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IN VIVO ANALYSIS OF KALLIKREIN-RELATED PEPTIDASE 6 (KLK6) FUNCTION IN OLIGODENDROCYTE DEVELOPMENT AND THE EXPRESSION OF MYELIN PROTEINS

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Abstract—Oligodendrocytes are important for not only nerve conduction but also central nervous system (CNS) development and neuronal survival in a variety of conditions. Kallikrein-related peptidase 6 (KLK6) is expressed in oligodendrocytes in the CNS and its expression is changed in several physiological and pathological conditions, especially following spinal cord injury (SCI) and experimental autoimmune encephalomyelitis. In this study, we investigated the functions of KLK6 in oligodendrocyte lineage cell development and the production of myelin proteins using KLK6-deficient (KLK6$^{-/-}$) mice. KLK6$^{-/-}$ mice were born without apparent defects and lived as long as wild-type (WT) mice. There was no significant difference in the numbers of oligodendrocyte precursor cells and mature oligodendrocytes in the adult naive spinal cord between WT and KLK6$^{-/-}$ mice. However, there were fewer mature oligodendrocytes in the KLK6$^{-/-}$ spinal cord than in the WT spinal cord at postnatal day 7 (P7). Expression of myelin basic protein (MBP) and oligodendrocyte-specific protein/claudin-11, major myelin proteins, was also decreased in the KLK6$^{-/-}$ spinal cord compared with the WT spinal cord at P7–21. Moreover, after SCI, the amount of MBP in the damaged spinal cords of KLK6$^{-/-}$ mice was significantly less than that in the damaged spinal cords of WT mice. These results indicate that KLK6 plays a functional role in oligodendrocyte development and the expression of myelin proteins.

Key words: protease, spinal cord, development, myelin, spinal cord injury.

INTRODUCTION

Oligodendrocytes are glial cells mainly located in the white matter of the central nervous system (CNS). The most important function of oligodendrocytes is to synthesize the myelin sheath, a lipid-rich and multilamellar structure ensheathing axons in the CNS. The myelin sheath plays a functional role in the insulation of axons and faster conduction of electrical signals, namely, saltatory conduction. The myelin sheath also functions to protect axons. For example, it protects axons against NO-induced blockade of nerve conduction (Redford et al., 1997). In addition to forming the myelin sheath, oligodendrocytes themselves are important for the maturation of axons (Colello et al., 1994; Sanchez et al., 1996). Moreover, oligodendrocytes produce several growth factors that promote the survival of neurons, such as insulin-like growth factor 1 (IGF-1), neurotrophins and glial cell line-derived neurotrophic factor (GDNF) (Du and Dreyfus, 2002).

Oligodendrocytes are derived from oligodendrocyte precursor cells (OPCs). OPCs are present not only in the developing CNS, but also in the adult brain and spinal cord. Several factors, including platelet-derived growth factor (PDGF) (McKinnon et al., 1993; Calver et al., 1998; Tokumoto et al., 1999; Glaser et al., 2005) and fibroblast growth factor (FGF) (McKinnon et al., 1990, 1993; Calver et al., 1998), affect the proliferation and differentiation of OPCs.

Demyelination, degeneration and loss of myelin, occurs in several pathological conditions, for example, metabolic diseases (Schiffmann and van der Knaap, 2004), neurotrauma (Lu et al., 2000; Park et al., 2004), infections (Hardy et al., 2011; Berger, 2011) and demyelinating diseases (Ontaneda et al., 2012). In most cases of CNS demyelination, oligodendrocyte death and remyelination failure occur, leading to secondary neuronal damage and degeneration. Therefore, an understanding of the mechanisms underlying oligodendrocyte development and myelination may lead

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Abbreviations: APC, adenomatous polyposis coli; BMS, Basso Mouse Scale; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; IGF-1, insulin-like growth factor 1; IHC, immunohistochemistry; KLK, kallikrein-related peptidase; MBP, myelin basic protein; OPC, oligodendrocyte precursor cell; OSP, oligodendrocyte-specific protein; PDGF, platelet-derived growth factor; PB, phosphate buffer; PBS, phosphate-buffered saline; PLP, proteolipid protein; RT-PCR, reverse transcription-polymerase chain reaction; SCI, spinal cord injury; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TUNEL, TdT-mediated dUTP nick-end labeling.

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to the development of new therapies for demyelinating disorders.

Kallikreins and kallikrein-related peptidases (KLKs) are serine proteases, and there are 15 family members in humans (Diamandis et al., 2000a, 2004). KLKs are known to serve a variety of physiological functions such as the regulation of blood pressure, semen liquefaction and skin desquamation (Borgono et al., 2004; Sotiropoulou et al., 2009). KLKs also contribute to a wide range of pathological processes, especially tumorigenesis, angiogenesis and metastasis (Borgono and Diamandis, 2004; Borgono et al., 2004; Sotiropoulou et al., 2009). On the other hand, it is reported that KLKs play some pathogenic roles in the CNS (Diamandis et al., 2000b; Yousef et al., 2003).

