Published in final edited form as:

Gene Expr Patterns. 2003 March ; 3(1): 109–113. doi:10.1016/s1567-133x(02)00065-0.

Expression of the Flk1 receptor and its ligand VEGF in the developing chick central nervous system

Takao Hashimoto¹, Xiang-Mei Zhang¹, Xian-Jie Yang^{*}

Jules Stein Eye Institute and Department of Ophthalmology, Molecular Biology Institute, University of California School of Medicine, 100 Stein Plaza, Los Angeles, CA 90095, USA

Abstract

The receptor tyrosine kinase Flk1 is known to mediate signals of vascular endothelial growth factor (VEGF) during vasculogenesis and hematopoiesis. We demonstrate by in situ hybridization that in addition to endothelial cells, chick Flk1 mRNA is also expressed in the notochord and in the neural epithelial cells of the ventral diencephalon, hindbrain, and spinal cord. During the development of the avascular chick retina, Flk1 mRNA is detected in the proliferative zone of the neural epithelium, whereas the VEGF ligand is expressed by differentiated retinal ganglion cells. Moreover, expression patterns of Flk1 in the retina are conserved among chick, quail and mouse, thus suggesting a distinct role of Flk1 and VEGF in the development of the vertebrate central nervous system.

Keywords

VEGF; Flk1 (VEGFR2); Chick; Retina; Brain; Notochord; Central nervous system; Diencephalon; Hindbrain; Neural progenitor; Development

1. Results and discussion

The receptor tyrosine kinase Flk1 (KDR, VEGFR2) plays a critical role in vasculogenesis and hematopoiesis (Shalaby et al., 1995, 1997). Activation of the kinase activity of Flk1 is triggered by binding to its cognate ligand vascular endothelial growth factor (VEGF), which affects endothelial cell proliferation, permeability, and migration (Millauer et al., 1993; Carmeliet et al., 1996; Ferrara, 1999; Gerber et al., 1999; Shibuya, 2001). Accumulating evidence suggest that VEGF and Flk1 may function in the nervous system to influence properties of neural progenitor cells, as well as differentiation and survival of neurons (Yang and Cepko, 1996; Soker et al., 1998; Silverman et al., 1999; Sondell et al., 1999, 2000; Yourey et al., 2000; Oosthuysen et al., 2001; Ogunshola et al., 2002; Louissaint et al., 2002; Jin et al., 2002).

As a necessary step toward understanding the function of Flk1 during neural development, we cloned the full-length chick Flk1 cDNA using RT-PCR based on conserved protein motifs within the kinase domains of the mouse (Matthews et al., 1991) and the quail Flk1

^{*}Corresponding author. Tel.: +1-310-825-7020; fax: +1-310-794-2144. yang@jsei.ucla.edu (X.J. Yang).

¹These two authors contributed equally to this work.

(Eichmann et al., 1996). Sequence analysis indicates that chick Flk1 encodes a protein of 1348 amino acid residues with the structure of a typical receptor tyrosine kinase, i.e. with a N-terminal signal peptide followed by an extracellular domain, a single transmembrane domain, and an intracellular protein tyrosine kinase domain. The predicted chick Flk1 protein shares 97, 69, and 71% amino acid similarities to quail, mouse, and human Flk1 (Terman et al., 1992), respectively.

The expression of Flk1 in chick embryonic heart tissues based on a partial cDNA has been previously reported (Sugishita et al., 2000). In this study, RNA probes corresponding to different regions of the full length chick Flk1 cDNA were used in in situ hybridization to characterize expression patterns of Flk1 during embryogenesis. As expected, Flk1 mRNA was detected in the developing vasculature of stage 13 embryos (48–52 h incubation) (Fig. 1A). In addition, strong Flk1 hybridization signals were observed in the axial mesodermal tissue, the notochord, as previously reported in quail (Wilting et al., 1997). Moreover, the floor plate of the neural tube also showed Flk1 hybridization signals. At stage 24 (embryonic day 4.5, E4.5) intense Flk1 hybridization signals were found in the perineural vascular plexus surrounding the spinal cord and capillaries invading the neural tissue as well as in the notochord, while signals in the floor plate was relatively weak in comparison (Figs. 1B,C). In the diencephalon, Flk1 mRNA was expressed in two patches of cells near the ventricular surface lateral to the midline (Fig. 1D). Whole-mount in situ hybridization of stage 24 embryos revealed expression of Flk1 in two stripes of cells flanking the midline in the ventral hindbrain (Figs. 1F,G), which were absent in control embryos hybridized to the sense probe (Fig. 1E). Analyses at two subsequent stages further confirmed these Flk1 expression patterns in the developing brain. At E5, Flk1-positive cells were detected in the ventral diencephalon (Figs. 1I,J) and in the ventral hindbrain at the position opposite to the mid-hindbrain junction, the isthmus (Figs. 1I, K,H). In addition, a subset of cells located in the ventral hindbrain further posterior to the isthmus also showed Flk1 expression (Fig. 1L). At E6, the expression of Flk1 mRNA persisted in the ventral hindbrain (Figs. 1O,P) and in the diencephalon near the optic chiasm (Figs. 1M,N,Q,R).

