Expression of c-myc, c-Ki-ras and c-Ha-ras Oncogene Products in Peripheral Blood Mononuclear Cells from Patients with Myasthenia Gravis

Shigeru ARIMORI and Zhi-Jun SONG*

The oncogene products of c-myc, c-Ki-ras and c-Ha-ras and the subsets of natural killer cells in the peripheral blood mononuclear cells (PBMC) from 39 patients with myasthenia gravis (MG) and 40 healthy individuals were studied by immunocytochemical procedures. The MG patients showed an increased expression of c-myc, c-Ki-ras and c-Ha-ras oncoproteins in both lymphocytes and monocytes compared to those of healthy individuals ($P<0.01$, $P<0.005$ and $P<0.001$, respectively). On the contrary, the number of Leu-7⁺Leu-11c⁺ cells of MG patients was less than that of healthy controls ($P<0.05$). The oncogene expressions in PBMC of MG patients correlated positively with white blood cell counts, total natural killer cell counts (NK), Leu-7⁺Leu-11c⁺ of NK cell subset, γ -globulin, erythrocyte sedimentation rate and serum immunoglobulins; the oncogene expressions were negatively correlated with the Leu-7⁺Leu-11c⁻ NK subset. Thus, the increased expressions of c-myc, c-Ki-ras and c-Ha-ras oncoproteins accompanying the decreased Leu-7⁺Leu-11c⁺ NK cell in MG PBMC are likely responsible for the acceleration of the clinical manifestations in MG patients.

(Internal Medicine 32: 519–522, 1993)

Key words: immunocytochemistry, lymphocytes, monocytes, NK cell subsets, two-color flow cytometry

Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by antiacetylcholine receptor antibody (anti-AchR Ab) (1). Thymic hyperplasia and thymoma are found in 98% and 13% to 48% of MG patients, respectively (2, 3). Thymic hyperplasia consists of proliferation of not only epithelial cells and T lymphocytic cells but also activated B cell clone (3). Lymphoblastic lymphoma and other extrathymic malignancies can be also associated with MG (4). Clarification of the causal pathogenesis in MG has been discussed mainly from the viewpoint of thymology including T cell abnormalities (5) and anti-AchR Ab in PBMC (6). Regarding these pathogenetic backgrounds it has been considered that the clinical severity in MG patients is correlated with the progression of the immune deficiency. Recently, we demonstrated a higher incidence of HLA class II DPB1*020 and HLA-DR53 in younger onset female with MG (7). Such genetical deviations is also expressed on the oncogene activation of the thymus and thymoma of MG patients (8).

Here, we report increased expression of c-myc, c-Ki-ras and c-Ha-ras oncogene products accompanied by a decreased NK cell subset, Leu-7⁺Leu-11c⁺ cells, in MG PBMC which was correlated with higher ESR, WBC and immunoglobulins. Based on these findings, the oncoproteins of MG PBMC appear to be a useful clinical tool for determining the severity and prognosis of MG patients.

Materials and Methods

MG patients and healthy controls

The study encompassed 39 patients with MG (8 males and 31 females; mean age 47.5 ± 14.3 years, range 13–72) and 40 healthy individuals (19 males and 21 females; mean age 49.1 ± 9.1 years, range: 33–66). The diagnosis of MG was based on muscle weakness which responded to the anti-cholinesterase drug, edrophonium chloride (Tensilon test), waning phenomena in repetitive electromyographical examination (EMG), and the presence of autoantibody (IgG) to human acetylcholine receptors (anti-AchR Ab) according to the criteria of Research

From the Tokai University Medical and Health Care Administration Center, Isehara, and *the Fourth Department of Internal Medicine, Tokai University School of Medicine

Received for publication October 19, 1992; Accepted for publication June 2, 1993

Reprint requests should be addressed to Dr. Shigeru Arimori, Tokai University Medical and Health Care Administration Center, Isehara, Kanagawa 259-11, Japan

Committee of Neuro immunological Diseases of Japanese Ministry of Health and Welfare (9). Thymectomy had been performed on 11 of 39 MG patients. Thymic hyperplasia was confirmed by histologic examination in those thymectomized MG patients. The thymoma was ruled out in nonthymectomized MG patients by using a computer tomography scanning (CT), magnetic resonance imaging analysis (MRI), Tarium scintigraphy and chest X-ray film.

