

Neoplastic epithelial cells express α -subunit of muscle nicotinic acetylcholine receptor in thymomas from patients with myasthenia gravis

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We studied the expression of mRNAs coding for the α -subunit of the muscle nicotinic acetylcholine receptor (AChR) in thymomas from patients with myasthenia gravis (MG). Northern blot analysis failed to detect the expression, but amplification of mRNAs derived from thymomas by reverse transcription and polymerase chain reaction produced the DNA fragments containing the nucleotide sequence coding for part of the α -subunit. We further revealed that the α -subunit mRNA was derived from neoplastic epithelial cells of thymoma. Our results support the hypothesis that AChR expressed in thymoma is a candidate for the primary antigen which induces autoimmune responses to muscle AChR. The close relationship between MG and thymoma may be at least in part explained by this hypothesis.

Thymoma; Myasthenia gravis; Acetylcholine receptor; mRNA expression; Polymerase chain reaction; Epithelial cell

1. INTRODUCTION

Myasthenia gravis (MG) is classified as an autoimmune disease in which the autoantibodies accelerate the degradation of the nicotinic acetylcholine receptor (AChR) at the muscle endplates, impairing the neuromuscular transmission [1]. However, a fundamental question remains unanswered as to where the locus responsible for the initiation of the antibody production to muscle AChR is present. Clinical evidence implicating the thymus in MG includes a high incidence of thymoma in patients with MG and the effectiveness of thymectomy as a treatment for many of them [2]. At the cellular level, much attention has been paid to myoid cells in the thymus, because these cells are suggested to have AChR on their surfaces [3].

However, the myoid cell seems to be overemphasized as a candidate for an antigen presenting cell. There is no difference in the myoid cell frequency between the thymuses from MG patients and control subjects. Our working hypothesis is that the neoplastic changes of epithelial cells start to express proteins, which may trigger an autoimmune response against AChR at the muscle endplates in MG patients. In the present study, we have provided direct evidence for the expression of mRNA coding for a part of α -subunit of muscle

nicotinic AChR in neoplastic epithelial cells from thymomas.

2. MATERIALS AND METHODS

2.1. RNA preparation

Total RNA was prepared from biopsied specimens of kidney and muscle, and autopsied brain tissues from non-MG patients. Test samples from patients with MG included 5 thymomas and 5 hyperplastic thymuses obtained at surgery. Peripheral leukocytes from MG patients, and neoplastic epithelial cells isolated from 2 individual thymomas of MG patients were cultured under conditions described previously [4]. The specimens were homogenized in a buffer containing guanidine thiocyanate, and layered over a cushion of CsCl and centrifuged [5]. After extraction with phenol and ethanol precipitation, the total RNAs were subjected to Northern blot analysis and amplified by polymerase chain reaction (PCR) experiments.

2.2. Northern blot analysis

30 μ g of total RNA from control muscle and MG patients' thymomas and hyperplastic thymuses were fractionated by 1% agarose gel electrophoresis, and transferred to nylon membranes (Amersham). A cDNA clone for the α -subunit of human muscle AChR was labeled with [³²P]dCTP by the random primer method [6]. After incubation with a [³²P]cDNA probe, the membranes were washed according to the method of Merlie et al [7].

2.3. Oligonucleotide primers for amplification of α -subunit mRNA

Based on the published nucleotide sequence of the α -subunit gene of nicotinic AChR of human muscle [8], two oligonucleotide primers encompassing the putative acetylcholine binding site were prepared on Applied Bio-systems model 381 A DNA synthesizer (Bioapplied) and purified on a NENSORB column (Dupont). The forward primer hybridizes to nucleotides 375–398 of the antisense strand of exon 5 of the α -subunit gene, while the reverse primer anneals to nucleotides 596–619 of the sense strand of exon 6. The sequences of these primers

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Abbreviations: AChR, acetylcholine receptor; MG, myasthenia gravis; PCR, polymerase chain reaction; bp, base pairs

are (5'-AAGCTACTGTGAGATCATCGTCAC-3') and (5'-TGACGAAGTCGGTAGCGTATGTCCTCA-3'), respectively.