To distinguish the Flk1 expression signals in the neural epithelium from its expression in the endothelial cells of the sprouting capillaries, we performed immunostaining of a chick endothelial cell marker, type II TGF β receptor (Brown et al., 1999). Staining patterns of this marker delineated the developing blood vessels, which showed distinct patterns from the Flk1 expression observed in the neural epithelium (Fig. 1T). Thus, the chick Flk1 receptor is expressed by neuronal cells in the developing brain in addition to the endothelial cells. Furthermore, since the Flk1 positive cells are located near the ventricular zone, they are likely a subset of neural progenitor cells.

In the retina, Flk1 positive cells were first detected in a subset of cells in the central region at stage 22 (Figs. 2A,B), coinciding with the commencement of neuronal differentiation. During the proliferative period of the chick retina, Flk1 hybridization signals persisted in the ventricular zone (Figs. 2D,E,G), where PCNA-positive progenitor cells were located (Fig. 2F). After the completion of retinal histogenesis, Flk1 mRNA signals were present in the inner nuclear layer (INL) and the outer nuclear layer (ONL) (Figs. 2H,I). These hybridization signals likely represent the expression of Flk1 in Muller glial cells, since their

cell bodies are located in the INL whereas their processes extend through the ONL. Alternatively, chick Flk1 is expressed by both Muller glia and photoreceptor cells.

We next characterized the expression of the VEGF ligand in the retina during development. The onset of the VEGF protein expression began at E3.5 coinciding with neuronal differentiation in the retina (data not shown). At E4, most VEGF proteins were concentrated in the inner retina occupied by the nascent ganglion cells, which was the only cell type stained positive for the LIM-homeo domain transcription factor Islet1 at this stage (Figs. 3A–C). Co-staining of E9 retina by antibodies against the POU-domain transcription factor Brn3a, which was expressed by a subset of ganglion cells (Xiang et al., 1997), and Islet1, which was expressed by ganglion cells as well as a subset of amacrine and photoreceptor cells at E9, showed that VEGF was distributed in the vicinity of ganglion cells (Figs. 3D–F). Since both Brn3a and Islet1 are nuclear proteins, whereas VEGF is secreted and could diffuse from the cells producing it, these immunostaining patterns suggest that ganglion cells are the major sites of VEGF synthesis in the developing retina. By E17 when all retinal cell types and laminar layers were formed, VEGF protein was present not only in the ganglion cell layer but also in the INL and ONL, possibly due to its expression by the Muller glia at this stage (Fig. 3G) (Yang and Cepko, 1996).

The avian retina differs from the mammalian retina as it remains avascular throughout development and adulthood (Gerhardt et al., 1996). However, similar complementary expression patterns of Flk1 and VEGF are present in developing chick and mouse retinas (Yang and Cepko, 1996). Moreover, similar Flk1 expression has been observed in the quail retina (Wilting et al., 1997). These data thus suggest a potential role of this ligand and receptor pair in retinal neurogenesis of some vertebrate species. The expression of Flk1 in other regions of the central nervous system is consistent with the increasing evidence that VEGF signaling is involved in neurogenesis and neuronal survival.