Preparation of PBMC

The PBMC were isolated from heparinized peripheral blood by Ficoll-Conray density gradient sedimentation (specific gravity 1.077). After washing with phosphate buffered saline (PBS, pH 7.4, 0.15 M), the concentration of cells was adjusted to 10^6 cells/ml with PBS. Cytospins were made on glass slides using a cytospin centrifuge (Shandon Cytospin 2, Shandon Southern Products Ltd., Runcorn, U.K.). The PBMC were composed of $88.78 \pm 5.85\%$ (mean $\pm$ SD) lymphocytes, $9.78 \pm 5.29\%$ monocytes, and $1.31 \pm 1.16\%$ granulocytes, with cell viability over 94%.

Two-color flow cytometry for determinations of NK cell subset

The PBMC were suspended in PBS containing 5% heat-inactivated fetal calf serum and 0.1% sodium azide (10^7 cells/ml) for the estimation of the subsets of NK cells. One hundred μ l of PBMC suspension was added to 10 μ l of fluorescein-isothiocyanate (FITC)-labeled anti-Leu-7 monoclonal antibody (MoAb), followed by 10 μ l of phycoerythrin (PE)-labeled anti-Leu-11c MoAb (Becton-Dickinson Immunocytometry Systems, CA, U.S.A.). The same volume of PBMC suspension was mixed with 20 μ l of Simultest control antibodies. The mixtures were protected from light and incubated for 30 min at 4°C, washed with 0.1% sodium azide-PBS, and preserved in 1% paraformaldehyde-PBS at 4°C for 16 h. The cell suspensions were filtered through nylon mesh filter. Five thousand cells were then scanned by two-color flow cytometry using a fluorescence activated cell sorter (FACScan, Becton-Dickinson, CA, U.S.A.) to determine the percentages of laser-activated positive cells. Lymphoid cells were gated by the setting of appropriate forward and 90° light scatter beams. Positive thresholds were determined with reference to the negative controls prepared for each sample. These controls always contained more than 99% negative cells.

Antibodies used for immunocytochemistry of oncoproteins

For the primary antibody, monoclonal mouse anti-human-c-myc (c-myc) oncoprotein antibody (Cambridge Research Biochemistry Co., Cambridge, U.K.), polyclonal rabbit anti-human-c-Ki-ras (c-Ki-ras) and anti-human-c-Ha-ras (c-Ha-ras) oncoprotein antibodies (provided kindly by Professor P.K. Nakane, Nagasaki University School of Medicine, Nagasaki, Japan) were used. Horseradish peroxidase (HRP) labeled goat anti-mouse IgG (Kirkegaard & Perry Lab. Inc., Maryland, U.S.A.) and goat anti-rabbit IgG (Biocellular Lab., Tokai University, Tokyo, Japan) served as the second antibody.

All antibodies against c-myc, c-Ki-ras, c-Ha-ras oncoproteins

were diluted with PBS containing 1% bovine serum albumin before use to 1:300, 1:100, and 1:50.

