Expression of OX40 (CD134) on CD4+ T-cells from patients with myasthenia gravis

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Summary

Myasthenia gravis (MG) is commonly regarded as the prototype of an antibody-mediated, organ-specific autoimmune disease. Antibodies against the acetylcholine receptor (AChR) on the muscle endplate trigger its typical clinical manifestations of weakness and fatiguability. T–B cell interactions are thought to play a crucial role in the pathogenesis of MG. OX40 (CD134), a costimulatory molecule that is expressed on activated CD4+ T-cells, might contribute to the development or pathogenesis of immune-mediated diseases such as rheumatoid arthritis and graft-versus-host disease. In the present study, we investigated the expression of OX40 on CD4+ T-cells from patients with MG and healthy individuals. Results from 36 MG patients and 28 healthy controls revealed that more freshly isolated CD4+ T-cells from MG patients expressed OX40 than cells from healthy individuals. High levels of antibodies against the AChR, thymic hyperplasia and onset at an early age were associated with elevated expression of OX40. Upon activation by various concentrations of anti-CD3 antibodies, CD4+ T-cells from MG patients showed a tendency toward higher levels of OX40 expression than cells from healthy individuals. Given the role of OX40 in the immune system, we conclude that OX40 might contribute to the development of MG.

Keywords: autoimmune disease, CD4+ T-cell, hyperplasia, myasthenia gravis, OX40 (CD134)

Introduction

Myasthenia gravis (MG) is an autoantibody-mediated disease and antibodies against the acetylcholine receptor (AChR) on the muscle endplate trigger its typical clinical manifestations. The autoantibody production is a T-cell dependent phenomenon both in human and experimental MG [1,2]. Thus, T–B cell interactions are essential events in the pathogenesis of MG. Among costimulatory pathways, there are two major families: the B7 family, including CD28 and CTLA-4, and the Tumour necrosis factor/Tumour necrosis factor receptor superfamily (TNF/TNFR). CD28 provides positive signals whereas CTLA-4 provides inhibitory signals to the T cell activation. We have earlier shown that MG patients had abnormally low expression of CTLA-4 on freshly isolated and stimulated T-cells in the peripheral blood [3]. Moreover, CTLA-4Ig was able to ameliorate experimental autoimmune MG [4]. In addition to the B7 family, TNF/TNFR superfamily members, such as CD27, OX40 (CD134), CD40L (CD154), 4–1BB (CD137), are

equally important for the effective generation of many types of T cell responses. These molecules appear to provide signals that allow continued cell division initially regulated by CD28, and/or to prevent excessive cell death [5]. Blockade of CD40 ligand by anti-CD40L antibodies suppressed chronic experimental autoimmune MG [6].

OX40, a member of TNFR family, is a 50-kD cell surface glycoprotein expressed on activated CD4+ T-cells [7]. Engagement of OX40 enhanced proliferation and cytokine production by CD4+ T-cells as well as the long-term survival of CD4+ T-cells by promoting Bcl-xL and Bcl-2 expression [8]. In addition, OX40 ligation enhanced cell turnover of antigen-activated CD4+ T-cells *in vivo* [9], and led to TRAF2 (Tumour necrosis factor receptor-associated factor 2) and TRAF5- mediated NF-κB activation [10]. OX40 ligand (OX40L) is expressed on APCs, such as activated B-lymphocytes [11,12] and dendritic cells [13]. Blocking of OX40–OX40L interaction with polyclonal anti-OX40 antibody resulted in a profound decrease of antigen-specific, T-cell-dependent-antibody production [14]. Mice lacking

OX40L showed impairment of APC function and a reduction in T cell proliferation and production of both Th1 and Th2 cytokines [15,16].

