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LABORATORY STUDY

Increased oxidative DNA damage, lipid peroxidation, and reactive oxygen species in cultured orbital fibroblasts from patients with Graves' ophthalmopathy: evidence that oxidative stress has a role in this disorder

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Abstract

Purpose **We investigated the oxidative stress in orbital fibroadipose tissues and cultured orbital fibroblasts from patients with Graves' ophthalmopathy (GO).**

Methods **The content of 8-hydroxy 2′-deoxyguanosine (8-OHdG), an important biomarker of oxidative DNA damage, was measured in orbital fibroadipose tissues and cultured orbital fibroblasts from patients with GO and compared with age-matched normal controls. A product of lipid peroxidation, malondialdehyde (MDA), and intracellular reactive oxygen species (ROS) in cultured orbital fibroblasts was also determined.**

Results **There was no significant difference in the 8-OHdG content of orbital fibroadipose tissues between patients with GO and age-matched normal controls ($P = 0.074$). However, the levels of 8-OHdG and MDA in GO orbital fibroblasts were significantly higher than those of normal controls ($P = 0.0026$ and $P < 0.001$, respectively). In addition, GO orbital fibroblasts had higher contents of superoxide anions and hydrogen peroxide compared with those of normal controls ($P = 0.0133$ and 0.0025, respectively).**

Conclusions **Orbital fibroblasts represent the most abundant cell type among orbital connective tissues and exhibit great differences in their phenotypes. Increased oxidative DNA damage and lipid peroxidation, as well as higher intracellular ROS levels in GO orbital fibroblasts may have a role in the pathogenesis of GO.**

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Keywords: lipid peroxidation; oxidative DNA damage; reactive oxygen species; orbital fibroblasts; Graves' ophthalmopathy

Introduction

Graves' ophthalmopathy (GO), the most frequent extrathyroidal manifestation of Graves' disease, may lead to functional disability (diplopia, exposure keratopathy, and vision impairment) and social disturbance (cosmesis) of affected individuals.[1–3] Despite recent progress in the understanding of its pathogenesis, clear and indisputable mechanisms have not been established and treatment is often not satisfactory.[4–9] It may represent a complex interplay among orbital fibroblasts, immune cells, cytokines, autoantibodies, genetics, and environmental factors.[9–13] Recently, there is growing evidence that a change of reactive oxygen species (ROS)

metabolism has been implicated in the aetiopathogenesis of several autoimmune disorders including Graves' disease and GO.[14–16] Bednarek *et al*[17,18] reported that the ROS and antioxidant enzymes were increased in peripheral blood of hyperthyroid patients and euthyroid patients with infiltrative ophthalmopathy. Lu *et al*[19] detected signals of oxygen free radicals in GO retroocular fibroblasts by using electron paramagnetic resonance spectroscopy. However, the contribution of ROS to the pathogenesis of GO has not yet been clarified. The accumulation of ROS can greatly damage cells by reacting with proteins, DNA, and membrane phospholipids. In our previous study, we showed that the urinary 8-hydroxy 2′-deoxyguanosine (8-OHdG) level, an important biomarker of oxidative DNA damage, is increased in patients with GO and is correlated with the clinical evolution of GO, especially the disease activity.[20] Furthermore, this biomarker of oxidative DNA damage could be reduced after treatment with systemic steroid in patients with active GO.[21] In this investigation, we determined the oxidative DNA damage in orbital fibroadipose tissues and cultured orbital fibroblasts from patients with GO and compared them with the controls. In addition, lipid peroxidation and intracellular ROS levels in cultured orbital fibroblasts were also determined.