Immunocytochemical staining for oncoproteins

The cytospun slides were fixed with 4% paraformaldehyde in 0.01 M PBS, pH 7.4, at 4°C for 1 min, followed by methanol for 1 h and 1% hydrogen peroxide in methanol for 30 min. Then the slides were washed in PBS at 4°C. The slides were immersed in 10% normal goat serum and 1% normal human serum for 30 min each to prevent nonspecific reactions, followed by the addition of 35 μ l of either anti-c-myc, c-Ki-ras and c-Ha-ras antibodies, or normal mouse serum (NMS) and normal rabbit serum (NRS) as a negative control. The slides were then put in moist chambers for 2 h at room temperature. After washing with PBS, the slides were reacted with 35 μ l of the appropriate HRP-labeled second antibody for 2 h at room temperature. They were then washed in PBS again and immersed for 10 min in DAB solution made with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Wako Co., Osaka, Japan), 0.1 M phosphate buffer, pH 7.2, 1% nickel ammonium sulfate (J.T. Baker Chemical Co., NJ, U.S.A.), 1% cobalt chloride (Fisher Scientific Co., NJ, U.S.A.) and hydrogen peroxide. The slides were washed with distilled water and immersed in 1% kernechtrot (Merck Co. Ltd., NY, U.S.A., kernechtrot 0.1 g and aluminium sulfate 5 g in 100 ml of distilled water) for 30 min; they were then dehydrated in ethanol and xylol, and mounted.

Observation of the slides and clinical parameters

The oncoprotein-stained slides were compared with positive and negative controls using separated free cells from colon cancer and/or hepatic carcinoma, and negative controls which were treated by NMS, NRS, second antibody or PBS alone. In general, the positive staining of c-myc, c-Ki-ras and c-Ha-ras oncoproteins was brown to dark brown. Negative cells showed no immunocytochemical staining. More than 500 lymphocytes and monocytes were counted separately under a light microscope ($\times 1,000$) and the positive percentages were calculated. In addition, oncogene expressions were compared with the clinical parameters of age, sex, ESR, WBC and lymphocyte counts, NK cells including Leu-7⁺Leu-11c⁻, Leu-7⁺Leu-11c⁺ and Leu-7⁻Leu-11c⁺ subsets, serum IgG, IgA and IgM measured by laser nephelometry (Behring werke Nephelometer Co., Berlin, Germany) and autoantibodies (rheumatoid factor, antinuclear factor, thyroid and microsome antibodies as well as anti-AchR Ab). Statistical analysis was done by Student's unpaired t-test and correlation coefficient analysis.

Results

The subsets of NK cells in PBMC

The Leu-7⁻Leu-11c⁺ NK subset decreased significantly in MG PBMC in comparison with healthy PBMC ($P < 0.05$). The lymphocyte count and absolute numbers of Leu-7⁺Leu-11c⁻ and Leu-7⁺Leu-11c⁺ NK cells in MG PBMC were also decreased but remained statistically within the same ranges as the healthy controls (Table 1).

The expression of c-myc oncoprotein in PBMC

The c-myc oncoprotein of PBMC from both MG and healthy controls, expressed fine to coarse granules in the nucleus, and linear along the nuclear and cytoplasm membranes.

The positive percentages of c-myc in both lymphocytes and monocytes from MG PBMC were 3 and 2 times higher than those from healthy PBMC, respectively ($P<0.001$ and $P<0.01$) (Table 2).

Expression of c-myc in MG PBMC showed no correlation with age, sex, duration of disease, history of thymectomy, anti-nuclear factor, microsome and thyroid tests, and anti-AchR Ab by coefficient analysis. However, WBC ($r=0.43$, $P<0.004$), IgA ($r=0.32$, $P<0.03$) and ESR ($r=0.29$, $P<0.04$) was positively correlated, and Leu-7⁺Leu-11c⁻ percentage ($r=-0.27$, $P=0.05$) was negatively correlated with c-myc expression in MG lymphocytes.

The expression of c-Ki-ras oncoprotein in PBMC

The c-Ki-ras oncoprotein stained the PBMC from both MG patients and healthy controls as a coarse granule or diffuse in the cytoplasm, occasionally including the nuclear membrane.

The percentages of c-Ki-ras positive lymphocytes and monocytes from MG PBMC were significantly higher than healthy PBMC, $P<0.005$ and $P<0.001$, respectively (Table 2).

The c-Ki-ras positive MG monocytes correlated positively

with the percentages of serum γ -globulin and lymphocytes ($r=0.30$, $P<0.04$) as well as serum IgA ($r=0.35$, $P<0.02$). The c-Ki-ras expression in MG lymphocytes correlated positively with the total NK cells ($r=0.30$, $P<0.03$) and Leu-7⁺Leu-11c⁺ percentage ($r=0.29$, $P<0.04$).