Due to its involvement in T–B cell interactions, the OX40 signal is strongly implicated in autoimmune disease. Signals from an agonistic antibody against OX40 could break an existing state of tolerance in CD4+ T-cells [17,18]. This suggests that a similar mechanism operating *in vivo* could lead to autoimmunity. Moreover, excess interactions of OX40/OX40L resulted in autoimmune-like inflammatory bowel disease (IBD) [19]. Expression of OX40 and/or OX40L has been demonstrated in tissues in several autoimmune disorders such as experimental autoimmune encephalomyelitis (EAE) [20,21], atherosclerosis [22], experimental IBD [23], human proliferative lupus nephritis [24], rheumatoid arthritis [25], human IBD [26], human inflammatory muscle disease [27] and human graft-*versus*-host disease (GVHD) [28]. Blocking the OX40:OX40L pathway ameliorated ongoing EAE [29], diabetes in NOD mice [30], murine models of asthma [31], collagen-induced arthritis [25], murine graft-*versus*-host disease [32] and experimental IBD [23].

In MG patients, OX40 was up-regulated in thymic tissues adjacent to germinal centres and might interact with OX40L in germinal centres to enhance anti-AChR antibody production in MG [33].

In the present study, we have tested CD4+ T-cells from peripheral blood mononuclear cells (PBMC), both freshly isolated and after stimulation with anti-CD3 antibody. The results indicate an involvement of OX40 in MG.

Materials and methods

Patients and healthy individuals

Thirty-six patients with clinically defined MG and 28 healthy age- and sex-matched controls (HC) were enrolled in the study. The diagnosis of the disease was based on a typical case history and clinical investigation, a positive response to edrophonium, a decremental response following repetitive nerve stimulation and the presence of antibodies against AChR. Samples from patients and healthy controls were taken between 0900h and 1400h. The analysis of samples was performed blinded. All MG patients were recruited in the MG centre at the Neurological Department in Karolinska Hospital. No patient was treated with immuno-suppressive drugs. Patient data are shown in Table 1. Patients were

Table 1. The percentage (%) of CD4+OX40+ T-cells of total CD4+T-cells from MG patients ($n = 36$) and healthy controls (HC) ($n = 28$).

	No. of patients	CD4+OX40+ T-cells	P-value *versus* HC
MG	36	7·7 (7·0–10)	0·012
Stage			
I	6	8·0 (3·1–15·9)	ns
II	28	7·3 (6·5–9·6)	0·022
III	2	12 (9·0, 15·1)	–
Thymic histopathologyy			
Thymoma	0	–	
Hyperplasia	16	9·1 (6·3–10·36)	0·035
Normal	2	8·9 (6·0, 8·8)	ns
Unthymectomized	18	7·2 (6·3–11·34)	ns
Gender			
Female	21	7·4 (6·5–10·4)	ns
Male	15	7·9 (6·1–11·1)	ns
Age of onset			
Early onset (< 40)	22	8·4 (6·7–10·8)	0·022
Late onset (> 40)	14	7·2 (5·8–10·6)	ns
Serum anti-AChR antibody (arbitrary unit)			
0	6	6·1 (2·6–12·3)	ns
> 0 (> 0–5 plus > 5)	30	8·4 (7·1–10·3)	0·008
> 0–5	22	7·3 (6·2–10·1)	0·045
> 5	8	10·3 (6·7–13·7)	0·009
HC	28	5·8 (4·8–7·3)	

Subgroups of MG patients were classified by stage, thymic histopathology, age of onset, gender and levels of anti-AChR antibodies. The numbers of CD4+OX40+ T-cells in CD4+ T-cells are shown as the median and the 5th to 95th confidence intervals. *P*-values obtained by the Mann–Whitney test between healthy individuals, total numbers of MG patients and subgroups of MG patients are listed in the table.

classified into different groups according to disease severity, thymic histology, age at onset, gender and levels of antibodies against the AChR. The Osserman-Oosterhuis classification was used to grade the disease [34]. Stage I, II and III indicate ocular MG, generalized disease and severe acute disease, respectively. Anti-AChR antibodies in sera from MG patients were determined by standardized radio-immuno-precipitation assay (RIA). The levels of the antibodies were given as arbitrary units [35].

