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Collagen Q – A potential target for autoantibodies in myasthenia gravis

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Abstract

Myasthenia gravis (MG) is an autoimmune disorder caused by autoantibodies targeting proteins expressed at the neuromuscular junction (NMJ). In most cases the targets are acetylcholine receptor (AChR), muscle-specific tyrosine kinase (MuSK), or occasionally low-density lipoprotein receptor-related protein 4 (LRP4), but there is still a group of patients, often called seronegative MG (SNMG), with unknown antibody targets. One potential target is collagen Q (COLQ), which is restricted to the NMJ and is crucial for anchoring the NMJ-specific form of acetylcholinesterase (AChE). 415 serum samples with a clinical diagnosis of MG and 43 control samples were screened for the presence of COLQ autoantibodies using a cell-based assay (CBA) with HEK293 cells overexpressing COLQ at the cell surface. COLQ antibodies were detected in 12/415 MG sera and in one/43 control samples. Five of the COLQ-Ab + individuals were also positive for AChR-Abs and 2 for MuSK-Abs. Although the COLQ antibodies were only present at low frequency, and did not differ significantly from the small control cohort, further studies could address whether they modify the clinical presentation or the benefits of anti-cholinesterase therapy.

Keywords

Collagen Q (COLQ); Myasthenia gravis (MG); Neuromuscular junction (NMJ); Cell-based assay (CBA); Autoantibodies; Neuroimmunology

1 Introduction

Myasthenia gravis (MG) is a classic autoimmune disorder resulting from the presence of autoantibodies targeting neuromuscular junction (NMJ) proteins, and leading to defects in neuromuscular transmission with fatiguable muscle weakness. In most cases the antibodies recognise acetylcholine receptor (AChR) or muscle-specific tyrosine kinase (MuSK) [1], but there are still individuals, often referred to as seronegative MG (SNMG), in whom AChR and MuSK autoantibodies are not detected in present assays. Additional antibody targets include low-density lipoprotein receptor-related protein 4 (LRP4) [2–4], agrin (AGRN) [5]

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and acetylcholinesterase (AChE) [6]. Despite some evidence of antibodies to these proteins [2–6], there remain patients presenting with an autoimmune MG with no specific antibody identified.

Collagen Q (COLQ) is a protein crucial for anchoring and concentrating AChE at the NMJ, where its expression is restricted to the extracellular matrix and accessible to circulating antibodies. Indeed, mutations in *COLQ* can underlie one form of congenital myasthenic syndrome (CMS) [7,8]. Although COLQ possesses a number of features which make it a potential target for autoantibodies in MG, the presence of these have not been reported.

Traditionally autoantibodies have been detected by radioimmunoprecipitation assays (RIA) or in some cases by enzyme-linked immunosorbent assays (ELISA) or fluorescence immunoprecipitation assays (FIPA). These assays can be sensitive and highly specific, but do not necessarily detect the most pathogenic antibodies. Recently cell-based assays (CBAs) have been established in order to look for antibodies that bind to the extracellular domains of proteins that are naturally expressed on the cell surface. To apply this technique to proteins that are not membrane tethered, it is necessary to fuse them with a transmembrane protein or domain. Here we expressed COLQ fused with the transmembrane domain of contactin-associated protein-like 2 (CASPR2) and looked for antibodies in MG patients and controls.

2 Materials and methods

2.1 Ethics statement

The MG samples were archived from therapeutic plasmaphereses in the 1980s and 1990s when written consent was not required, but verbal was obtained. Ethical approval for use of pre 2006 stored patient samples without patient written consent was obtained from the Oxfordshire REC C 09/H0606/74. Samples from healthy individuals were obtained with written consent and ethical approval from the Oxfordshire REC Rf 07/Q1604/28.

2.2 Cloning of pcDNA-COLQ-CASPR2TM

Construct encoding *COLQ* in pcDNA™3.1/Hygro(+) (Invitrogen, V87020) was kindly provided by Dr Janet Kenyon. COLQ cDNA was engineered into pcDNA-*Lgi1-CASPR2TM* to replace the leucine-rich glioma inactivated 1 (*Lgi1*) cDNA. The C-terminus of COLQ was chosen for the fusion with the transmembrane domain (TM) of contactin-associated protein-like 2 (CASPR2TM). A construct with a Myc tag, introduced immediately downstream the N-terminal signal peptide, was also cloned to control for the cell surface expression of the protein.