In the CNS, two KLKs, KLK6/protease M/neurosin and KLK8/neuropsin, are constitutively and abundantly expressed. KLK8 mRNA is constitutively expressed in the neurons of the limbic system of the adult mouse brain (Chen et al., 1995; Yoshida and Shiosaka, 1999; Shiosaka and Yoshida, 2000). We previously reported that oligodendrocytes express KLK8 in cases of spinal cord injury (SCI) (Terayama et al., 2004) and experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (Terayama et al., 2005b). Furthermore KLK8-deficient mice show milder symptoms and less oligodendrocyte loss than wild-type (WT) mice following SCI (Terayama et al., 2007) and those with EAE (Terayama et al., 2005b). These results suggest that KLK8 plays functional roles in the pathogenesis of SCI and immune-mediated demyelination.

KLK6 has been identified in human cancer cells and in the brain (Anisowicz et al., 1996; Yamashiro et al., 1997; Yousef et al., 2003). KLK6 is constitutively expressed in oligodendrocytes in the CNS and its expression is enhanced after SCI and in EAE (Yamanaka et al., 1999; Blaber et al., 2004; Terayama et al., 2004, 2005a). We previously reported that knockdown of KLK6 in cultured oligodendrocytes using RNAi suppressed the expression of the major myelin proteins myelin basic protein (MBP) and proteolipid protein (PLP) (Bando et al., 2006). These reports suggested the possibility that KLK6 plays functional roles in myelin formation in physiological and pathological conditions. However the functions of KLK6 *in vivo* have not been elucidated.

In the current study, we produced KLK6-deficient ($KLK6^{-/-}$) mice and investigated the function of KLK6 in oligodendrocyte lineage cell development and the expression of myelin proteins.

EXPERIMENTAL PROCEDURES

Animals

pZsGreen vector (Clontech Laboratories, Mountain View, CA, USA) was used for production of the targeting vector for mouse *KLK6* gene (GeneBank ID: NC_000073) deletion. The short arm of *KLK6* gene including exon 1 and exon 2, β galactosidase gene, neomycin-resistance gene and the long arm of *KLK6* gene including exon 5 and exon 6 were inserted at the NotI site. The targeting vector was designed to replace a site containing part of exon 3 and all of exon 4 of *KLK6* gene, including the histidine of the catalytic site. The targeting vector was introduced into C57BL/6 ES cell lines and homologously recombined cells were selected using G418. The $KLK6^{-/-}$ mice were created from these mutant ES cell lines. These mutant mice and WT mice were maintained on a C57BL/6 background. Deletion of the KLK6 allele was confirmed by PCR using genomic DNA prepared from the tails and primers specific for the WT allele (sense, 5′-TTTCCAGGCTGCCCTCTACACC-3′ and antisense, 5′-TTCAGGGTCCCGGAACCTATGG-3′) and the mutated allele (sense, 5′-GAGGCGCGTAAGCTTCCTAGG-3′ and antisense, the same primer used for the WT allele). The body weights of WT and $KLK6^{-/-}$ mice were measured every week from 3 weeks to 10 weeks after birth ($n > 10$ of each genotype at all time points). At postnatal day 70, mice ($n > 5$ in each genotype) were killed by excess ether anesthesia and their brains were removed and weighed. All experiments were carried out in accordance with the guidelines in the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and as required by Asahikawa Medical University. All efforts were made to minimize the numbers of animals used and their pain and suffering.

Reverse transcription-polymerase chain reaction (RT-PCR)

For detection of mRNA, total RNA from adult brain and spinal cord was extracted from samples using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and aliquots containing 2 μg of total RNA were used for RT with AMV-reverse transcriptase (Promega, Madison, USA), according to the manufacturer's instructions. Aliquots from the RT reaction were then amplified by PCR. The primers used and their product sizes were as follows: KLK6 sense, 5′-CCCAGATACCATTCAGTGT-3′, antisense, 5′-CGTGGGGGAGAACTGGATGT-3′ (315 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-CTACATGGTCTACCTGTTCCAGT-3′, antisense, 5′-AGTTG TCATGGATGACCTTGG-3′ (380 bp) and KLK8 sense, 5′-CCCA CTGCAAAAAACAGAAG-3′, antisense, 5′-TGTCAGCTCCATTG CTGCT-3′ (405 bp). Thirty, 25 and 33 PCR cycles were used for KLK6, GAPDH and KLK8, respectively. The reaction conditions were as follows: KLK6, denaturation at 94 °C for 15 s, annealing at 57 °C for 15 s and extension at 72 °C for 45 s; GAPDH and KLK8, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The reaction products were separated by electrophoresis in 1.5% agarose gels and visualized using a transilluminator after staining with ethidium bromide. For quantitative analysis, the LightCycler rapid thermal system (Roche Diagnostics, Indianapolis, IN, USA) was used according to the manufacturer's protocol. The primers used and PCR conditions were the same as above. A serially-diluted cDNA from mouse naive spinal cord was used as a standard. The quantified data were analyzed with the LightCycler analysis software.

Western blot analysis

Mice at 1, 4, 7, 14, 21 and 70 postnatal days were killed by excess ether anesthesia ($n = 4–5$ of each genotype at all time points). Spinal cords were removed and homogenized in assay buffer (50 mM Tris–Cl, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P40 and 150 mM NaCl). The homogenates were clarified by centrifugation and the supernatant fractions were used in subsequent experiments. After determination of the protein concentration (Micro BCA Protein Assay, Thermo Scientific, Rockford, USA), 20 μg of protein was loaded onto each lane of 15% polyacrylamide gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore, Massachusetts, USA). Immunoblotting was carried out using anti-MBP (SMI-94,