Antibodies

The following monoclonal antibodies (mAbs) were purchased from Becton & Dickinson (Mountain View, CA, USA): FITC-labelled anti-OX40 (clone ACT35); PE-labelled anti-CD69 (clone FN50), anit-CD25 (clone M-A251), anti-CD28 (clone 28·2), anti-CTLA-4 (CD152, clone 14A2.H1); PerCP-labelled anti-CD4 (clone RPA-T4), anti-CD3 (clone HIT3a). Irrelevant isotype-matched mouse mAbs were FITC-IgG1 (clone JDC-1) and PE-IgG1 (clone G17-1).

Preparation and culture of mononuclear cells

Heparinized blood samples were collected from controls and MG patients. PBMC were separated by Ficoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). The cells were collected and washed three times with RPMI-1640 (Gibco, Paisley, UK) containing antibiotics.

Increasing levels of immobilized anti-CD3 antibodies were used to stimulate PBMC. Briefly, anti-CD3 antibody (OKT3) was coated on 24 well cell culture plates (Sarstedt, USA) and plates were incubated at 4 °C overnight. Plates were then washed three times with PBS before adding RPMI-1640 medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine and 10% (V/V) FCS (Life Technologies, Rockville, MD, USA). PHA (Life Technologies) was used as a positive control.

Flow cytometry

Cell suspensions were triple stained with FITC, PE- and PerCP-conjugated mAbs. Samples were incubated with mAb for 30 min at room temperature in the dark. Only cell surface molecules were detected and no intracellular staining was done in the present study. The cells were washed three times with PBS and analysed in a Becton-Dickinson FACScalibur flow cytometer using CellQuest software (Becton-Dickison). For freshly isolated PBMC, only small lymphocytes were gated and analysed. For stimulated PBMC, a larger gate was set according to the forward and side scatter.

Statistical analysis

The nonparametric Mann–Whitney test was used to compare the expression of OX40 on CD4+ T-cells in patients with MG, subgroups of MG and HC. Reported *P*-values were two-tailed and considered statistically significant at $P < 0.05$.

Results

OX40 expression on freshly isolated PBMC from MG and HC

The percentage of CD4+OX40+ T-cells among blood CD4+ T-cells in all MG patients are shown in Table 1 and Fig. 1. Results from 36 MG patients and 27 healthy controls revealed that more CD4+OX40+ T-cells in freshly isolated PBMC from MG patients than from healthy individuals ($P = 0.012$, Table 1 and Fig. 1). There was no difference in the mean fluorescence intensity (MFI) of OX40 between patients and healthy controls (data not shown).

Patients were divided into subgroups according to the stage, thymic histology, age of onset and levels of serum anti-AChR antibodies levels for additional analysis (Table 1). Patients with generalized disease, onset before 40 years of age, thymic hyperplasia and high levels of anti-AChR antibodies had more cells expressing OX40 ($P = 0.022$, 0·022, 0·035 and 0·008 *versus* HC, respectively). Patients without antibodies against the AChR antibodies had the same frequencies of OX40+ T cells as healthy controls (Table 1).

Figure 2 shows representative plots of OX40 expression on CD4+ T-cells from a MG patient. The expression of the surface molecules CD28, CTLA-4, CD25 and CD69 on CD4+ T-cells were analysed and shown in Fig. 2b–d). All CD4+OX40+ T-cells expressed CD28 (100%) but very few of them expressed CTLA-4(CD152) (< 1%) (Fig. 2b,c). CD25, normally expressed on activated T cells, was coexpressed on 91% of CD4+OX40+ T-cells (Fig. 2d), which is in agreement with previous studies [33]. In contrast, only 54·5% of CD4+OX40- T-cells from this patient expressed CD25 (Fig. 2d). CD69 could not be demonstrated on the surface of
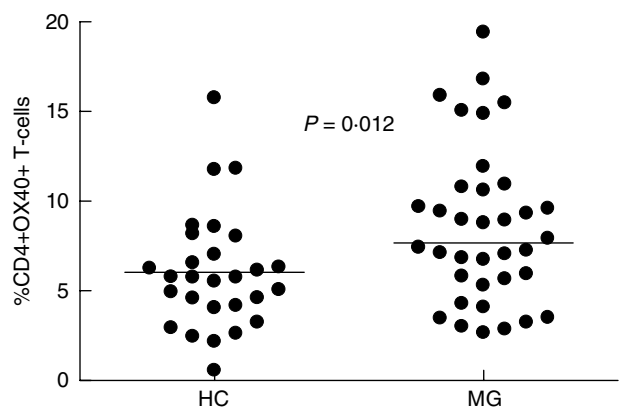