2.4. Reverse transcription and PCR of RNA

The first strands of cDNA were synthesized from 3.0 µg of total RNA prepared from 5 thymomas, hyperplastic thymuses, and neoplastic epithelial cells from 2 thymomas using the Amersham cDNA Synthesis System according to the manufacturer's directions. The first strands were added to the PCR reaction buffer to a total volume of 100 µl, and amplified in the presence of the two synthesized primers using Taq polymerase under the conditions as specified by Perkin Elmer Cetus. The PCR run consisted of 30 cycles of denaturation at 94°C, annealing at 42°C and extension at 74°C; 60 s for each step. 3 µg of total RNA obtained from brain, kidney and leukocytes were amplified in the same manner for dot blot analysis.

15 ng of muscle RNA and 280 ng of thymoma and hyperplastic thymus RNA were subjected to serial doubling dilutions and amplified in the same manner to estimate the relative abundance of α -subunit mRNA expression in the tissues. PCR products from these RNAs were precipitated with ethanol, fractionated on 6% polyacrylamide gel, and visualized by staining with ethidium bromide.

2.5. Hybridization and sequence analyses

One-tenth of PCR products from RNA of thymic tissues, and cultured neoplastic epithelial cells was fractionated on a 6% polyacrylamide gel, and electrophoretically transferred to nylon membranes. An one-tenth of PCR products of total RNA from brain, muscle, thymoma, kidney, leukocytes and neoplastic epithelial cells was dotted on the nylon membrane. The membranes were hybridized with α -subunit cDNA probe, washed in 0.1 \times SSC at 65°C and

autoradiographed. The fragments of 245 base pairs(bp) amplified from RNA of neoplastic epithelial cells were cloned into the X13 vector and sequenced by the dideoxy chain termination method [9].

3. RESULTS AND DISCUSSION

The expression of mRNA coding for the AChR α -subunit was detected in control muscles but not in 5 thymomas and 5 hyperplastic thymuses from patients with MG by Northern blot analysis (Fig. 1). Since sufficient amounts of intact mRNAs were not obtained from the surgical specimens, we must take into account the possibility that the sensitivity of Northern blot analysis was not sufficiently high for detecting α -subunit mRNA of limited amounts. We used a more sensitive method of PCR combined with reverse transcriptase reaction. PCR only requires that the average molecular weight of the template RNA is slightly higher than the largest fragment to be amplified, and is capable of amplifying reverse transcribed mRNA by several orders of magnitude.

We could detect the amplified band of 245 bp in 4 thymomas and in 3 hyperplastic thymuses. Hybridization of the band with α -subunit cDNA probe indicated that the thymic tissues contained α -subunit mRNA (Fig. 2). Dot blot analysis showed that PCR product of

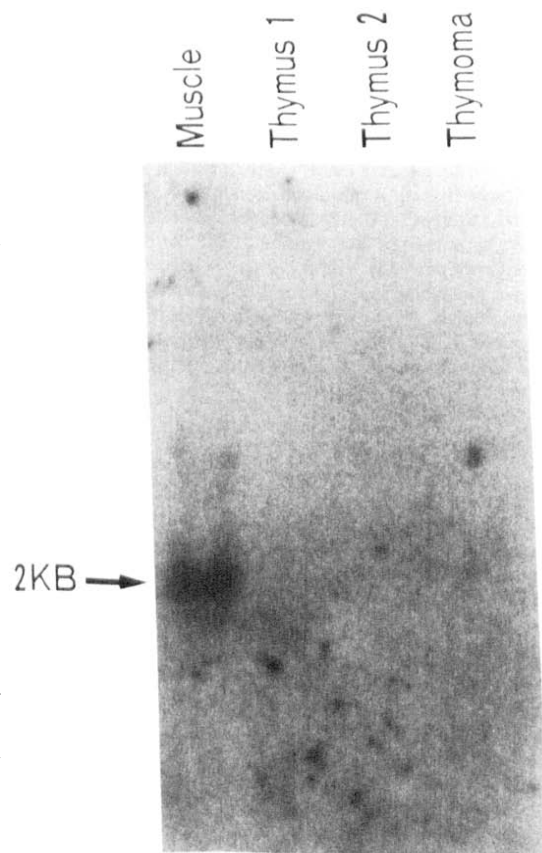


Fig. 1. Northern blot analysis of total RNA from muscle and thymic tissues.