Materials and methods

Tissue acquisition and cell culture

Orbital tissue samples used for the experiments reported here were obtained from 10 patients with GO as surgical waste during decompression surgery or from apparently normal orbital tissues in 7 age and sex-matched patients undergoing surgery for noninflammatory conditions. Orbital tissues were immediately frozen and stored at −80°C until analysis. Orbital fibroblast cultures were obtained from four patients with GO during decompression surgery and from apparently normal orbital tissues in three patients undergoing surgery for noninflammatory conditions. The primary culture of orbital fibroblasts was established as previously described.[22] Fibroblasts were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 μg/ml pyruvate, 50 μg/ml uridine, and antibiotics within 200 U/ml penicillin G, 200 μg/ml streptomycin sulphate, and 0.5 μg/ml amphotericin B. Medium covering the cultures was changed every 3–4 days, and monolayers were maintained in a 5% CO_2, humidified incubator at 37°C. Cultured orbital fibroblasts were used from the early passages and the culture at the same number of passage was used for the same set of experiments. All patients with GO achieved stable euthyroidism with medication (carbimazole, methimazole, or propylthiouracil) for at least 6 months before surgery. The tenets of the Declaration of Helsinki principles were followed, and the study received approval from the Institutional Review Board of Taipei Veterans General Hospital.

Determination of 8-OHdG in orbital tissues and cultured orbital fibroblasts

The DNA damage was evaluated by the 8-OHdG content in total DNA, which was determined by using the 8-OHdG ELISA kit from Japan Institute for the Control of Aging (Fukuroi, Japan) according to the manufacturer's instructions.[23] Total DNA from orbital tissues and cultured orbital fibroblasts was isolated by phenol/chloroform extraction with butylated hydroxyl toluene (freshly prepared in ethanol) as previously described.[24] After precipitating with ice-cold 75% ethanol, we air-dried and dissolved isolated DNA in distilled water. An aliquot of 50 μg DNA was then first digested with 6 U of nuclease P1 (Roche, Mannheim, Germany) in a solution of 200 mM sodium acetate (pH 5.3) at 37°C for 2 h, followed by reaction with 2 U of calf intestine alkaline phosphatase (Roche) at 37°C for 2 h. The hydrolysate was filtered through the Millipore Ultra free C3LGC (Millipore, Billerica, MA, USA) at 10 000 *g* for 10 min to remove enzymes and other macromolecules. The sample was then ready to perform the ELISA. The detection range of the 8-OHdG concentration is 0.125–10 ng/ml.

Determination of lipid peroxidation in cultured orbital fibroblasts

The lipid peroxidation product, malondialdehyde (MDA), in cultured orbital fibroblasts was measured by a spectrophotometric assay kit (MDA-586; OxisResearch Inc. Portland, OR, USA) according to the manufacturer's instructions. The MDA was quantified in the reaction with a chromogenic regent *N*-methyl-2-phenylindole to form an intensely coloured carbocyanine dye with a maximum absorption at 586 nm.[25] The method is specific for MDA instead of other lipid peroxidation products such as 4-hydroxyalkenal because they cannot produce significant absorbance at 586 nm under the experimental conditions. An MDA standard curve was established by using the MDA samples at the concentration range of 0–50 μM and the MDA levels in orbital fibroblasts were normalized to cell numbers (10^6 cells). The results are expressed as mean ± SD of the results from three independent experiments.

Determination of intracellular ROS in cultured orbital fibroblasts

The intracellular levels of H_2O_2 and superoxide anions in orbital fibroblasts were measured by using the probes 2′,7′-dichlorofluorescein (DCF, 10 μM) and dihydroethidine (DHE, 10 μM) (Molecular Probes, Invitrogen, Eugene, OR, USA), respectively.[26] After trypsinization, we washed cells with PBS buffer (pH 7.4) followed by resuspension in 0.5 ml of PBS buffer (pH 7.4), and then subjected them to analysis on a flow cytometer (Model EPICS XL-MCL; Beckman Coulter, Miami, FL, USA). The excitation wavelength was set at 488 nm and the intensity of emitted fluorescence of a total of 10 000 cells was recorded at 530 nm on channel FL1 for DCF and at 585 nm on channel FL2 for DHE. Data were acquired and analysed using the Cell Quest software (Becton Dickinson, San Jose, CA, USA) and each value of GO orbital fibroblasts was presented as a relative value, which was calculated by taking the intracellular ROS levels of the human fibroblast CCD cell line as 100%. CCD skin fibroblasts were purchased from ATCC (Manassas, VA, USA) and ATCC number is CCD-1112SK. It was cultured from the foreskin of a healthy newborn.