Fig. 1. OX40+ expression on freshly isolated CD4+ T-cells from MG patients ($n = 36$) and healthy controls (HC) ($n = 28$) ($P = 0.012$). The vertical lines indicates the median levels of CD4+ T-cells expressing OX40.
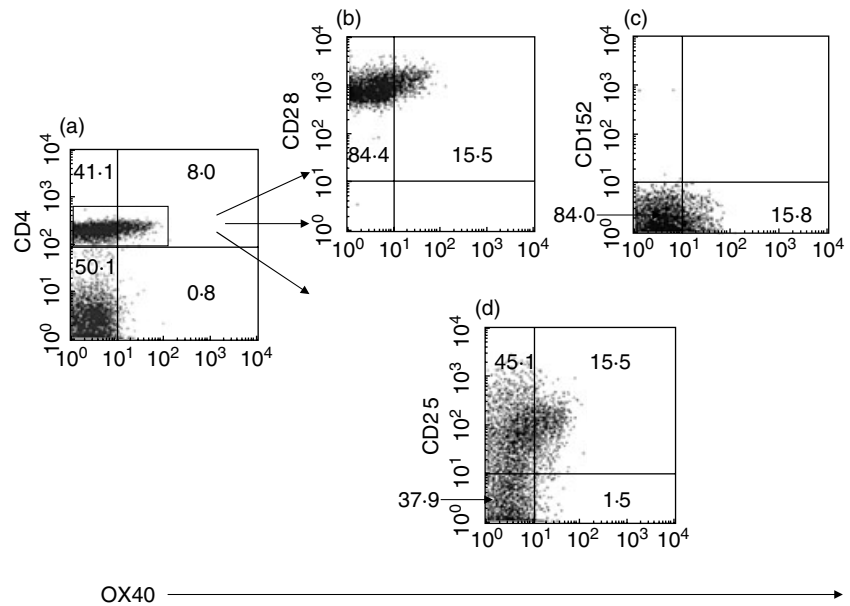
Fig. 2. Representative examples of flow cytometry plots showing (a) CD4+OX40+ T-cells from one MG patient (15·5% of total CD4+T-cells expressed OX40) and surface expression of (b) CD28 (100%) (c) CD152 (CTLA-4) (< 1%) (d) CD25 on CD4+OX40+ T-cells. CD4+OX40+ T-cells exhibited elevated CD25 (91%) expression compared with CD4+OX40- T-cells (54·5%).

CD4+OX40 T-cells (data not shown). All CD4+OX40+ T-cells from patients and controls displayed the same pattern as the cells from the MG patient shown in Fig. 2. CD8+ cells expressing OX40+ were rare in both patients and controls (data not shown).

OX40 expression on activated CD4+ T-cells from MG and HC

According to previous kinetic studies, OX40 expression on CD4+ T-cells was demonstrated within 24 h of stimulation with anti-CD3 antibodies and peaked between 48 and 72 h [7]. In this study, we stimulated PBMC with increasing concentrations of anti-CD3 antibodies for 24 and 96 h. Data from a few patients and healthy controls showed a similar kinetic pattern (data not shown). CD4+ T-cells from five MG patients showed a tendency towards elevated OX40 expression compared to cells from five healthy individuals after stimulation of 24 h (Fig. 3a). Consistent with previous findings, anti-CD3 antibody alone induced expression of OX40 on CD4+ T-cells [7] (Fig. 3b). PHA was used as a positive control. Stimulated CD4+OX40+ T-cells coexpressed the activation markers CD69 (89% of total OX40+ T-cells), and CD152 (18% of total OX40+ T-cells) (Fig. 3c,d). There was no difference after 96 h of stimulation (data not shown).