2.3 HEK293 cell culture and transfection

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, D6429) supplemented with 10% foetal calf serum (Sigma, F2442) and antibiotics (Invitrogen, 15240-062). Transfections were performed using polyethylenamine (PEI). Transfection mixes used for one well of a 6-well plate contained 3 μg DNA, 1.25 μl 20% glucose, 1.5 μl PEI, nuclease-free water up to 6.5 μl and 1 ml growth medium.

2.4 A cell-based assay for the detection of COLQ antibodies

Cells plated on poly-L-leucine-coated coverslips were transfected with pcDNA-*COLQ-CASPR2TM*, pcDNA-*Myc-COLQ-CASPR2TM* or pcDNA™3.1(+) vectors, respectively. 2 days after the transfection, the cells were incubated for 1 hour with sera diluted 1:20 in blocking solution (DMEM, 1% BSA, 20 mM HEPES) or a mouse monoclonal anti-Myc antibody (Cell Signalling, 2276), fixed with 3% paraformaldehyde and then incubated for one hour with Alexa Fluor 568 secondary antibodies (1:500) against human or mouse IgG, respectively. The immunostaining of the cells was analysed by widefield or confocal fluorescence microscopy using an Olympus X71 Fluorescence Microscope and SimplePCI software, or a Zeiss 780 Inverted Microscope and ZEN lite software, respectively.

2.5 Serum samples

Serum samples that were tested were either archived SNMG who were negative on a standard radioimmunoprecipitation assays for detection of autoantibodies to either AChR and MuSK or from patients with a clinical diagnoses of MG sent to Oxford to be analysed by CBAs for clustered AChR or MuSK antibodies (n = 415). Control serum samples were either healthy laboratory workers (n = 22) or disease control serum samples obtained from patients with established epilepsy identified either on admission or attending specialist epilepsy clinics at a large teaching hospitals, approximately 60% of whom had focal seizures and the remaining had generalized epilepsy (n = 21).

3 Results

3.1 A cell-based assay for the detection of anti-COLQ antibodies

To detect anti-COLQ antibodies, HEK293 cells transfected with the construct encoding COLQ-CASPR2TM were incubated with serum samples and Alexa Fluor 568 anti-human IgG secondary antibodies. HEK293 cells transfected with pcDNA-*Myc-COLQ-CASPR2TM* labelled with an anti-Myc antibody were used to control for the cell surface expression of the protein. The assay was used to screen 415 MG sera for the presence of antibodies to COLQ. The results of those assays were not known at the time of testing for COLQ antibodies. Red fluorescence at the surface of COLQ-CASPR2TM expressing cells, but not mock-transfected cells, indicated the presence of COLQ autoantibodies in the samples (e.g. Fig. 1A). The scores (0–3), corresponding to the intensity of the fluorescence at the cell surface of COLQ-CASPR2TM transfected cells, are shown in Fig. 1B. 12 out of 415 samples were found to be COLQ + ve, based on the mean ± 3SDs of the scores assigned to the healthy and epilepsy control samples (2.9%). In the control group a relatively weak (score = 1.5) COLQ binding was detected in only one out of 43 individuals (0 out of 22 in the healthy control, 1 out of 21 in the epilepsy patients). In addition 5 of the 12 MG samples were also positive on CBA or for binding to AChR and two in the RIA to MuSK. For patients confirmed as SNMG by assay (negative on CBA or RIA for antibodies to the AChR, MUSK, LRP4 or AGRN) 5 out of 149 were COLQ + ve (3.4%). To control against the possibility of a non-specific binding to CASPR2-TM (though this was considered unlikely since the CASPR2-TM domain would be hidden within the membrane) the COLQ + ve samples were tested using assays in which the NMJ proteins AGRN and LRP4 were

similarly expressed at the cell surface by fusion with CASPR2-TM. The COLQ + ve samples were negative in all these assays.

Four COLQ positive samples, with sufficient volume available, were titrated. The titres of the analysed samples were 1:100, 1:100, 1:100 and 1:20, respectively (Fig. 1C).

To confirm the specificity of the COLQ antibodies, the samples were preadsorbed against COLQ on COLQ-CASPR2TM-expressing HEK293 cells. As a control, the sera were incubated with HEK293 cells overexpressing MuSK at their surface. The binding of the serum antibodies to COLQ-HEK293 cells was abrogated by adsorption against COLQ-HEK293 cells but not against MuSK-HEK293 cells. (Fig. 1D).

3.2 Clinical presentation of COLQ antibody positive cases

All the positive patients were female, and all but two (out of the ten with known age of onset) had disease onset below 40 years (defined as early-onset MG). Three had purely ocular disease but the others had generalised (n = 7) or ocular/bulbar involvement (n = 2). The patients were treated with steroids or AChE inhibitors, and some data on the response to these therapies were available. Seven individuals responded well to steroids and in four out of nine patients, for whom the data were available, the response to AChE inhibitors was considered poor (Table 1).