The expression of c-Ha-ras oncoprotein in PBMC

c-Ha-ras was expressed in both MG and healthy PBMC as a fine granular appearance in the cytoplasm, and occasionally linear in the nuclear membrane. The nucleus was occasionally stained diffusely.

The percentages of c-Ha-ras positive lymphocytes and monocytes in MG PBMC were significantly higher than those in healthy PBMC, $P<0.001$ and $P<0.01$, respectively (Table 2). This c-H-ras expression in MG monocytes was positively correlated with serum IgM ($r=0.37$, $P<0.02$). The percentages of Leu-7⁺Leu-11c⁺ were positively correlated and those of Leu-7⁺Leu-11c⁻ were negatively correlated with c-Ha-ras expressions in MG monocytes ($r=0.28$, $P<0.05$) and lymphocytes ($r=-0.33$, $P<0.03$), respectively.

Discussion

Thymic hyperplasia, the germinal center formation in the thymus, is an important pathognomonic feature in MG with regards to the anti-AchR Ab formation (1). This germinal center in the thymus is considered to be a B cell clone of the T cell organ and to be representative of autoimmunity (2). This idea suggests thymic hyperplasia to be an inflammatory expression of the thymus, i.e., thymitis. Usually chronic inflammations promote a malignant transformation; such thymitis is a so called prethymomatic state. The malignant transformation of lymphoid tissue, especially of the thymus, is controlled by oncogene activations or/and antisense gene deletions (8). In a preliminary study we demonstrated that hyperplastic thymus and thymoma show accelerated cell cycles and higher expressions of oncogene products, especially those of c-myc, c-Ki-ras, c-Ha-ras and c-N-ras, in not only epithelial cells but also thymic lymphocytes (8). These oncoproteins expressed correspondingly on clinical severity and positiveness of anti-AchR Ab (8). On the other hand MG had subset abnormalities of T and B cells in not only the thymus but also in the PBMC (5).

Here we found increased c-myc, c-Ki-ras and c-Ha-ras oncogene expressions accompanied by a decreased Leu-7⁺Leu-11c⁺ NK subset in MG PBMC.

The c-myc protooncogene exists on the long arm of chromosome 8(8q24) (10). c-myc protooncogene activated by virus or nonviral noxa, such as epidermal growth factor, platelet derived growth factor or fibroblastic growth factor, produces c-myc mRNA and converts c-myc oncogene to c-myc oncoprotein. The c-myc oncoprotein promotes cell cycle of the normal and malignant cells from G0 to G1 and S phase. The molecular weight of c-myc oncoprotein is 62K daltons. This oncoprotein can bind to double- or single-stranded DNA in the nucleus, and highly increases in the nucleus of proliferating cells not only normal cells but also malignant cells (10). The c-Ki-ras and c-

Table 1. Subsets of NK Cells in PBMC of 39 MG Patients and 35 Healthy Controls (Mean $\pm$ SE)

	MG	Control	P value
Lymphocytes	1853.97 $\pm$ 143.32	2148.49 $\pm$ 110.90	>0.1
Leu-7 ⁺ Leu-11c ⁺	137.93 $\pm$ 15.78	189.34 $\pm$ 15.39	<0.05
Leu7 ⁺ Leu11c ⁺	273.74 $\pm$ 43.88	310.42 $\pm$ 43.07	>0.5
Leu7 ⁺ Leu11c ⁻	163.89 $\pm$ 21.31	203.33 $\pm$ 19.82	>0.1

NK: natural killer, PBMC: peripheral blood mononuclear cell, MG: myasthenia gravis.