Statistical analysis

Comparisons of the values of 8-OHdG between GO and normal orbital fibroadipose tissues and the differences in 8-OHdG, MDA, and intracellular ROS between GO and normal cultured orbital fibroblasts were performed by the Student's unpaired *t*-test. Correlations between 8-OHdG contents in GO orbital fibroadipose tissues and thyroid-stimulating antibody hormone (TSH) receptor antibody levels of patients with GO were determined by Spearman's correlation coefficients. In all analyses, $P<0.05$ was considered statistically significant. Statistical analyses were performed using the Stata statistical software (Stata Corp., College Station, TX, USA).

Results

8-OHdG content in orbital fibroadipose tissues

The mean 8-OHdG level in GO orbital fibroadipose tissues (4.53 ng of 8-OHdG per mg DNA) was slightly higher than that of normal controls (2.39 ng of 8-OHdG per mg DNA) (Table 1). However, the difference did not reach a statistically significant level ($P=0.0736$). Among 10 patients with GO, there were positive correlations between the TSH receptor antibody levels and 8-OHdG contents in orbital fibroadipose tissues ($\rho=0.86$, $P=0.012$).

Table 1 The 8-OHdG levels in fibroadipose tissues from GO patients and normal subjects

	GO patients (n = *10*)	*Normal subjects* (n = *7*)	P-*value*
Mean 8-OHdG (ng/mg DNA)	4.53 ± 2.60	2.39 ± 0.62	0.0736

8-OHdG content in cultured orbital fibroblasts

Table 2 shows that the mean 8-OHdG content in GO orbital fibroblasts (3.34 ng of 8-OHdG per mg DNA) was significantly higher than that of normal cultures (2.09 ± 0.28 ng of 8-OHdG per mg DNA, $P=0.0026$).

MDA content in cultured orbital fibroblasts

As shown in Table 2, the mean MDA level in GO orbital fibroblast cultures was significantly higher than that of normal cultures (327.55 *vs* 247.19 nmol per 10^6 cells, $P<0.001$).

Intracellular ROS in the orbital fibroblasts

The GO orbital fibroblast cultures had significantly higher levels of superoxide anions and H_2O_2 than did normal cultures (Table 3).

Discussion

Oxidative stress occurs when the generation of ROS exceeds the cellular ability to efficiently neutralize and eliminate them, and ultimately leads to the widespread oxidative damage to all components of the cell, including

Table 2 The levels of 8-OHdG and MDA in cultured orbital fibroblasts from GO patients and normal subjects

	GO patients (n = *4*)	*Normal subjects* (n = *3*)	P-*value*
Mean 8-OHdG (ng/mg DNA)	3.34 ± 0.31	2.09 ± 0.28	0.0026
Mean MDA (nmol per 10^6 cells)	327.55 ± 10.54	247.19 ± 9.74	0.0002

Table 3 The intracellular levels of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in cultured orbital fibroblasts from GO patients and normal subjects

	GO patients (n = *4*)	*Normal subjects* (n = *3*)	P-*value*
O_2^- (relative ratio)	101.93 ± 3.41	93.02 ± 1.50	0.0133
H_2O_2 (relative ratio)	126.65 ± 4.24	105.39 ± 0.73	0.0025

DNA, lipid, and proteins. Oxidative damage of biomolecules can theoretically contribute to disease development. The content of 8-OHdG, one of the most abundant oxidative products of DNA, has been observed to be significantly elevated in various ocular pathologies, such as in trabecular meshworks of patients with primary open-angle glaucoma[27] and pterygium tissues.[28] ROS can also attack polyunsaturated fatty acids in cell membranes and induce formation of lipid peroxidation products, and MDA is a stable end product of peroxidation of lipids caused by ROS.[29] Previously, increased MDA levels were detected in several ocular diseases, including aqueous humours of patients with primary open-angle glaucoma[30] and lens epithelial cells of patient with pseudoexfoliation syndrome.[31] This is the first study to explore changes in oxidative DNA damage, lipid peroxidation, and intracellular ROS production in GO orbital fibroblasts.