4 Discussion

A CBA was developed for the detection of COLQ binding in patient sera and detected antibodies to COLQ in ~ 3% of the sera. This was at a similar frequency as found in our control samples (1/43), though the small number makes this result difficult to assess. The antibodies were at relatively low titres (1:20–1:100) but preadsorption indicated that they were specific for COLQ.

Although their frequency in patients without AChR or MuSK antibodies is low, these antibodies could still play a role in the disease. COLQ anchors AChE in the extracellular matrix and it is possible that the COLQ antibodies disrupt the concentration of asymmetric AChE complexes [9,10], and reduce the amounts of AChE. In CMS patients with genetic defects in the gene for *COLQ* [7,8,11,12], there is thought to be impaired formation of AChE-COLQ complexes, or lack of attachment to the NMJ [10]. The AChE-COLQ complexes are anchored by two mechanisms. First, two heparan-binding domains of COLQ interact with the heparan sulphate proteoglycan–perlecan [13,14] in the matrix. Second, the C-terminal fragment of COLQ interacts with MuSK [15]. These interactions have been shown to be affected by *COLQ* mutations in CMS and by MuSK antibodies in MG, and hence the mechanism could be analogous [16–18]. The impaired COLQ function could lead to a prolonged lifetime of acetylcholine (ACh) in the synaptic cleft, an increased duration of endplate currents and an increased ion flux, resulting in an endplate myopathy and compromised neuromuscular transmission or a desensitisation of the endplate receptors [19].

In the CMS patients with mutations in COLQ, there is a loss of asymmetric AChE at the NMJ and many patients do not show any benefit from AChE inhibitors [11]. Indeed four of

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the patients had poor responses to these drugs, but five had responded. Nevertheless, even though these antibodies may not be directly pathogenic, they could contribute to the highly varied clinical presentations in MG patients, and/or modify the response to AChE inhibitors and warrant further investigation.

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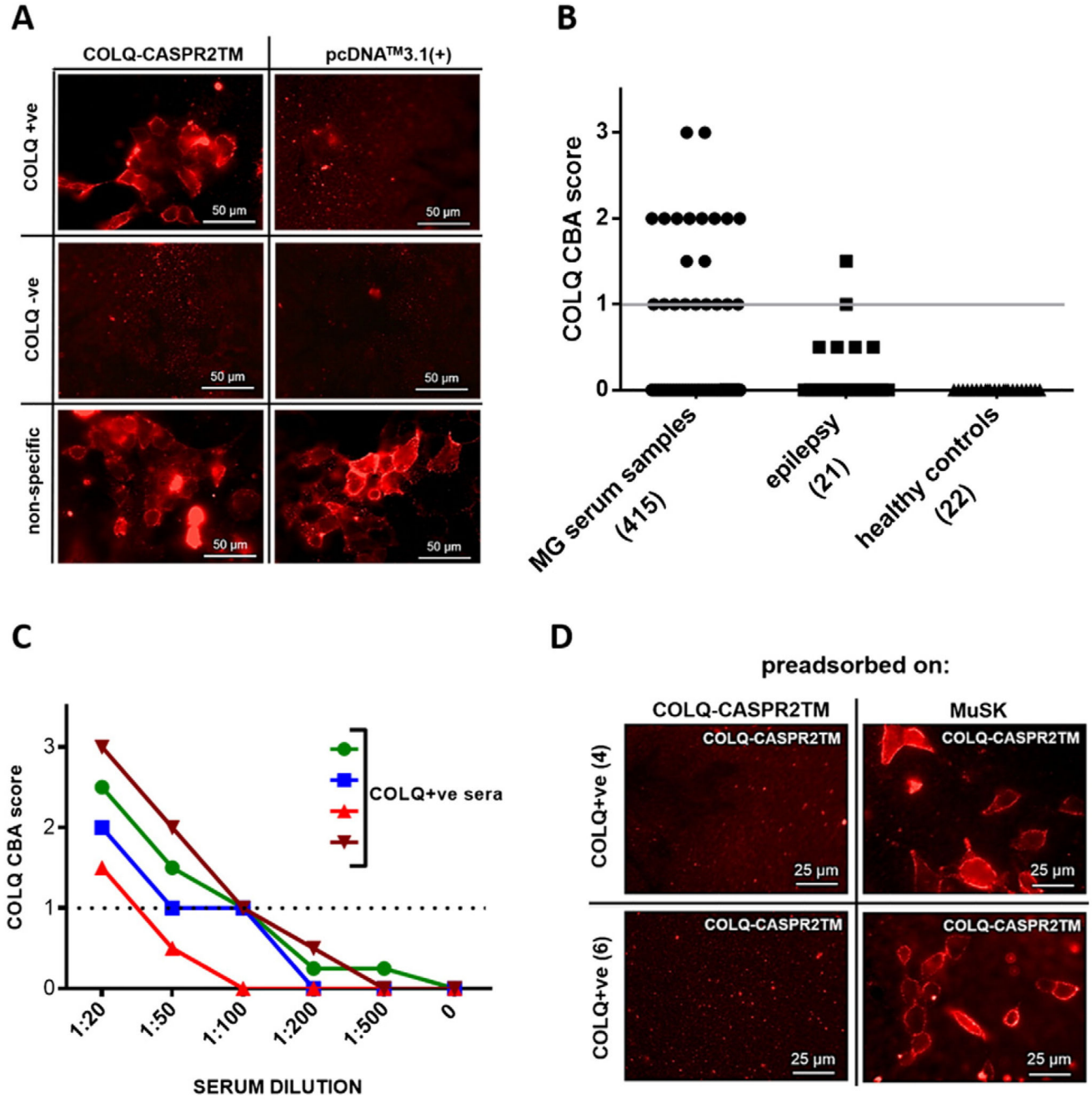

