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Expression of vascular endothelial growth factor receptors Flt-1 and Flk-1 in embryonic rat forebrain

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Abstract

To define better the putative targets of vascular endothelial growth factor (VEGF) in the developing brain we have examined the ontogeny of the two VEGF tyrosine kinase receptors, Flt-1 and Flk-1, in embryonic rat forebrain. Semiquantitative reverse transcriptase-polymerase chain reaction and immunoblot analysis showed expression of both receptors in the forebrain at all embryonic ages studied. Messenger RNAs for Flt-1 and Flk-1 appeared along most of the ventricular zone of the lateral ventricle as early as embryonic day (E) 13. Messages gradually became restricted to a limited ventricular zone at E20. Expression of VEGF receptors was also observed in the cerebral cortex, hippocampus and thalamic nuclei. In the cortex, expression of mRNA for both receptors was detected in the cortical plate around E15, and became relatively weak and restricted to the deeper layers of the cortical plate at E20. These data suggest that VEGF may contribute to early developmental processes including the proliferation, differentiation and maturation of specific neuronal populations via specific VEGF receptors in the developing rat forebrain.

Keywords: Ventricular zone; Cerebral cortex; *In situ* hybridization histochemistry; Development; Flt-1; Flk-1

Vascular endothelial growth factor (VEGF) has been regarded as a major regulator of angiogenesis during development, tumor growth and tissue regeneration [6,8]. VEGF exerts its function through two high-affinity receptor tyrosine kinases, the fms-like kinase (Flt-1, VEGF receptor-1) and the fetal liver kinase (Flk-1, VEGF receptor-2) [14].

Recent studies have shown that VEGF stimulates neurogenesis in both developing and adult nervous systems in several experimental paradigms [7,10,18]. Jin et al. [10] showed in their *in vivo* study that exogenous administration of VEGF increased proliferation within the adult hippocampus and the subventricular zone, and that these regions expressed Flk-1. In addition, cultured neural stem cells and mouse embryonic cortical neurons expressed VEGF and its receptors [10,13] and VEGF stimulated neurogenesis *in vitro* through Flk-1 receptors [10]. These observations suggest that VEGF may be involved in several aspects of nervous system development via specific VEGF receptors.

Even though the role of VEGF during development has been extensively studied, there are few reports dealing with the expression of VEGF receptors in the developing brain. Yang et al. [19] demonstrated the expression of Flt-1 and Flk-1 in the cortical and hippocampal neurons of neonatal and adult rat brain. The neuronal expression pattern of the two VEGF receptors showed a diverse pattern during postnatal development, suggesting that these receptors might be involved in the developing brain. To date, however, there has been no information regarding the distribution patterns of VEGF receptors in the embryonic rat brain.

In order to define better the putative targets of VEGF actions in the developing brain we examined the ontogeny of Flt-1 and Flk-1 expression using *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) and immunoblot analysis during brain development in embryonic rat forebrain. Particular attention was paid to receptor distribution in the cerebral cortex and hippocampus.

Pregnant Sprague Dawley rats with the exact dates of conception known were purchased from BioKorea (Osan, Korea). The day of vaginal plug discovery was considered embryonic day 0 (E0). For *in situ* hybridization histochemistry, 24 rats of different ages (embryonic days E13, E15, E17 and E20; $n = 6$ for each time point) were used. Embryonic pups were removed from the mothers, anesthetized with 4% (w/v) chloral hydrate (1 ml/100 g body weight, intraperitoneally), and sacrificed by decapitation followed by dissection of brains. The brains were immersed for

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Table 1
Oligonucleotide pairs for RT-PCR

Gene	Oligonucleotide primers	Position	Size	Genebank
Flt	Sense: 5′-aac aac agg acc atg cac Antisense: 5′-gct tca gtt ttc gga tga	2022–2589	568	D28498
Flk	Sense: 5′-cca atg aag ggg aac tg Antisense: 5′-tga ctg ctg gtg atg ct	2611–3145	535	U93306
GAPDH	Sense: 5′-cgatcccgctaacatcaaat Antisense: 5′-ccacagtcttctgagtggca	264–589	326	M17701

5 h at 4 °C in fixative containing 4% (v/v) paraformaldehyde buffered with 0.1 M phosphate buffer (PB, pH 7.2). Dissected brains were equilibrated with 30% (w/v) sucrose in 0.1 M PB and frozen until used. All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea and were in accordance with the US National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996).

Semiquantitative RT-PCR analysis was carried out as described previously [4,5]. In brief, total RNA was extracted from the forebrain tissue, including the cortex and the dorsal hippocampus, of rats of varying ages (E13, E15 and E17) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using Reverse Transcriptase M-MLV (Takara Korea Biomedical Inc., Korea) according to the manufacturer's instructions. Equal amounts (1 μl) of the reverse transcription products were then PCR-amplified using Perfect Premix Version 2.1 (Ex Taq version; Takara Korea Biomedical Inc.). Amplification commenced with denaturation at 94 °C for 4 min followed by 25–30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The final extension was made at 72 °C for 10 min. One pmol of each primer was used in the amplification reaction. Ten microliters of each PCR reaction product was electrophoresed into 1.5% (w/v) agarose. For the semiquantitative measurements we coamplified the Flk-1 and Flt-1 mRNAs with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and optimized the number of PCR cycles to maintain amplification in the linear range. Three animals were used for PCR at each time point. The primers used are listed (Table 1).

