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EXPRESSION OF DISCOIDIN DOMAIN RECEPTOR 1 DURING MOUSE BRAIN DEVELOPMENT FOLLOWS THE PROGRESS OF MYELINATION

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Abstract—Discoidin domain receptor 1 is a tyrosine kinase receptor expressed in a variety of tissues including the brain. This study describes mRNA and protein expression of discoidin domain receptor 1 in mouse brain during development and provides new insights into its role during gliogenesis and neurogenesis. We performed *in situ* hybridization for discoidin domain receptor 1 in mouse brains at embryonic day 18, postnatal days 5, 9, 15, 21 and adulthood and observed a diffuse pattern in the proliferative areas during embryogenesis. From postnatal day 5 onwards, a defined cellular expression pattern of discoidin domain receptor 1 was observed, mainly located in white matter tracts and following a spatio-temporal pattern that overlapped the progress of myelination. Next, we performed double-labeling reactions (*in situ* hybridization followed by immunohistochemistry) that confirmed that discoidin domain receptor 1 was expressed by mature oligodendrocytes. We observed that cells positive for discoidin domain receptor 1 also expressed carnosine and anti-adenomatous polyposis coli, two mature oligodendrocyte markers. Based on the localization of discoidin domain receptor 1 specifically in the white matter fiber tracts during postnatal development, we suggest that discoidin domain receptor 1 participates in the development and maintenance of the myelin sheath. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: carnosine, discoidin domain, myelin, oligodendrocytes.

Tyrosine kinase receptors (RTKs) are important mediators of intracellular signal transduction pathways that govern growth, differentiation and developmental signals (Blume-Jensen and Hunter, 2001). Discoidin domain receptors, DDR1 and DDR2, are a novel subfamily of RTKs. In their extracellular region both contain a discoidin domain, a homology region that was first described in the lectin discoidin I from the slide mold *Dictyostelium discoideum* (Springer et al., 1984). Other proteins that contain the discoidin motif are: neuropilins and neurexins, X-linked juvenile retinoschisis gene one (XLRS-1), and blood coagulation factors V and VIII (Vogel, 1999). Neuropilins and neurexins are involved in the development of the nervous system. Neuropilins are receptors of semaphorins mainly involved in axon growth (Nakamura et al., 2000) and neurexins mediate cell-to-cell contacts (Peles et al., 1997).

So far, five isoforms of DDR1 (from DDR1a to e) generated by alternative splicing of a single *DDR1* gene have been reported (Alves et al., 2001). Compared with DDR1a, the b-isoform has an in-frame insertion of 111-bp coding for additional 37 amino acids (aa) in the juxtamembrane region and DDR1c an extra six aa in the kinase domain. Upon activation of the kinase function, all three isoforms transmit signals by recruiting different adaptor proteins to phosphotyrosine docking sites. The other two isoforms, d and e, presumably lack kinase activity due to a truncation or in frame deletion, respectively. Important progress in understanding the function of DDR1 was made with the finding that DDR1 is activated by collagen, the main component of extracellular matrix. Whereas DDR1 is activated by all the collagens so far tested, DDR2 is only activated by fibrillar collagens (Shrivastava et al., 1997; Vogel et al., 1997).

The mechanism of DDR1 activation is similar to other RTKs: stimulation with native collagen induces dimerization and a subsequent transphosphorylation. However unlike other RTKs, activation follows slow kinetics: it takes up to 18 h to reach the maximum receptor phosphorylation (Vogel et al., 1997). The large number of isoforms and the slow kinetics of DDR1 activation might, however, lead to a more complex regulatory mode of signal transduction.

The removal of DDR1 functions in mice resulted in viable, but smaller, animals. Female DDR1-null-mice revealed abnormalities in the blastocyst implantation pro-

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Abbreviations: aa, amino acids; APC, anti-adenomatous polyposis coli; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CA II, carbonic anhydrase II; DDR1, discoidin domain receptor 1; DDR2, discoidin domain receptor 2; E, embryonic day; EDTA, ethylenediamine tetraacetic acid; eGL, external granular layer; GFAP, glial fibrillary acidic protein; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; NBT, nitroblue tetrazolium; NeuN, neuron-specific nuclear protein; NGS, normal goat serum; NG2, NG2 chondroitin sulfate proteoglycan; P, postnatal day; PB, phosphate buffer; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; RTKs, tyrosine kinase receptors; RT-PCR, reverse transcription–polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, sodium chloride–sodium citrate; TBS, Tris-buffered saline.

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cess and defective differentiation of mammary ducts resulting in lactational failure (Vogel et al., 2001). *In vitro* studies performed with several cell types from DDR1 null mice demonstrated that DDR1 is an important mediator of cell and extracellular matrix communication since it is involved in (i) cell proliferation and adhesion (Curat and Vogel, 2002), (ii) cell migration (Hou et al., 2001; Kamohara et al., 2001), and (iii) extracellular matrix degradation through matrix metalloproteinase (MMP) activation (Hou et al., 2002).