Fig. 1. CBA for the presence of anti-COLQ autoantibodies in patient sera.

A. HEK293 cells were transfected with pcDNA-*COLQ-CASPR2TM* or pcDNA™3.1(+) vectors, respectively, and stained with patient sera, and an Alexa Fluor 568 anti-human IgG secondary antibody. The panel shows example staining of COLQ + ve, COLQ-ve serum samples or the ones exhibiting unspecific cell surface binding. B. COLQ CBA scores in the cohort of serum samples. The dot plot presents the binding scores, reflecting the intensity of the fluorescence obtained with the patient and control sera. The numbers in brackets indicate a number of individuals in a particular subgroup. The grey line indicates the cut-off of the

CBA (score = 1), based on mean ± 3SDs of all controls. C. Titration of COLQ + ve sera. HEK293 cells were transfected with the pcDNA-*COLQ-CASPR2TM* plasmid, stained with anti-COLQ sera diluted 1:20, 1:50, 1:100, 1:200, and 1:500, and binding detected with Alexa Fluor 568 anti-human IgG secondary antibody. The intensity of the fluorescence was scored (0–3). Different symbols represent serum samples from different individuals. The dotted line indicates the cut-off of the CBA (score = 1), based on mean± 3SDs of all controls. D. Preadsorption of anti-COLQ antibodies from COLQ + ve sera. HEK293 cells were transfected with COLQ-CASPR2TM-encoding plasmid and incubated with two different COLQ + ve serum samples (from patients #4 and #6), preadsorbed against either COLQ-CASPR2TM or MuSK expressing cells, respectively. Binding of the patients' antibodies to the COLQ-CASPR2TM expressing cells was not found after adsorption against COLQ-CASPR2TM-expressing cells, but adsorption against MuSK-expressing cells did not reduce binding of the patients' antibodies.

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Table 1

Clinical information for the COLQ antibody + ve cases.

	Gender	Age of onset	Form of MG	EMG (decrement or jitter)	Reported response to AChE inhibitors	Response to steroids	Anti AChR or anti-MuSK antibodies	COLQ CBA score
1	F	15	Oculobulbar	Abn	Good	Yes	–	3
2	F	23	Generalised	Abn	Poor	ND	–	3
3	F	23	Ocular	Abn	Poor	ND	Clustered AChR	2
4	F	33	Generalised	Abn	Poor	ND	Clustered AChR	2
5	F	31	Ocular	Abn	Good	ND	Clustered AChR	2
6	F	20	Generalised	Abn	Good	Yes	AChR	2
7	F	39	Ocular	Abn	Good	Yes	Clustered AChR	2
8	F	NA	Generalised	ND	ND	Yes	MuSK RIA	2
9	F	NA	Oropharyngeal Respiratory	ND	ND	ND	MuSK RIA	2
10	F	65	Generalised	ND	ND	Yes	–	2
11	F	6	Generalised	Abn	Good	Yes	–	1.5
12	F	45	Generalised	Abn	Poor	Yes	–	1.5

Abn – abnormal, ND – not done, NA – not available, RIA - radioimmunoprecipitation assay.