target bands was measured using molecular analytic image software. To determine the point of equivalence, at which the densities of the competitor and target bands were equal, the log of the ratio of the competitor (C) to the target (T) band densities was plotted against the log of the competitor concentration. The resulting curve was rectilinear with a slope of 1, demonstrating that the competitor was amplified with the same efficiency as the target mRNA. The point of equivalence lies at C/T = 1.

Validation of the RT-PCR assay. To validate the RT-competitive PCR assay, we made a dose response curve with a known amount of RNA. The initial concentration of the RNA stock solution was determined by absorbance measurement at 260 nm before serial dilutions in water (from pmol/μL to amol/μL). The slope obtained measured 1.09 ($r = 0.99$) demonstrating that the RT-quantitative PCR assay allowed an accurate quantification of RNA over a wide range of concentrations.

Statistical analysis

Comparisons among groups were performed using Kruskal-Wallis analysis of variance (ANOVA) by ranks analysis followed by the Dunn test as *post hoc* comparisons of mean pairs, and using the Mann-Whitney *U* test. Statistical differences were considered significant for $p < 0.05$.

Results

The results of the PPAR-γ gene expression experiments in patients with GO and normal controls are shown in Figure 2 and Table 2. PPAR-γ gene expression in adipose tissue from patients with GO and normal controls was 87.1 ± 75.8 and 24.0 ± 4.9 amol/micro l (mean ± standard deviation), respectively. The results were significantly greater in patients with GO than in controls ($p < 0.05$). Specimens from patients with inactive and active GO yielded 58.8 ± 40.6 aM/μg and 150.8 ± 103.9 aM/μg, respectively.

The PPAR-γ gene expression in adipose tissue was significantly greater in patients with the active form of GO than controls (150.8 ± 103.9 aM/μg total RNA and 24.0 ± 4.9 aM/μg total RNA respectively, $p < 0.05$). There was no significant difference in PPAR-γ gene expression between patients with inactive ophthalmopathy (58.8 ± 40.6 aM/μg RNA total) and controls (Table 2, Fig. 2).

Discussion

The mechanism involved in the increase of adipocyte tissue in GO remains unclear. Adipogenesis is a complex process involving the interplay of several transcription factors. The finding in GO of high levels of PPAR-γ expression in orbital adipose tissue, PPAR-γ being one of the most important adipogenic transcription factors, may contribute to our understanding of the phenomenon. Our *in vivo* findings are in accordance with data published by Pasquali and colleagues (18), who evaluated the level of expression of PPAR-γ 2 in primary cultures of fibroblasts from GO and normal retroorbital tissues. They concluded that the mRNA expression

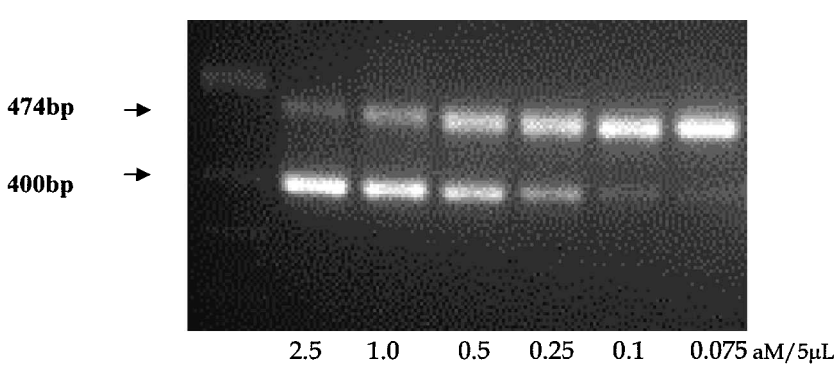

FIG. 1. Representative result of competitive reverse transcription-polymerase chain reaction (RT-PCR). The PCR products were analysed in 2% agarose gel, stained with ethidium bromide. The upper band is the target type product (474 bp) and the lower band the mutant product (400 bp). Lanes 1–6: decreasing diluition of the mutant (2.5; 1.0; 0.5; 0.25; 0.1; 0.075 aM/5 μL)

Table 2. PPAR-γ Gene Expression (aM/μg total RNA) from Orbital Adipose Tissue in Patients with Graves' Ophthalmopathy (GO) and Controls

	Active go Patients 1 to 4	*Inactive go Patients 5 to 13*	*Controls*
	159	106	28
	288	28.4	22
	40	77	28
	116	51	18
		12	
		48	
		136	
		47	
		23.7	
M ± SD	150.8 ± 103.9	58.8 ± 40.6	24.0 ± 4.9

M, mean; SD, standard deviation.

was significantly higher in GO than in normal orbital fibroblast cultures.

Because orbital adipocytes from patients with GO have shown expression of thyrotropin (TSH) receptor (3,19), a presumed major antigen involved in the pathogenesis of this disease, some studies have explored the link between adipogenesis and induction of TSH receptor expression in orbital fibroblasts. Sorisky and colleagues (5) revealed that cultured orbital preadipocytes can be induced *in vitro* to differentiate into adipocytes bearing a functional TSH receptor. Smith and colleagues (10) showed *in vitro* that fibroblasts derived from the perimysium of orbital muscles do not differentiate into adipocytes while those derived from connective/adipose orbital tissue are capable of adipocytic differentiation when incubated under identical culture conditions. To our knowledge, our study is the first to show an increase in PPAR-γ expression *in vivo* in patients with GO (20), an observation that seems to confirm the involvement of the PPAR-γ transcription factor in the pathogenesis of this disorder.

