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LOTUS suppresses axon growth inhibition by blocking interaction between Nogo receptor-1 and all four types of its ligand

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ABSTRACT

Axon growth inhibitors such as Nogo proteins, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), and B lymphocyte stimulator (BLyS) commonly bind to Nogo receptor-1 (NgR1), leading to enormous restriction of functional recovery after damage to the adult central nervous system. Recently, we found that lateral olfactory tract usher substance (LOTUS) antagonizes NgR1-mediated Nogo signaling. However, whether LOTUS exerts antagonism of NgR1 when bound by the other three ligands has not been determined. Overexpression of LOTUS together with NgR1 in COS7 cells blocked the binding of MAG, OMgp, and BLyS to NgR1. In cultured dorsal root ganglion neurons in which endogenous LOTUS is only weakly expressed, overexpression of LOTUS suppressed growth cone collapse and neurite outgrowth inhibition induced by these three NgR1 ligands. LOTUS suppressed NgR1 ligand-induced growth cone collapse in cultured olfactory bulb neurons, which endogenously express LOTUS. Growth cone collapse was induced by NgR1 ligands in *lotus*-deficient mice. These data suggest that LOTUS functions as a potent endogenous antagonist for NgR1 when bound by all four known NgR1 ligands, raising the possibility that LOTUS may protect neurons from NgR1-mediated axonal growth inhibition and thereby may be useful for promoting neuronal regeneration as a potent inhibitor of NgR1.

Introduction

Neurons fail to re-elongate damaged axons to their original targets in the adult central nervous system (CNS). This failure has been ascribed to repulsive axon guidance molecules (Niclou et al., 2006) and axonal growth inhibitory molecules in the glial scar (Yiu and He, 2006) and in myelin (Schwab, 2010; Yiu and He, 2006). Among these obstacles, the molecular mechanism underlying myelin-associated inhibition of axonal growth has been investigated most extensively (Schwab, 2010; Yiu and He, 2006). Nogo proteins (GrandPré et al., 2000), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994), and oligodendrocyte myelin glycoprotein (OMgp) (K.C. Wang et al., 2002) have been identified as the major inhibitors of axonal growth in myelin. Nogo receptor-1 (NgR1) is the common receptor for all of these axonal growth inhibitors (Domeniconi et al., 2002; Fournier et al., 2001; K.C. Wang et al., 2002) and is expressed in many types of neurons and their axons in the CNS (X. Wang et al., 2002). Recently, B lymphocyte stimulator (BLyS), which is a tumor necrosis factor superfamily member that is expressed in astrocytes in the CNS (Krumbholz et al., 2005), has also been identified as a functional ligand for NgR1 (Zhang et al., 2009). NgR1 forms a receptor complex with leucine-rich repeat and immunoglobulin domain-containing Nogo receptor-interacting protein 1 (LINGO-1) (Mi et al., 2004) and either the 75-kDa neurotrophin receptor ($p75^{NTR}$) (Yamashita et al., 2002) or tumor necrosis factor receptor superfamily member 19 (TROY) (Park et al., 2005). These co-receptors play a role in transmitting signals to intracellular molecules such as RhoA (Mi et al., 2004; Niederöst et al., 2002; Park et al., 2005; Yamashita et al., 2002) and its effector, Rho-associated, coiled-coil containing protein kinase (ROCK) (Niederöst et al., 2002). Binding of these four glial components to NgR1 triggers signal transduction to downstream molecules via the NgR1 co-receptors to induce growth cone collapse and neurite outgrowth inhibition (Schwab, 2010; Yiu and He, 2006). This signaling pathway, which is called Nogo signaling, enormously restricts the ability of neurons to regenerate their damaged axons in the CNS (Schwab, 2010; Yiu and He, 2006).

Accumulated evidence has shown that neutralizing antibodies against Nogo (Freund et al., 2006), an NgR1 antagonist that is specific

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for Nogo (GrandPré et al., 2002), soluble NgR1 peptides (Li et al., 2004), and genetic deletion of Nogo (Kim et al., 2003) or NgR1 (Kim et al., 2004) promote the histological and functional regeneration of damaged CNS axons. Furthermore, triple mutation of Nogo, MAG, and OMgp exhibits greater improvement in axonal regeneration in the injured CNS compared with single mutation of Nogo (Cafferty et al., 2010). These reports suggest that inhibition of the function of multiple glial components that bind to NgR1 may more effectively improve the ability of neurons to regenerate their damaged CNS axons, although whether inhibition of BLyS function contributes to the regeneration of damaged CNS axons in vivo remains unknown.

Recently, we identified lateral olfactory tract usher substance (LOTUS)/cartilage acidic protein-1B (Crtac1B) as a novel molecule that functions in axonal bundle formation by antagonizing NgR1 function by Nogo (Sato et al., 2011). However, whether LOTUS exerts antagonistic activity on NgR1 that is bound by the other three ligands remains unknown. We show here that LOTUS suppressed axonal growth inhibition that was mediated by NgR1 function by blocking the binding of these three types of NgR1 ligands. Our findings suggest that LOTUS functions as a potent endogenous antagonist for NgR1 when bound by all the known NgR1 ligands, raising the possibility that LOTUS may overcome the failure of damaged CNS neurons to regenerate due to NgR1 function.

