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Abstract

Myelin associated glycoprotein (MAG) has growth promoting effect on mouse cerebellar neurons. In the present study, we examined which isoform of MAG has the effect. cDNA for L-MAG and S-MAG was stably transfected into BALB/c 3T3 cells, on which cerebellar neurons were cultured. The neurons were stained with antibody against microtubule-associated protein-2 (MAP-2). Neurites of the neurons cultured on cells expressing L-MAG extended significantly further than those cultured on cells expressing S-MAG or on control cells. Therefore, intracellular domain of MAG may have the potential to affect MAG-neurite interaction.
Myelin associated glycoprotein (MAG) is an integral membrane protein of myelin-forming cells, namely oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). MAG is a transmembrane protein and belongs to the immunoglobulin superfamily [9]. MAG exists in the periaxonal myelin and may facilitate contact with the axon. Although its role in axonal growth is rather controversial, we and Turnley et al. observed a neurite promoting effect of MAG on perinatal mouse cerebellar neurons [5,10]. However, the difference in the effects of isoforms of MAG has not been studied. There are two isoforms of MAG, L-MAG and S-MAG, resulting from alternative splicing. L-MAG is abundant in the developing CNS and is reduced in expression level in the adult [9]. Thus, it is possible that L-MAG has axon promoting properties. Isoform-specific knockout mice lacking L-MAG had a similar phenotype to MAG gene-knockout mice in myelination but did not degenerate PNS myelin [3]. This result indicated that the two isoforms of MAG have different functions. We performed the present study to clarify the difference in the isoform-specific effects of MAG on neurite outgrowth.

L-MAG and S-MAG cDNAs were subcloned into pBCMGSneo. These plasmids or vector alone were transfected into BALB/c 3T3 cells by lipofection. Stable clones were isolated in the presence of 0.3 mg/ml G418 (Wako). Single cell clones from the bulk transfectants were derived by limiting dilution cloning. These transfectants were cultured in Dulbecco's modified essential medium (DMEM, Sigma) containing 10% fetal calf serum (FCS).
Before neuronal culture, BALB/c 3T3 cells stably transfected with L-MAG, S-MAG, or vector alone or BALB/c 3T3 cells alone were plated at 5 x 10^4 cells / cm^2 and cultured in DMEM containing 10% FCS for 2 days. Some of the cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with anti-MAG antiserum which recognizes the extracellular region of MAG. The antibody was fully characterized before [5]. Furthermore, the expression of MAG was quantified by ELISA with the same antibody. The cerebellar cells were dissociated from 1- to 2-day-old BALB/c mice. The cells were suspended in Ham's F12 / DMEM (1:1) containing 2% FCS, 5 mg/ml insulin, 5 mg/ml transferrin, 30 nM sodium selenite, 30 nM triiodothyronin, 20 nM progesterone and 10 nM hydrocortisone and plated at 1.5 x 10^5 cells/cm^2 on the transfected BALB/c 3T3 cells. Twenty four hours after plating, the cultures were fixed with 4% paraformaldehyde in PBS and incubated with a monoclonal antibody to a neuronal marker, microtubule-associated protein-2 (MAP-2, Sigma). After incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel), immunostaining was visualized with diaminobenzidine. Four microscopic fields were randomly selected. Neurites of 70-90 neurons per field were measured and categorized into 4 groups, namely, neurons with neurites shorter than their own cell bodies (0-1), with neurites 1-3 times longer (1-3), 3-5 times longer (3-5) and 5 times or longer (>5).

We produced BALB 3T3 cells expressing L-MAG or S-MAG. The expression was confirmed by immunostaining with antisera which recognizes the extracellular domain of MAG (Fig. 1A and B). The
cells transfected with vector alone or those not transfected gave no staining (Fig. 1C). There was no significant difference in the expression between L- and S-MAG (Fig. 1D).

To see the difference in the effect on neurite extension between the two isoforms of MAG, cerebellar neurons were cultured on the L- or S-MAG-transfected cells for 24 hours. MAP-2 immunostaining allowed us to visualize neuronal cell bodies and neurites (Fig. 2). Significantly more neurons cultured on L-MAG-expressing cells had long neurites (Fig. 2A). Here we define "long neurites" as those at least 3 times or longer than the cell diameter. Few primary neurons cultured on the cells transfected with S-MAG cDNA, vector alone or without transfection extended long neurites (Fig. 2B, C, D).

Fig. 3 shows the percentage of neurons which extended long neurites. On the cells expressing L-MAG, 51% of neurons extended long neurites (3 times or longer than their own cell body), compared to 15% neurons on the cells expressing S-MAG. There was no significant difference between neurons on S-MAG and mock transfected or untransfected cells. Whereas only 2.6% of neurons on S-MAG expressing cells extended neurites that were more than 5 times longer than their own cell body, 18% of neurons on L-MAG expressing cells did so. No significant difference was found between neurons on S-MAG- and mock-expressing cells.

In the present study, we showed that L-MAG but not S-MAG had growth promoting activity in neurons isolated from postnatal mouse cerebellum. More than half of the neurons cultured on L-MAG-expressing cells had long neurites, comparing 15% of those on S-MAG expressing cells.
This result can explain preferable effect of MAG on the axon elongation during development. L-MAG is predominantly expressed early in the development and disappears in the adult. On the contrary, S-MAG is the predominant isoform in the adult [4, 7]. Thus, there may be physiological merit if myelin containing L-MAG in the developing stage permits axonal outgrowth. Although our methods could not distinguish between axons and dendrites, myelin may permit axonal elongation with L-MAG in the early stage and can become repulsive against axonal elongation with S-MAG in the adult, inhibiting unnecessary axonal sprouting in the complete network. There are two possible explanations for the different responses of the neurites on the fact that the two isoforms are identical in the extracellular domains but differ in the length of the intracellular domain. One is that the difference in the intracellular domain affects the conformation of the extracellular domains. The other is that there is a difference in the response of the MAG-expressing cells, for example, in the expression of adhesion molecules after they make contact with neurites. Further study is necessary to identify the molecules responsible for L-MAG's effect on neurites.

Another point of interest is that we confirmed that MAG has a promoting activity rather than inhibitory on neurite elongation of young cerebellar neurons. We and Turnley et al. observed a neurite promoting effect of MAG on postnatal mouse cerebellar neurons and embryonic mouse cerebellar neurons [5, 10]. However, there have been many studies suggesting MAG’s inhibitory effect on neurite extension [2, 6, 8]. The difference could be attributed to species or the conditions for the culture
especially the tropic factors in the serum as was reported by Cai et al. [1].
References


Figure legends

Figure 1. The expression of MAG by transfected BALB/c 3T3 cells. Immunostaining for the extracellular region of MAG shows that L-MAG (A) and S-MAG (B) were expressed to the same extent. The cells transfected with vector alone gave no staining (C). (D) Quantification of expressed MAG. There was no significant difference between L- and S-MAG.

Figure 2. Neurite extension on MAG-expressing cells. Primary cerebellar neurons were cultured for 24 hours on BALB/c 3T3 cells with cDNA for L-MAG (A), with cDNA for S-MAG (B), with vector alone (C) or without transfection (D). After fixation, cells were immunostained with anti-MAP-2 antibody.

Figure 3. Quantitation of the number of neurons with various lengths of neurites. Cultured neurons were stained as shown in Fig. 2 and the lengths of the neurites were measured. The length of the longest neurite of a cell was divided by the diameter of the cell body. The cells were categorized into 4 groups according to their neurite length / cell body diameter.
Fig. 1

Fig. 2

Fig. 3