Our study shows a significantly elevated PPAR-γ expression in patients with active GO compared to controls. This finding could be related either to the inflammatory process of the disease that is present during the active stage or to some other factor, such as drugs and medications that could be taking place concomitantly. PPAR-γ expression can be induced by several substances that have been shown to be agonists of this receptor, including antidiabetic medications such as thiazoladinediones (3,21,22) and nonsteroidal anti-inflammatory drugs such as indomethacin (23). None of our patients used any of the drugs known to be associated with increased expression of PPAR-γ, although some received corticosteroid treatment for severe orbitopathy. Because *in vitro* studies show that dexamethasone is important in adipocyte differentiation by regulating the expression of nuclear factors such as PPAR-γ (24), previous use of prednisone might be considered a possible confounding factor in our results. However, we believe that this is unlikely. Although one of the patients with active GO (case 1) was taking prednisone at the time the orbital samples were obtained and had been taking it for 2 months, a second patient (case 2) had been taking it only for 2 weeks and was not undergoing corticosteroid treatment at the time of surgery. Furthermore, one patient (case 4) with active GO did not receive it for 6 months before surgery and had elevated levels of PPAR-γ. On the other hand, although none of the patients with inactive GO were taking corticosteroids at the time of surgery, four of them had received corticosteroid treatment for prolonged periods of time in the past and presented much lower levels of PPAR-γ.

An analysis of the clinical data presented in Table 1 also seems to exclude other treatment modalities and thyroid status as possible confounding factors. In the group with active GO one patient presented with hypothyroidism, another hyperthyroidism and two were euthyroid. PPAR-γ was elevated in patients with hypothyroidism (case 1), hyperthyroidism (case 2), and euthyroidism (case 3). Therefore, thyroid function would not seem to have had any influence on the PPAR-γ results. In the group with inactive GO one

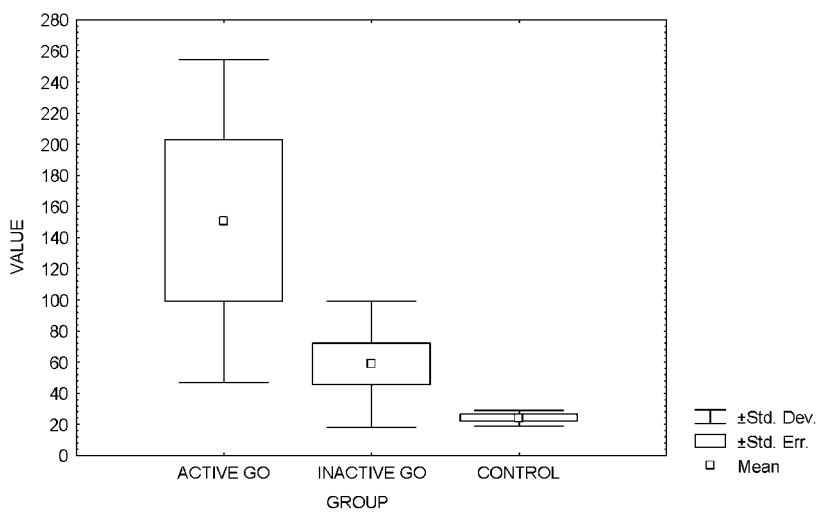

FIG. 2. Graphic representation of peroxisome proliferator-activated receptor (PPAR)-γ mRNA results in controls as well as in patients with active and inactive Graves' ophthalmopathy (GO). Value = mean PPAR-γ mRNA level (amol/μg total RNA). Std. Dev., standard deviation, Std. Err, standard error of the mean.

patient had hypothyroidism and another hyperthyroidism while 7 patients were euthyroid.

Because PPAR-γ has been demonstrated by immunofluorescence to be present in orbital preadipocytes and its role in adipogenesis has been well documented (5,10), an increased amount of this transcription factor in the orbit of patients with GO can be considered an expected finding. Recently, Starkey and colleagues (20) documented worsening of proptosis and increase in fat volume of the orbit in a patient with previously stable GO after the introduction of PPAR-γ agonist thiazolidinedione. Their findings emphasize the importance of PPAR-γ in the pathogenesis of the disease. GO is, however, generally a self-limiting condition and differentiation of fibroblasts to adipocytes probably occurs only during a certain period of time, while progression of eye symptoms occur. The higher expression levels of PPAR-γ in orbital tissues from patients with the active stage of GO compared with those from patients with the non-active stage of the disease and normal orbital adipose/connective tissue specimens are in accordance with such an expectation. PPAR-γ decrease probably correlate with clinical stabilization of symptoms.

The main factors leading to PPAR-γ increase in the orbit of patients with GO not taking PPAR-γ agonists are unknown. Our study, showing high expression of the transcription factor in orbital tissues from patients in the active stage of GO indicate a possible relationship between elevated PPAR-γ levels and the inflammatory process that is triggered by the autoimmune process that is present in the active congestive stage of GO (25). This assumption is in accordance with recent experimental reports that link PPAR-γ with an anti-inflammatory action. For instance, some studies indicated that PPAR-γ ligands reduced the colonic inflammatory process in animal models and inhibited induced cytokine synthesis of mononuclear cells *in vitro* (26,27). Furthermore, Feinstein and coworkers (27) reported that oral use of the PPAR-γ agonist pioglitazone prevents experimental autoimmune encephalomyelitis in mice. Their study indicates that PPAR-γ exerts an anti-inflammatory effect on glial cells and reduces the proliferation and activation of T cells (28). Because PPAR-γ seems to have anti-inflammatory action it is likely that the high levels observed in the active stage of GO is induced by the inflammation produced by the autoimmune process. Orbital adipose tissue enlargement could therefore occur as a secondary event, resulting from raised levels of PPAR-γ.

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