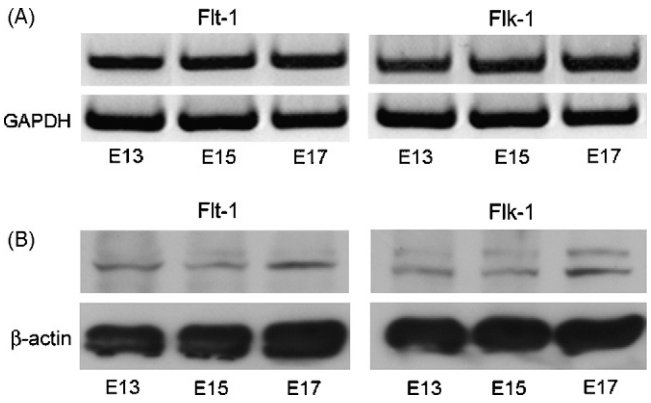

Fig. 1. RT-PCR analysis (A) and immunoblotting (B) for Flt-1 and Flk-1 in the rat forebrain at different ages (E13, E15 and E17). (A) The amplification products of Flt-1 and Flk-1 mRNAs were of lengths 568 bp and 535 bp, respectively. As an internal standard, GAPDH mRNA was measured. (B) Protein extracts were prepared from the forebrain tissue and identical amounts of total protein (50 μg/lane) were applied to the gel, and immunoblotted with specific antibody to Flt-1, Flk-1 or anti-β-actin antibody.

Specific sequences for Flt-1 and Flk-1 were prepared using RT-PCR, and antisense and sense riboprobes were labeled with digoxigenin (DIG) using *in vitro* transcription, as described in detail previously [4,5]. Coronal cryostat sections (25 μm thick) were hybridized with antisense or sense probes diluted in hybridization solution (150 ng/ml) at 52 °C for 18 h. Hybridization was visualized using an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche, Germany; diluted at 1:2000) with 4-nitroblue tetrazolium chloride (0.35 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.18 mg/ml) as substrates. Tissue sections were visualized using a microscope and pho-

Fig. 2. Representative photomicrographs demonstrating the expression patterns of Flt-1 (A–I) and Flk-1 (J–L) in the embryonic rat forebrain. (A–C) E13 forebrain. (A and C) Hybridization signals were observed in the ventricular zone of the lateral ventricle (LV) and third ventricle (3V). OE: olfactory epithelium. (B) Section as in (A) hybridized to sense-stranded probe. (D) E15 forebrain. Labeling was observed in the developing cortical plate in addition to the ventricular zone. (E and F) E17 forebrain. Flt-1 expression was observed in the ventricular zone of the lateral ventricle, the vascular endothelial cells of the choroid plexus and in the developing cortex. (inset in E) Higher magnification view of the boxed area in (E) showing Flt-1 expression in the vascular endothelial cells of the choroid plexus (chp). VZ: the ventricular zone of the lateral ventricle. (F) Higher magnification view of the boxed area in E showing Flt-1 expression in the ventricular zone and in the cortical plate (CP), but not in the marginal zone (MZ). Labeling was weak in the intermediate zone (IZ). Asterisks indicate labeled blood vessels. (G–I) E20 forebrain. (G) Expression of Flt-1 mRNA was observed within a limited ventricular zone of the lateral ventricle. (inset in G) Higher magnification view of the boxed area in (G). Note that labeling was restricted to the columnar cells of the striatal ventricular zone, and was not observed in the septal ventricular zone (asterisks). (H) Section as in (G) hybridized to sense-stranded probe. (inset in H) Higher magnification view of the boxed area in H. (I) Hybridization signals were observed in the hippocampus including the pyramidal cell layer (pcl), in the ventricular zone surrounding the temporal horn of the lateral ventricle and in thalamic nuclei. DG: the dentate gyrus; LHb: the lateral habenular nucleus. (J) E13 forebrain. Hybridization signals for Flk-1 were observed in the ventricular zone of the lateral ventricle. (inset in J) Section as in J hybridized to sense-stranded probe. (K) E17 forebrain. Flk-1 expression was observed in the ventricular zone of the lateral ventricle and in the developing cortex. (L) E20 forebrain. Flk-1 was expressed within a limited ventricular zone of the lateral ventricle with intensely labeled cells in the subventricular zone at the edge of the lateral ventricle. (inset in L) Higher magnification view of the boxed area in L. Note that labeling was restricted to the columnar cells of the striatal ventricular zone, and was not observed in the septal ventricular zone (asterisks). Scale bars: 500 μm for D, E, G–I and L; 300 μm for A–C, J, K and inset in J; 60 μm for F, insets in E, G, H and L.