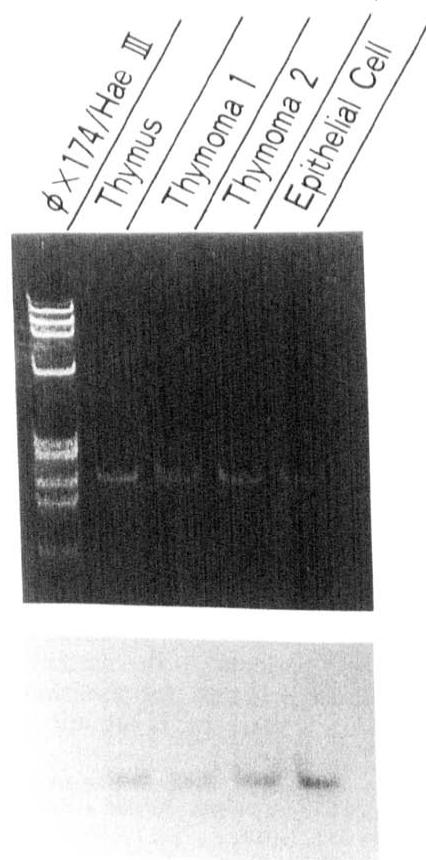


Fig. 2. Polyacrylamide gel electrophoresis of PCR products of RNA derived from thymic tissues and neoplastic epithelial cells (upper) and Southern blot analysis (bottom).

RNA from the brain contained no fragments that bind to the α -subunit cDNA probe. Amplified RNA from the kidney showed a faint signal on autoradiograph indicating a low abundance of α -subunit mRNA expression in the tissue (Fig. 3). Minimal doses of template RNAs were 1.9 ng from muscle (Fig. 4) and 35 ng from thymoma and hyperplastic thymus (data not shown) to identify the bands by ethidium bromide staining on a polyacrylamide gel. From these results, the relative abundance of α -subunit mRNA of muscle to thymic tissues was estimated about 35 to 1.9.

Next, we investigated the origin of the α -subunit mRNA in the thymic constituents. Dot blot analysis of the amplified leukocyte RNA from MG patients was negative (Fig. 3). The amplified products using total RNA from cultured epithelial cells obtained from 2 thymomas were confirmed as an authentic copy of α -subunit by Southern blot (Fig. 2) and sequence analyses. The product of 245 bp contained the nucleotide sequence identical to that of α -subunit cDNA (125-206) including the coding region for the acetylcholine binding site (Cys-Cys) (Fig. 5) [10,11]. The sequence was distinct from those of α -subunit cDNAs of neuronal AChR, because the homology of the amino acid sequences between the muscle and neuronal receptors was rather low in the amplified region [12]. These results indicate that the muscle α -subunit mRNA is expressed in the neoplastic epithelial cells derived from MG-associated thymomas.

The specific expression of the α -subunit mRNA in neoplastic epithelial cells was confirmed by the following procedures. The passages of the neoplastic epithelial cells in culture excluded the contamination by myoid cells, because the myoid cells could not be cloned under our culture conditions. All cultured cells were im-

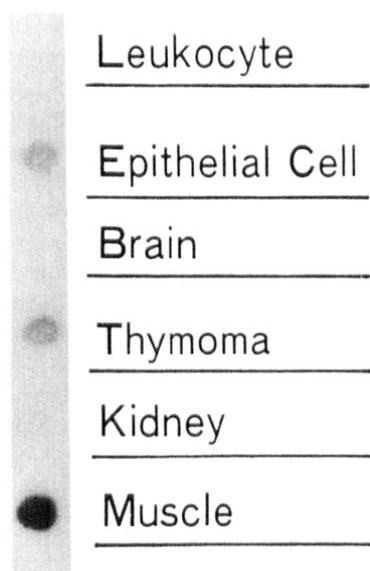


Fig. 3. Dot blot analysis of PCR-amplified RNA.