Results

LOTUS blocks the binding of MAG, OMgp, and BLyS to NgR1

We previously showed that LOTUS overexpression together with NgR1 in COS7 cells blocks the binding of Nogo66, which is the functional domain of NogoA involved in axon growth inhibition (GrandPré et al., 2000), to NgR1 (Sato et al., 2011). To examine whether LOTUS blocks the binding of the other three NgR1 ligands to NgR1, we performed a binding assay with alkaline phosphatase (AP)-fused MAG (MAG-AP), OMgp (AP-OMgp), or BLyS (AP-BLyS) to NgR1, which were overexpressed together with LOTUS in COS7 cells (Fig. 1A). Cell surface expression of NgR1 and/or LOTUS was confirmed with double immunocytochemistry with antibodies against NgR1 and LOTUS applied to unfixed COS7 cells under nonpermeabilizing conditions (Fig. 1A). The binding levels of MAG-AP, AP-OMgp, and AP-BLyS to NgR1 overexpressed in COS7 cells were quantified by measuring the reaction product of *p*-nitrophenyl phosphate (*p*NPP) and normalized to the binding level of MAG-AP (10 nM), AP-OMgp (10 nM), or AP-BLyS (2 nM) in COS7 cells overexpressing NgR1 alone, respectively (Fig. 1B–D). The binding of MAG-AP and AP-OMgp to COS7 cells overexpressing LOTUS alone was not detected at any dosage (10, 20, or 50 nM). MAG-AP (100.0 ± 0.0% vs. 48.6 ± 7.6% in Mock control at 10 nM, $^{**}P < 0.01$; 177.7 ± 7.6% vs. 91.6 ± 11.1% in Mock control at 20 nM, $^{**}P < 0.01$; 406.0 ± 39.3% vs. 216.6 ± 37.9% in Mock control at 50 nM, $^{**}P < 0.01$) and AP-OMgp (100.0 ± 0.0% vs. 45.5 ± 3.7% in Mock control at 10 nM, $^{**}P < 0.01$; 163.2 ± 8.5% vs. 74.1 ± 2.6% in Mock control at 20 nM, $^{**}P < 0.01$; 334.3 ± 14.7% vs. 174.7 ± 12.5% in Mock control at 50 nM, $^{**}P < 0.01$) were clearly detected in COS7 cells overexpressing NgR1 alone in a dose-dependent manner, whereas the binding of MAG-AP (60.2 ± 3.8% vs. 100.0 ± 0.0% in NgR1 alone at 10 nM, $^{**}P < 0.01$; 110.6 ± 3.7% vs. 177.7 ± 7.6% in NgR1 alone at 20 nM, $^{**}P < 0.01$; 245.5 ± 25.0% vs. 406.0 ± 39.3% in NgR1 alone at 50 nM, $^{*}P < 0.05$) and AP-OMgp (60.5 ± 5.9% vs. 100.0 ± 0.0% in NgR1 alone at 10 nM, $^{**}P < 0.01$; 105.0 ± 10.5% vs. 163.2 ± 8.5% in NgR1 alone at 20 nM, $^{**}P < 0.01$; 199.4 ± 20.4% vs. 334.3 ± 14.7% in NgR1 alone at 50 nM, $^{**}P < 0.01$) was completely inhibited to negative control levels (Mock control or LOTUS alone) in COS7 cells overexpressing both NgR1 and LOTUS (Fig. 1B, C). The binding of AP-BLyS to COS7 cells without overexpression of LOTUS or NgR1 was not detected at any dosage (2, 5, or 10 nM). AP-BLyS was detected in COS7 cells overexpressing LOTUS alone (53.9 ± 4.2% vs. 14.5 ± 1.7% in Mock control at 2 nM, $^{**}P < 0.01$; 172.9 ± 14.0% vs. 33.1 ± 2.5% in Mock control at 5 nM, $^{**}P < 0.01$; 324.5 ± 14.2% vs. 48.9 ± 4.4% in Mock control at 10 nM, $^{**}P < 0.01$) (Fig. 1D). AP-BLyS was detected more clearly in COS7 cells overexpressing NgR1 alone in a dose-dependent manner (100.0 ± 0.0% vs. 14.5 ± 1.7% in Mock control at 2 nM, $^{**}P < 0.01$; 247.3 ± 7.4% vs. 33.1 ± 2.5% in Mock control at 5 nM, $^{**}P < 0.01$; 434.5 ± 18.3% vs. 48.9 ± 4.4% in Mock control at 10 nM, $^{**}P < 0.01$), and the binding of AP-BLyS was also completely inhibited to the negative control level (LOTUS alone) in COS7 cells overexpressing both NgR1 and LOTUS (75.8 ± 2.0% vs. 100.0 ± 0.0% in NgR1 alone at 2 nM, $^{**}P < 0.01$; 200.1 ± 10.9% vs. 247.3 ± 7.4% in NgR1 alone at 5 nM, $^{*}P < 0.05$; 371.1 ± 10.0% vs. 434.5 ± 18.3% in NgR1 alone at 10 nM, $^{*}P < 0.05$) (Fig. 1D). Thus, LOTUS overexpressed with NgR1 blocked the binding of these three NgR1 ligands to NgR1.