Table 2. Expressions of Oncoproteins in PBMC of 39 MG Patients and 40 Healthy Controls

% of positive oncoproteins (Mean $\pm$ SE)			
	MG	Control	P value
c-myc			
Lymph	13.38 $\pm$ 1.87	4.88 $\pm$ 0.73	<0.001
Mo	10.10 $\pm$ 1.90	4.25 $\pm$ 0.75	<0.01
c-Ki-ras			
Lymph	7.13 $\pm$ 1.09	3.30 $\pm$ 0.55	<0.005
Mo	7.74 $\pm$ 0.97	3.20 $\pm$ 0.54	<0.001
c-Ha-ras			
Lymph	7.38 $\pm$ 0.99	3.23 $\pm$ 0.44	<0.001
Mo	9.10 $\pm$ 1.17	5.35 $\pm$ 0.68	<0.01

PBMC: peripheral blood mononuclear cells, MG: myasthenia gravis, Lymph: lymphocytes, Mo: monocytes.

Ha-ras studied in the present study are two members of the ras family which include Ki-, Ha- and N-ras. The human c-Ki-ras gene is located in the short arm of chromosome 12(p12.1-pter) and c-Ha-ras gene is in the short arm of chromosome 11(p15.1-15.5) (11). The point mutation of the ras oncogenes by insertion of RNA virus codon activates ras gene to ras oncoprotein, and binds to GDP (guanosine-5'-diphosphate) in the cytoplasmic membrane. This chain reaction of ras activation works as an initial trigger of malignant transformation of human cells under the cooperation of the deletion of antisense gene or/ and other oncogene activation (11). These oncogene activations can be identified by using Southern blotting (12), PCR-RFLP method (13), *in situ* hybridization (14), and also immunocytochemical staining (8, 14) as employed in this paper.

Observed c-myc oncoprotein in PBMC is located mainly in the nucleus. The ras family was expressed stronger in the cytoplasm than nucleus. These oncoproteins in PBMC were observed in T and B cells, monocytes, and granular cells as well. This paper showed definitely higher expressions of c-myc as well as ras family oncoproteins in PBMC of MG than normal. These data are compatible with thymic expression of c-myc and ras family oncoproteins which are reported to express more in the epithelial cells of the thymoma than in thymic hyperplasia (8). Out of 11 thymectomized patients, the oncoprotein expressions of both thymus and PBMC were studied concurrently in 3 patients. Synchronized high percentage of c-myc oncoprotein was shown in 2 MG patients (both Type IIb females and one of them complicated with idiopathic thrombocytopenic purpura), and of c-Ki-ras in 2 (Type IIb and Type IIa females). These 3 patients showed higher c-N-ras expressions in the thymic epithelial cells at the same time. This paper included only MG PBMC with thymic hyperplasia thus we could not discuss about the differences between MG PBMC associated with thymoma and with hyperplastic thymus.

The triggers to initiate thymitis and thymoma are not clear yet. As one of the triggers, viral infection in the thymus is thought to be highly responsible (15). Virus can initiate cell proliferation and cause malignant transformations *in vitro* as well as *in vivo* intermingled with accelerated expressions of c-myc and ras oncoproteins (10, 11) and with a decrease of NK cells.

In the present study, PBMC from patients with MG and from healthy individuals were monitored for the numbers of NK cells, Leu-7⁺Leu-11c⁻, Leu-7⁺Leu-11c⁺, Leu-7⁻Leu-11c⁺ cells as NK cell subsets by two-color flow cytometry. A definitely decreased Leu-7⁻Leu-11c⁺ was observed in MG PBMC. A Leu-7⁻Leu-11c⁺ subset has been confirmed to be highly active in destroying target cells such as virus-derived transformed cells and malignant transformed cells (16). Consequently, a decrease of Leu-7⁻Leu-11c⁺ cells in MG patients allows proliferation of virus-infected cells (15) as well as oncoprotein positive cells, PBMC, as shown in this paper.

The clinical severity of MG was evaluated according to the grade and duration of muscle weakness, responsiveness against antiacetylcholine esterase drug and laboratory examinations which indicated inflammatory and autoimmune reactions, i.e.,

ESR, WBC, immunoglobulins and anti-AchR Ab and so forth.