2. Experimental procedures

White Leghorn chicken eggs were purchased from Spafas, Inc. and embryos were staged according to Hamburger and Hamilton (1951). RT-PCR was initially performed using E6 chick retinal cDNAs and degenerate primers towards the Flk1 kinase domain. The full-length chick Flk1 cDNA was obtained from E4.5 chicken head total RNA by 5′- and 3′-RACE (Hashimoto et al., 1997; Clontech). Amino acid similarity was calculated by Lipmann-Pearson's method (Lipman and Pearson, 1985) using DNASTAR program (DNASTAR Inc.). Whole-mount and section in situ hybridization were performed using conditions as previously described (Riddle et al., 1993; Yang and Cepko, 1996; Yang, 2001). Digoxigenin-labeled chick RNA probes corresponding to: (a) the protein kinase domain; (b) the intracellular domain and the 3′ untranslated region; (c) the extracellular domain; or a combination of (b) and (c) were used. Identical hybridization patterns were observed for all probes. Immunocytochemistry was performed by using antibodies against amino acid residue 77–148 of the TGFβ type II receptor (Exalpha Biologicals), followed by binding to biotinylated secondary antibodies, coupled with the ABC Elite kit and horseradish peroxidase reaction (Vector Laboratories) using 3, 3′-Diaminobenzidine (DAB) as chromogen. Immunofluorescent staining was performed by incubating with anti-Brn3a

(Chemicon), anti-Islet1 (clone 4D5, Developmental Studies Hybridoma Bank; Yamada et al., 1993), or anti-PCNA (clone PC10, Sigma) antibodies followed by binding to Alexa 488 (Molecular Probes) or Texas Red (Jackson ImmunoResearch Laboratories) conjugated antibodies in the presence of 4', 6-diamidino-2-phenylindole (DAPI). In situ hybridization and immunostaining signals were visualized using a Nikon E800 microscope and captured with a SPOTII digital camera (Diagnostic Instruments Inc.).

Acknowledgements

This work was in part supported by a grant from Japan Eye Bank Association to T. H. and grants from the Research to Prevent Blindness Foundation, the March of Dimes Birth Defect Foundation, the Karl Kirchgessner Foundation, and the National Institute of Health (EY12270) to X.-J. Y.

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Fig. 1.

In situ hybridization detection of Flk1 mRNA expression at various developmental stages. Panels A–D, M, N, Q–T show transverse sections; panels I–L, O, P show sagittal sections; panel H shows a frontal section parallel to the floor of the hind brain; panels E, F, G show dorsal view of whole-mount hindbrain preparations, with the roof of the hindbrain removed. Panels E and S have been hybridized with sense RNA probes. Panels J and K, N, P, and R show enlarged views of boxed regions in I, M, O, and Q, respectively. Panel T shows immunocytochemical staining of the type II TGF β receptor. Labeled RNA probes used in A–D and E–S were derived from (a) and (b and c) regions, respectively (see Section 2 for details). Yellow arrows indicate sites of Flk1 hybridization signals in non-endothelial cells, green arrowheads point to blood vessels. Scale bars represent 500 μ m in E, F, I, M, O, Q, and 100 μ m in the rest of the panels. Abbreviations: *di*, diencephalon; *fp*, floor plate; *hb*,

hind brain; *ist*, isthmus; *le*, lens; *no*, notochord; *on*, optic nerve; *opc*, optic chiasm; *ret*, retina; *rhb*, roof of hindbrain; *sc*, spinal cord; *tec*, tectum; *tel*, telencephalon.

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Fig. 2.

Expression of Flk1 mRNA in the embryonic chick retina. All panels show in situ hybridization signals of antisense Flk1 probes except panel C, which has been hybridized with sense RNA probes, and Panel F, which shows immunostaining of E8 retina against PCNA, a progenitor cell marker. All panels were hybridized with RNA probes derived from the (b) and (c) regions of chick Flk1, except panel G, which was hybridized with probe (c) (see Section 2 for details). Green arrowheads point to blood vessels. Scale bars represent 200 μ m in panel A and 50 μ m in the rest of the panels. Abbreviations: *di*, diencephalon; *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *le*, lens; *onl*, outer nuclear layer; *pe*, pigmented epithelium.

Fig. 3.

Expression of VEGF in the developing chick retina. Panels A, D, and G show immunofluorescent staining of VEGF protein in the retina at indicated stages. Panel B and C show the same field as A stained for Islet1 (green) and DAPI, respectively. Panel E and F show merged images of E9 staining signals of VEGF (red) and Brn3a (green), and VEGF (red) and Islet1 (green), respectively. Panels G' shows DAPI staining of the corresponding panel G. Panel H shows the staining pattern of the anti-VEGF antibody preincubated with the peptide antigen. White arrowhead in B indicates auto-fluorescence. Scale bars represent 50 μ m in A–C and 100 μ m in D–H. Abbreviations: vs, ventricular surface; others, see Fig. 2 legend.