DDR1 is overexpressed in several neoplastic tissues of patients with brain (Weiner et al., 2000), lung (Laval et al., 1994), ovarian or breast cancer (Alves et al., 2001; Barker et al., 1995). Northern and *in situ* mRNA analysis showed DDR1 expression in a number of different cells and tissues, including lung, kidney, breast, heart and muscle, but the highest levels of DDR1 mRNA are found in the brain (Barker et al., 1995; Zerlin et al., 1993). Embryonic and adult mouse brain shows expression of DDR1 in proliferative areas, including areas of primary neurogenesis (Zerlin et al., 1993; Sanchez et al., 1994) and areas of postnatal secondary neurogenesis (Bhatt et al., 2000). Within the developing cerebellar cortex, Bhatt et al. (2000) demonstrated that DDR1 is involved in the axonal outgrowth of granular cells. Although it has been suggested that DDR1 is expressed by a subpopulation of glial cells located in the white matter areas in the adult murine brain (Zerlin et al., 1993; Sanchez et al., 1994), the nature of the specific cell types expressing DDR1 is presently unclear. No further studies have been made in postnatal stages, when important neurogenesis and gliogenesis changes occur. In this paper we provide new insights into the role of DDR1 in the CNS during early and late postnatal development. We studied the distribution pattern of DDR1 mRNA in the brain by *in situ* hybridization and by conducting double labeling using immunohistochemistry in conjunction with *in situ* hybridization.

EXPERIMENTAL PROCEDURES

Animals

All experimental protocols carried out in this study were approved by the Institutional Animal Care and Use Committee of the University of Barcelona, Spain, and conform to National Institutes of Health guidelines. Efforts were made to minimize suffering and animal numbers. A total of 78 OF1 mice were used in this study. Mice were analyzed at different ages (embryonic day (E) 18, postnatal day (P) 0, P5, P7, P9, P15, P21 and adult). The mating day was considered E0. The day of birth was considered P0. Mice were anesthetized with chloral hydrate (350 mg/kg).

Brain extracts and Western blot analysis

Twenty-four mice were decapitated and the brains were extracted and sonicated with a blender in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.5), 100 mM NaCl, 1.5 mM $MgCl_2$, 5 mM of EDTA, 10% glycerol, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/mL aprotinin. Samples were centrifuged at 13,000 r.p.m. for 10 min at 4 °C and the supernatants were stored in aliquots of 50 μg/mL at −80 °C.

Immunohistochemistry and *in situ* hybridization techniques

E18 and postnatal animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. Brains were cryoprotected in PB–sucrose 30% and frozen in dry ice for immunohistochemistry studies. For *in situ* hybridization studies, the cryoprotection buffer was PB 0.1 M–4%-PFA, 30% sucrose. Serial coronal sections of 50 μm (for P0, P5, P7, P9) or 30 μm (for P15, P21 and adult) of a total of 27 mice were cut with a cryostat and stored at −20 °C in a 40% PB 0.1 M, 30% glycerol and 30% ethylene glycol buffer.

Total RNA isolation and real time reverse transcription–polymerase chain reaction (RT-PCR) analysis

Twenty-four mice were decapitated and the brains were extracted and kept at −80 °C until use. The brains were extracted and sonicated with a blender in RNAase free lysis buffer (Applied Biosystems, Barcelona, Spain). Samples were kept for 1 h at 4 °C.

Cell culture

Human oligodendroglial cell line MO3.13 was obtained from Eucell Bank (Barcelona, Spain) by permission of Dr. Niels Hellings (Buntinx et al., 2003). The cells were grown in wells in a Laboratory-Tek II Chamber slide system (Nunc, Barcelona, Spain) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen, Barcelona, Spain) in a humidified 5% CO_2 incubator at 37 °C. MO3.13 cells grown under these conditions show immunoreactivity for the oligodendrocyte markers: myelin-specific lipids galactosylceramide, 2′,3′-cyclic nucleotide 3′-phospodiesterase and myelin basic protein (MBP) (Buntinx et al., 2003).

Western blot

Fifty micrograms of total protein from each sample was boiled (under reducing conditions) at 100 °C for 5 min. Proteins were separated on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel (run at constant voltage, 125 mV for 1–2 h) and electrophoretically transferred (at 200 mV for 4 h) to a nitrocellulose membrane (Hybond P, Amersham Biosciences, Barcelona, Spain). After several washes in Tris-buffered saline (TBS, Tris–HCl 50 mM, 150 mM NaCl, pH 7.4), the membrane was incubated with a rabbit anti-DDR1 antibody (Abdulhussein et al., 2004) in TBS–0.05% BSA–0.02%-sodium azide at 4 °C overnight. On the following day, membranes were washed with TBST (0.05% Triton X-100 in TBS buffer) and TBS with 3% non-fat milk. Immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated antirabbit IgG (dilution 1:2000) (Dako, Barcelona, Spain) using an enhanced chemiluminescence system (ECL Plus, Amersham Biosciences). As a positive control, human embryonic kidney (HEK) cells transiently transfected with DDR1b were used (Alves et al., 2001).

In situ hybridization

Riboprobe preparation. A 1.2 kb fragment from a full length mouse DDR1 cDNA cloned in pBluescript SK± (kindly provided by Dr M. E. Hatten, Rockefeller University, New York, NY, USA) was used to make sense and antisense probes. The antisense riboprobe for mDDR1 was generated by linearization of this plasmid with *Hind*III and subsequent incubation with T7 RNA polymerase (Ambion, Madrid, Spain). The sense riboprobe was prepared by cutting with *Xba*I and transcribing with T3 RNA polymerase. The *in vitro* transcription was performed using Megascript kit T7/T3 (Ambion, Inc.) and the probes were labeled with digoxigenin-UTP (Roche Diagnostics, Barcelona, Spain).