Discussion

Up-regulation of OX40 has been demonstrated in several types of autoimmune disease. T-cells in the synovial fluid of patients with active rheumatoid arthritis displayed the OX40 surface antigen [36]. OX40 was highly expressed in the gastrointestinal tissue of patients with IBD [37]. Biopsies from thymic hyperplastic areas from MG patients showed that OX40+ cells around the germinal centres (GC) were more frequent than in normal thymuses. A considerable number of OX40+ cells were present in the thymic tissues adjacent to thymomas from MG patients [33]. A patient who developed MG 25 months after receiving an allogeneic bone marrow transplant displayed prominently increased CD4+OX40+ T-cells in the peripheral blood one month before the onset of MG [38]. However, correlation of peripheral blood OX40+ T cells with chronic graft-*versus*-host disease (GVHD) in patients who received allogeneic haematopoietic stem cell transplantation was also observed [28]. The last two reports prompted us to question whether up-regulation of OX40 on PBMC was due to the effect of transplantation or of MG, and whether enhancement of OX40 expression is a typical feature of human MG. Indeed, our results demonstrated that the circulating CD4+OX40+ T-cell populations were increased in patients affected with MG when compared to healthy controls. There was no difference in the MFI between patients and controls, indicating that the number of OX40 molecules per cell was not changed. The CD4+OX40+ T-cells exhibited high levels of CD25, suggesting they include the activated, autoreactive T-cells [39]. There was no CD69 expression on these cells, indicating that they did not represent recently activated T-cells. In addition, no expression of CTLA-4 on the surface of CD4+OX40+ T-cells was detected, implying that these T-cells were not regulatory cells. This was further substantiated using CD25 and OX40 double staining, which showed that the OX40+CD25+ T-cells were never CD25bright, which was to be expected for naturally arising regulatory T cells [40].

One of the crucial events in the development of MG is activation of self-reactive T-cells and abundant IgG
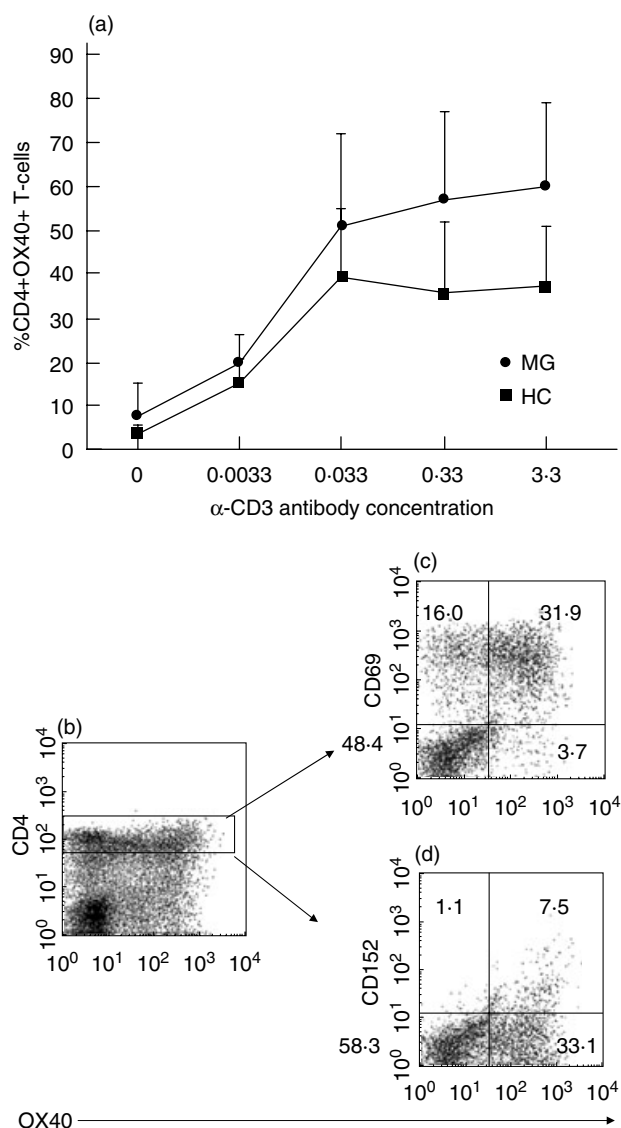
Fig. 3. (a) Induction of OX40 expression on CD4+ T-cells after 24 h (a). PBMC from MG (●, $n = 5$) and HC (■, $n = 5$) were stimulated in parallel with immobilized anti-CD3 antibody at the indicated coating concentrations for 24 hRepresentative examples of flow cytometry plots showing (b) CD4+OX40+ T-cells from one MG patient stimulated with anti-CD3 antibody for 24 h and surface expression of (c) CD69 (89%) (d) CD152 (CTLA-4) (18%) on CD4+OX40+ T-cells.