We observed correlations between the percentages of oncoprotein positive MG PBMC with NK cells and other clinical parameters of MG. The markedly increased expression of c-myc oncoprotein in the PBMC of MG patients was correlated with a lower percentage of Leu-7⁺Leu-11c⁻, and with higher ESR, WBC, or IgA. The increased total NK cells, percentage of Leu-7⁺Leu-11c⁺ cells, % of γ -globulin, and % of lymphocytes was correlated with higher c-Ki-ras. Further, the lower Leu-7⁺Leu-11c⁻ cells and higher Leu-7⁺Leu-11c⁺ cells and serum IgM was well correlated with the higher c-Ha-ras. Accordingly, thymic hyperplasia in MG was accompanied by a decrease in Leu-7⁻Leu-11c⁺ cells, and was closely correlated with the accelerated expressions of c-myc, c-Ki-ras and c-Ha-ras oncoproteins in MG PBMC and with deterioration of MG manifestation.

Acknowledgment: We thank Dr. Shigeo Hinohara, Director of Health Center, Associate Professor, Department of Health Development, Tokai University for kindly providing blood samples of healthy individuals. We also appreciate the suggestions and help of Professor P.K. Nakane of Nagasaki University School of Medicine, Nagasaki.

References

- 1) Arimori S. Pathogenesis and treatment of myasthenia gravis. *Tokai J Exp Clin Med* **11** (Suppl): 159, 1986.
- 2) Berrih-Aknin S, Morel E, Raimond F, et al. The role of the thymus in myasthenia gravis: Immunohistological and immunological studies in 115 cases. *Ann N Y Acad Sci* **505**: 50, 1987.
- 3) Arimori S, Ichikawa Y, Takaya M, et al. The thymus and autoimmune disorders. *Jap J Chest Dis* **23**: 24, 1985 (abstract in English).
- 4) Mortimer JE, Kidd P. Myasthenia gravis and lymphoblastic lymphoma. Antiacetylcholine receptor antibody as a tumor marker – a case report. *Cancer Invest* **7**: 327, 1989.
- 5) Ichikawa Y, Shimizu H, Yoshida M, Arimori S. Two color flow cytometric analysis of thymic lymphocytes from patients with myasthenia gravis and/or thymoma. *Clin Immunol Immunopath* **62**: 91, 1992.
- 6) Lindström J. An assay for antibodies to human acetylcholine receptor in serum from patients with myasthenia gravis. *Clin Immunol Immunopath* **7**: 36, 1977.
- 7) Horiki T, Moriuchi J, Inoko H, et al. HLA-DPB1 allele associates with early-onset myasthenia gravis in Japan. *Neurology* **43**: 771, 1993.
- 8) Yoshida M, Arimori S. Oncogene in the thymoma. *Path Clin* **11**: 40, 1993 (in Japanese).
- 9) Arimori S. Myasthenia gravis. *Nihon Rinsho* **48** (Suppl): 282, 1990 (in Japanese).
- 10) Persson H, Leder P. Nuclear localization and DNA binding properties of a protein expressed by human c-myc oncogene. *Science* **225**: 718, 1984.
- 11) Bos JL. Ras oncogenes in human cancer: a review. *Cancer Res* **49**: 4682, 1989.
- 12) Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**: 503, 1975.
- 13) Inoko H. PCR-RFLP method holds great promise for complete HLA class II genotyping. *Tissue Antigens* **36**: 88, 1990.
- 14) Izumi S, Moriuchi T, Koji T, et al. Localization of c-myc in HL-60 cells, neoplastic and normal tissues: an immunohistochemical and *in situ* hybridization study. *Acta Histochem Cytochem* **21**: 327, 1988.
- 15) Arimori S, Ichimura K, Tokunaga M, Morita K. Retrovirus like particles in human thymomas. *Tokai J Exp Clin Med* **15**: 219, 1990.
- 16) Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J Immunol* **131**: 1789, 1983.