Cloning and sequencing of complete thyrotropin receptor transcripts in pretibial fibroblast culture cells


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ABSTRACT. Pretibial fibroblasts are considered to be targets of autoimmune attack in pretibial myxedema. A possibility of the pathogenesis of pretibial myxedema is that T cells, reacting with thyrotropin (TSH) receptor, will be targeting to the pretibial fibroblasts where, in the presence of antigen (TSH receptor), they will secrete various cytokines and stimulate fibroblasts to secrete glycosaminoglycans. We have demonstrated that TSH and TSH receptor antibody can bind to fibroblasts and the presence of RNA encoding the extracellular domain of the TSH receptor in fibroblasts derived from skin lesions of two patients with pretibial myxedema. The present study was designed to determine whether there are complete TSH receptor transcripts in pretibial fibroblasts obtained from patients with pretibial myxedema. RNA was prepared from pretibial fibroblasts obtained from 11 patients with pretibial myxedema and from four normal subjects, then reverse-transcribed by polymerase chain reaction using three sets of primers (-11/+8 and +754/+773; +353/+373 and +1265/+1285; +1000/+1017 and +2284/+2301). The overlapped 2312 bp cDNA sequence was expected to contain the genetic sequences of the signal peptide (+1/+60), extracellular domain (+61/+1254), transmembrane domain (+1255/+2046), and cytoplasmic domain (+2047/+2292) of the TSH receptor. The sequences were determined using dideoxy sequencing method. All of the 2312 nucleotide sequences in 15 samples were consistent with the reported TSH receptor sequence of transcripts in thyroid. These data suggest that the complete TSH receptor transcripts are very possible to be present in the fibroblasts derived from pretibial skin.

INTRODUCTION

Graves's disease is an autoimmune disease. Typical clinical features include diffuse toxic goiter, ophthalmopathy and pretibial myxedema. Hyperthyroidism is related to the overproduction of thyroid hormones by the stimulation of thyrotropin (TSH) receptor antibodies on thyroid follicular cells through TSH receptors (1). However, the pathogenesis of Graves' ophthalmopathy and pretibial myxedema is still not very clear.

Orbital and pretibial fibroblasts are considered to be targets of autoimmune attack in Graves' ophthalmopathy and pretibial myxedema (2). A possibility of these two conditions' pathogenesis is that T cells which react with the TSH receptor will be targeting to the orbital and pretibial fibroblasts where, in the presence of antigen (extracellular domain of TSH receptor), they will secrete various cytokines, such as glycosaminoglycan-stimulatory lymphokine (3), which in turn stimulates fibroblasts to secrete glycosaminoglycans or to proliferate, to express MHC class II antigens (HLA DR) and to synthesize collagen and connective tissue (4).

However, there are controversies about whether there are TSH receptors in fibroblasts of Graves' ophthalmopathy and pretibial myxedema. RNA encoding the extracellular domain of the TSH receptor has been demonstrated in orbital (5, 6) and pretibial fibroblasts (5-7). However, Feliciello et al. (8) could demonstrate TSH receptor mRNA in retro-orbital tissue but not in fibroblasts. Paschke et al. (9) were not even able to demonstrate the extracellular domain of the TSH receptor in extraocular muscle, which may contain a significant proportion of fibroblasts.

We have demonstrated that TSH and TSH recep-
tor antibody can bind to fibroblasts and the presence of RNA encoding the extracellular domain of the TSH receptor in fibroblasts derived from skin lesions of two patients with pretibial myxedema (7). The present study was designed to determine whether there are complete TSH receptor transcripts in pretibial fibroblasts obtained from patients with pretibial myxedema.

MATERIALS AND METHODS

Patients and fibroblast cultures

Eleven subjects of Graves' disease with pretibial myxedema (3 males and 8 females; median age: 47 years, range: 23 to 63 years) were included in the present study. All also had Graves' ophthalmopathy. All had given informed consent for the procedures involved. Monolayer cultures of dermal fibroblasts were established by the explant method from biopsies of the affected pretibial skin as confirmed by histopathology. Biopsy of normal pretibial skin from four persons without pretibial myxedema was used for control. The explants were cultured until confluency in 25-cm² culture flasks with Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) at 37 °C in a 5% CO₂ humidified atmosphere. Fibroblast cultures were subcultured by trypsinization, and cells from passages 3 to 6 were then used for studies.

Ribonucleic acid (RNA) preparation

The RNA used in this experiment was prepared from fibroblast cells by the method of Chomczynski and Sacchi (10). Briefly, the cultured cells were lysed in a denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) and then extracted with a phenol/chloroform/isoamyl alcohol followed by isopropanol precipitation. The RNA precipitate was rinsed with 75% ethanol, vacuum dried, and stored at -70 °C until use.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The RNA preparation was reverse transcribed by Moloney murine leukemia virus ribonuclease H-reverse transcriptase (GIBCO BRL, Gaithersburg, MD) using random hexamer as a primer. Five micrograms of total RNA and 100 pmoles of random hexamers in a 11.3 μl of total water volume were denatured for 5 min at 90 °C, cooled on ice, and subsequently incubated for 1 hour at 37 °C with 8.7 μl of a premix containing 2 μl 10-fold PCR buffer (0.5 M KCl, 0.1 M Tris-HCl, pH 9.0, and 1.0% Triton X-100), 2 μl dNTPs (10 mM each), 3.2 μl MgCl₂ (25 mM), 20 U RNasin (Promega, Madison, WI), and 1 μl Moloney murine leukemia virus ribonuclease H-reverse transcriptase (200 U/μl). The cDNA synthesis was terminated by heating the reaction mixture for 5 min at 95 °C, and then by quick-chilling on ice. To 20 μl of the cDNA solution, 80 μl of a pool containing upstream and downstream primers (50 pmole each), 8 μl 10-fold PCR buffer, 2.8 μl MgCl₂ (25 mM), and 2.5 U Taq DNA polymerase (Promega, Madison, WI) was added. The PCR amplification was started with a cycle of 5 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C; followed by 38 cycles of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C; and finally ended with a cycle of 1 min at 94 °C, 2 min at 55 °C, and 10 min at 72 °C.

In order to determine whether there are complete TSH receptor transcripts in fibroblasts obtained from pretibial skin, three sets of primers (-11/+8 and +754/+773; +353/+373 and +1265/+1285; +1000/+1017 and +2284/+2301; +1 refers to the first nucleotide of start codon) were selected and used in this study. The positions of these primer sets in regard to the corresponding exons of TSH receptor gene is demonstrated in Figure 1. The overlapped 2312 bp cDNA sequence was expected to contain the genetic sequences of the signal peptide (+1/+60), extracellular domain (+61/+1254), transmembrane domain (+1255/+2046), and cytoplasmic domain (+2047/+2292) of the TSH receptor (11). The nucleotide sequences of the three primer sets were (1) 5'-TCCCGTGGAATAATGAGGCC-3' and 5'-CAGGTGTTTCTTGCTATCAG-3', (2) 5'-ACCTGATGCCCTCAAAGAGC-3' and 5'-GAGCCAGCAGACTAACGAACC-3', and (3) 5'-ATTGTTGGTACAAGGACA3' and 5'-GTAACTTACAAAACGCGT3'. The sequences of the first and the third primer sets were according to Bahn et al. (12) and Paschke et al. (9) respectively.

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Fig. 1 - Exonic representation of the human TSH receptor gene showing the positions of the three primer pairs used for PCR amplification of TSH receptor cDNA fragments.