production and T-cell-dependent antibody production. CTLA-4 exerts inhibitory effects to T-cell activation [43]. Additionally, CD95 is involved in activation-induced cell death, particularly in negative selection [44]. The low expression of CTLA-4 on T-cells [3], but accumulated CD95 on thymocytes that occur in MG patients may permit the escape of autoreactive T-cells from negative selection and result in robust T-cell activation upon autoantigen. All data listed here indicated the activated background of MG will help us to understand the contribution of costimulatory molecules in the disease development of MG.

Co-stimulation through OX40 is crucial for the induction of an allo-reactive T-cell response both in humans and mice and involved in T-cell help for B-cells in the development of IgG responses [12,14,45]. We found that MG patients with anti-AChR antibodies had elevated level of CD4+OX40+ T-cells (Table 1), compared with healthy controls. Up-regulation of OX40 might induce activation of autoreactive T-cells and T-cell dependent autoantibody production. This speculation was further confirmed by animal experiments, which showed that constitutive OX40–OX40L interactions triggered autoimmune-like disease [19], and that an agonistic anti-OX40 antibody allowed anergic, autoreactive T-cells to acquire effector cell functions [17,18]. Nevertheless, considering the low percentage of AChR-specific T-cells in PBMC, we have to conclude that many CD4+OX40+ T-cells in patients may actually be activated bystanders. More detailed investigations relating the up-regulation of OX40 on CD4+ T-cells and MG disease development in both mice and human are required to address the issue.

The association between OX40 with generalized disease, hyperplasia and early age onset is an interesting finding of the present study. In generalized MG, thymic hyperplasia is the most frequent and typical pathological change. The thymus is characterized by numerous and prominent lymph follicles with germinal centres and a surrounding T-cell zone, containing autoreactive activated T-cells, and also B cells producing anti-AChR antibodies. High expression of OX40 is present in inflammation diseases such as IBD [36], rheumatoid arthritis [25] and atherosclerosis [22]. Our finding further confirmed the crucial role of OX40 in inflammation/ immune activation.

Accumulating evidence indicates that reagents designed to block OX40–OX40L interactions *in vivo* could be a promising therapy for human autoimmune disease. Two different OX40-specific therapeutic interventions have successfully been used to treat animals with autoimmune diseases [21,29]. Unlike conventional immunotherapies, blockage/ inhibition of OX40–OX40L interactions seems to specifically inhibit the disease-responsible effector T-cell function, and therefore does not cause widespread immunosuppression. Given the increased expression of the OX40 molecule on CD4+ T-cells from MG patients, and the possibility of its contribution to disease development, blocking the OX40-OX40L pathway may provide a new therapy for human MG.

autoantibody production. Altered expression of costimulatory molecules, such as CTLA-4 [3], CD80 and CD86 [40] on CD4+ T-cells from peripheral blood, and CD95 on thymocytes [41] has been demonstrated in patients with MG. Costimulation through CD80 is believed to result in the development of Th1 immune responses characterized by cell-mediated tissue destruction, whereas CD86 promotes Th2 immune responses characterized by a strong humoral component [42]. The increase of these two molecules on T-cells from patients thus may lead to profound cytokine

Acknowledgements

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