LOTUS suppresses MAG-, OMgp-, and BLyS-induced growth cone collapse

NgR1 ligands induce growth cone collapse in chick embryonic day 13 (E13) dorsal root ganglion (DRG) neurons, which express NgR1 (Domeniconi et al., 2002; Fournier et al., 2001; K.C. Wang et al., 2002; Zhang et al., 2009), but LOTUS is only weakly expressed. We previously reported that LOTUS overexpression in cultured chick E13 DRG neurons renders the growth cone insensitive to collapse induced by Nogo66 (Sato et al., 2011). To examine whether LOTUS exerts an antagonistic action on growth cone collapse mediated by the other three NgR1 ligands, we performed a growth cone collapse assay with cultured chick E13 DRG neurons overexpressing LOTUS by infecting the cells with recombinant herpes simplex virus (HSV) to express Myc-tagged LOTUS (Myc-LOTUS) (HSV-Myc-LOTUS). DRG neurons were exposed to MAG-Fc, which was fused to the Fc portion of human IgG at the carboxyl terminus, OMgp, or BLyS. The expression of Myc-LOTUS was confirmed immunocytochemically with antibodies against Myc, and the growth cone morphology was visualized with F-actin staining with phalloidin (Fig. 2A). The growth cone collapse rates were shown as a percentage in the collapsed growth cones of all growth cones observed. Overexpression of Myc-LOTUS itself did not affect growth cone collapse (MAG-Fc, 25.8 ± 2.8% vs. 31.0 ± 1.2% in Mock control; OMgp, 25.8 ± 2.8% vs. 31.0 ± 1.2% in Mock control; BLyS, 25.9 ± 3.5% vs. 28.5 ± 1.9% in Mock control) (Fig. 2B–D). MAG-Fc (54.2 ± 3.0% vs. 31.0 ± 1.2% in Mock control, $^{**}P < 0.01$), OMgp (55.5 ± 3.3% vs. 31.0 ± 1.2% in Mock control, $^{**}P < 0.01$), and BLyS (50.0 ± 1.4% vs. 28.5 ± 1.9% in Mock control, $^{**}P < 0.01$) induced growth cone collapse in DRG neurons exposed to HSV of Mock vector, whereas growth cone collapse was not induced by these ligands in growth cones overexpressing Myc-LOTUS (MAG-Fc, 34.3 ± 3.7% vs. 54.2 ± 3.0% in Mock control, $^{**}P < 0.01$; OMgp, 40.5 ± 2.8% vs. 55.5 ± 3.3% in Mock control, $^{**}P < 0.01$; BLyS, 29.4 ± 3.2% vs. 50.0 ± 1.4% in Mock control, $^{**}P < 0.01$) (Fig. 2B–D). Thus, overexpression of LOTUS suppressed growth cone collapse induced by these three NgR1 ligands.

MAG, OMgp, and BLyS induce growth cone collapse in lotus-deficient mice

Our previous report showed that Nogo66 does not induce growth cone collapse in cultured mouse E13 olfactory bulb (OB) neurons, which express both LOTUS and NgR1, but Nogo66 induces growth cone collapse in cultured OB neurons from *lotus*-deficient mice at E13 (Sato et al., 2011). To examine whether endogenous LOTUS exerts an antagonistic action on growth cone collapse induced by the other three NgR1 ligands, we performed the growth cone collapse assay with cultured E13 OB neurons from *lotus*-deficient mice. OB neurons from wild-type mice exposed to the three NgR1 ligands did not show inducible growth cone collapse (MAG-Fc, 24.8 ± 1.7% vs. 19.4 ± 1.5% in wild-type mice; OMgp, 20.9 ± 1.6% vs. 19.4 ± 1.5% in wild-type mice; BLyS, 25.2 ± 1.6% vs. 27.6 ± 2.4% in wild-type mice), whereas the NgR1 ligands induced growth cone collapse in OB neurons from *lotus*-deficient mice (MAG-Fc, 34.0 ± 2.7% vs. 24.8 ± 1.7% in wild-type mice, $^{*}P < 0.05$; OMgp, 30.1 ± 2.4% vs. 20.9 ± 1.6% in wild-type mice, $^{**}P < 0.01$; BLyS, 35.8 ± 1.9% vs. 25.2 ± 1.6% in wild-type mice,