Expanded orbital connective tissue volume is a hallmark of GO and contributes to many of the clinical symptoms and signs of GO. Hondur *et al*[32] had shown increased oxidative stress and antioxidant activity in fibroadipose tissues of patients with GO. In this study, the oxidative DNA damage was only slightly increased in fibroadipose tissues of patients with GO, but significantly elevated in cultured orbital fibroblasts from them. In addition, the MDA level was also noted to be higher in GO orbital fibroblasts. Orbital fibroblasts represent the major target cells in the pathogenesis of GO, and participate actively in not only the early inflammation process but also the later remodelling process.[33] As orbital fibroadipose tissues include not only fibrous but also fatty tissues, and the percentage of fibroblasts in fibroadipose tissues from different patients is individualized and unpredictable. Therefore, the oxidative DNA damage in cultured orbital fibroblasts is more reliable and specific than that in fibroadipose tissues, and the increased oxidative DNA damage in cultured orbital fibroblasts is also in accordance with our previous findings in urinary levels of 8-OHdG.[20] Oxidative DNA damage and lipid peroxidation were increased in GO orbital fibroblasts as compared with normal fibroblasts in this study implies that oxidative stress in orbital fibroblasts may have an important role in the pathogenesis of GO. This distinct phenotype exhibited by orbital fibroblasts may underlie the susceptibility of the orbit to manifestations of GO.

Our results also showed increased intracellular superoxide anions and H_2O_2 in GO fibroblasts. GO is thought to be an inflammatory disorder of autoimmune background. It has been reported that oxidative stress is correlated with the extent of inflammation.[34] Whether the inflammation processes is the main factor responsible for the oxidative stress in GO fibroblasts remains to be elucidated. However, increased accumulation of ROS may cause more oxidative damage such as lipid peroxidation or oxidative DNA damage in the GO orbital fibroblasts. In addition, Burch *et al*[35] have shown that superoxide radical induced the proliferation of cultured orbital fibroblasts from patients with GO in a dose-dependent manner, whereas control normal fibroblasts showed no preference in response to superoxide generation. Low concentrations of H_2O_2 have also been found to stimulate cellular proliferations in a variety of cell types including the fibroblasts.[36–38] Moreover, Heufelder *et al*[39] showed that the expression of heat shock protein 72, an important factor in site-directed autoimmune response of GO, was strongly enhanced in GO fibroblasts by H_2O_2. In combination with previous observations, the elevated levels of superoxide anions and H_2O_2 in GO fibroblasts not only indicate a cellular oxidant/antioxidant imbalance in these cells but also further address the importance of ROS in the development and progression of GO.

Until now, impressive treatment for GO has been very limited, and systemic corticosteroid and radiotherapy remains the mainstay treatment for GO. We have previously reported that systemic corticosteroids are effective in reduction of both the clinical manifestation and oxidative DNA damage in patients with active GO.[21] In a small case series, oral antioxidants provided beneficial effect in the treatment of mild and moderately severe GO.[40] However, more basic work and clinical studies are warranted to provide more information about the role of antioxidants and corticosteroids in the treatment of GO, especially those patients with coexisting oxidative stress.

In conclusion, our data showed that oxidative DNA damage, lipid peroxidation, and ROS production in cultured GO orbital fibroblasts were increased compared to those of normal orbital fibroblasts. These orbital fibroblasts exhibit potentially important differences in their phenotypes that we believe account for the important role of oxidative stress in the pathogenesis of GO.

Summary

What was known before

- Recently, there is growing evidence that a change of reactive oxygen species (ROS) metabolism has been implicated in the aetiopathogenesis of several autoimmune disorders including Graves' disease and GO.

What this study adds

- This is the first study to explore changes in oxidative DNA damage, lipid peroxidation, and intracellular ROS production in GO orbital fibroblasts.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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