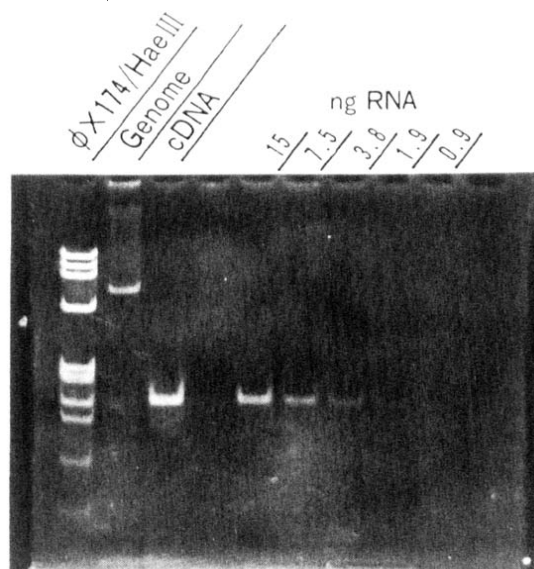


Fig. 4. PCR amplifications of genome and α -subunit cDNA, and reverse transcription and PCR of serially diluted RNA from muscle tissue. Doses of template RNA are shown at the top of the lanes.

munohistologically stained with antikeratin antibodies, indicating that they were of epithelial cell-origin. PCR produced an α -subunit fragment of 245 bp, ruling out the contamination of the genomic DNA, because the amplification of α -subunit genomic DNA resulted in the

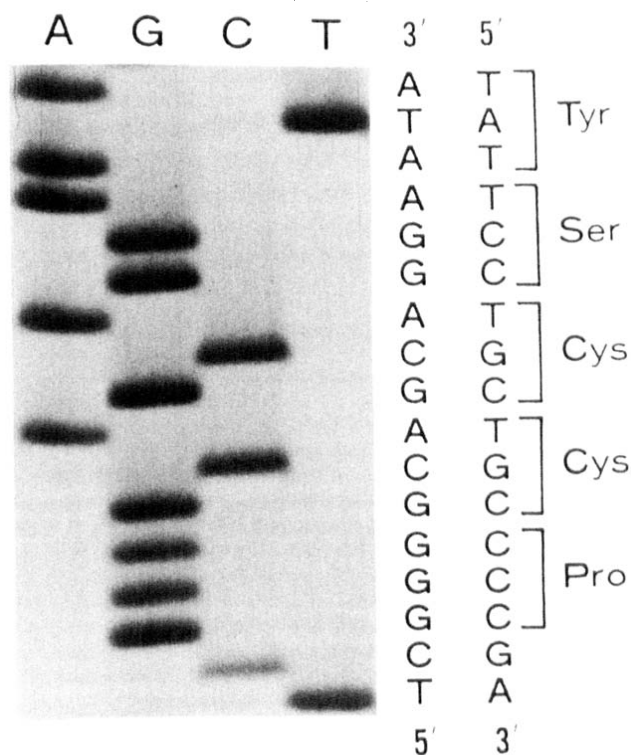


Fig. 5. Nucleotide sequence coding for the putative acetylcholine binding site.

product of 650 bp including exon 5, intron and exon 6 (Fig. 4). α -Subunit fragments were amplified using total RNA obtained from hyperplastic thymuses (Fig. 2). Since epithelial cells are not the main constituent in these thymuses, we cannot confirm whether the epithelial cells synthesize α -subunit mRNA. It may be possible that the α -subunit mRNAs were derived from myoid cells because the cells are suggested by immunological studies to possess the receptors.

In contrast to our observations of α -subunit mRNA expression in neoplastic epithelial cells, the mature AChRs are not evidenced on the cells. Marx et al. showed that the monoclonal antibody against the α -subunit of muscle AChR recognizes a protein quite distinct from any known AChR chain in epithelial cells [13]. The explanations for the discrepancy of the expressions at RNA and protein levels include: AChR subunit genes are rearranged, and RNA transcripts from the genes are out of phase inside the neoplastic epithelial cells [14]. Therefore AChRs are not expressed on the neoplastic cells. There is, however, no evidence supporting this explanation. Neither unusual rearrangements of the AChR gene nor major deletions are detected inside the AChR gene [15].

Alternatively, the posttranslational modifications of the epithelial AChR are different from that of muscle AChR, and the immunogenicities of the two receptors are different. Based on this hypothesis and our data, we speculate that the neoplastic changes of a large number of epithelial cells in thymomas start to express AChR, and the neoplastic cells provide loci responsible for the initiation of the antibody production to muscle AChR in thymoma associated MG patients.

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