Vascular Endothelial Growth Factor and Its Receptors Expression in the Rat Eye

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Vascular endothelial growth factor (VEGF) plays a pivotal role in the angiogenesis of embryonic development and tumorigenesis. Recent extensive studies show that VEGF also affects non-vascular cells. In the present study, we performed an immunohistochemical examination of cellular distribution of VEGF and its receptors, Flt-1 and Flk-1, in the retina in vivo and in vitro. Moreover, we addressed their expression in the cornea, iris, and ciliary body. In the retina, both Flt-1 and Flk-1 were localized to the axon of retinal ganglion cells (RGCs) and to the retinal pigment epithelium. In vitro experiments showed that both VEGF receptors seemed to be expressed in the cytoplasm of RGCs and Müller cells. In the extra-ocular tissues, ciliary body epithelium showed VEGF, Flt-1 and Flk-1 expression, while iris epithelium showed VEGF and Flk-1 but not Flt-1. Corneal endothelium only showed Flk-1. These data support the hypothesis that VEGF plays a role in many ocular tissues via Flt-1, Flk-1 or both.

Key words: Retinal ganglion cell axon, Müller cell, Vascular endothelial growth factor (VEGF), Flt-1, Flk-1

I. Introduction

Vascular endothelial growth factor (VEGF) is a prime regulator of angiogenesis, vasculogenesis, and vascular permeability [17]. VEGF exists as a homodimeric glycoprotein composed of one pair of six isoforms, generated by alternative splicing of the VEGF gene. VEGF serves as a chemotactant for monocytes and endothelial cells in vitro [7], and for angioblast migration in Xenopus [14]. In addition, it is reported that VEGF induces a rapid Ca²⁺ entry into cultured endothelial cells from bovine aorta, human umbilical vein, and bovine adrenal cortex [10, 15, 16]. Intracellular Ca²⁺ levels in HK293 cells were increased by VEGF stimulation, which was inhibited by excess soluble VEGF antibody [44].

So far two specific VEGF receptors that possess protein tyrosine kinase have been identified [12] such as Flt-1 (VEGF receptor-1) and Flk-1 (VEGF receptor-2), and a recent study showed another VEGF receptor which was identical to neuropilin-1 [35]. Although the full spectrum of Flt-1 functions is yet unknown, Flt-1 knockout mice exhibit altered vascular integrity [19]. Flk-1 is required not for the formation of hematopoietic/endothelial progenitors but rather for the subsequent migration and expansion of such cells in vitro [33].

VEGF and VEGF receptors are involved in angiogenesis not only during normal development but also under pathological conditions including rheumatoid arthritis, cardiovascular disease and malignant tumors [18]. Neovascular diseases of the retina are major causes of blindness which are often caused by hypoxia, and under such conditions VEGF is induced to initiate the formation of blood vessels in the retina [37, 38]. Intraocular VEGF levels are elevated in humans with proliferative diabetic retinopathy, iris neovascularization and retinopathy of prematurity [1, 5, 6, 9, 28, 31]. Moreover, patients with neovascular glaucoma had a significantly increased level of VEGF in the aqueous humor [40].

Various cell types in the eye, such as the retinal pigment epithelium (RPE), retinal ganglion cells (RGCs), astrocytes, and choroidal fibroblasts, express the mRNA for VEGF [2, 6, 26, 27, 30, 34]. These cells are capable of synthesizing VEGF and secreting it into intraocular fluid [40]. Several studies using in situ hybridization demonstrated Flt-1 and Flk-1 gene expression in the visual organs [13, 21, 37, 44, 45], but the distinct cellular distribution of both receptors is still unclear. In the present study, we determined Flt-1 and Flk-1 localization immunohistochemically in vivo and in...
vitro.

II. Materials and Methods

Animals

All animals used in this study were cared for and handled in compliance with the ARVO Statement for the Use of Animals in the Ophthalmology and Vision Research. 35 neonatal Sprague-Dawley (SD) rats and three adult female SD rats weighing about 350 g were used.

Antibodies

Rabbit macrophage antibody (Inter-Cell Technologies, Hopewell, NJ), mouse monoclonal Thy 1.1 antibody (T11D7e2; American Type Culture Collection, Rockville, MD) and goat anti-rabbit IgG (L+H chain) antibody (Southern Biotechnology Associates, Birmingham, AL) were used for RGC culture. Rabbit polyclonal VEGF antibody (sc-507; Santa Cruz Inc., Santa Cruz, CA) was raised against a peptide corresponding to amino acids 1–140 of VEGF of human origin. Rabbit polyclonal Flt-1 antibody (sc-316; Santa Cruz) was raised against carboxyl terminus of Flk-1 of human origin, and rabbit polyclonal Flk-1 antibody (sc-504; Santa Cruz) against carboxyl terminus of Flk-1 of mouse origin. Propidium iodide (Molecular Probes, Eugene, OR) was used for the counter staining of nuclei.

Retinal ganglion cell culture

RGCs were isolated according to the two-step panning method. Briefly, postnatal-day-2 (P2) rats were sacrificed to obtain approximately 60 eyes for each experiment. Retinas were separated from enucleated eyeballs, immersed in a solution containing 5 mg/ml of papain for 20 min in order to dissociate the cells and incubated with rabbit anti-macrophage serum for 5 min. Cell suspensions were treated in 100 mm petri dishes with goat anti-rabbit IgG (L+H chain) serum for 45 min. Subsequently, the suspension was spread over the dishes coated with monoclonal Thy 1.1 antibody for 60 min. The cells were collected after treatment with 0.125% trypsin for 10 min and incubated in 24 well plates. Cover glasses were coated with 0.1 mg/ml of polyornithine (Sigma Chemical Co., St. Louis, MO) for 5 hr or longer and then coated overnight with 5 µg/ml of EHS-laminin (Upstate Biotechnology, Lake Placid, NY). Dulbecco’s Modified Eagle’s Medium (DMEM) containing the following substrate were used: insulin (1.6×10⁻⁶ M), progesterone (4.0×10⁻⁸ M), selenite (6.0×10⁻⁸ M), transferrin (12.5×10⁻⁴ M), putrescine (2×10⁻⁴ M), hydrocortisone (1.0×10⁻⁷ M), cytidine-5’-diphosphocholine (5.2×10⁻⁶ M) and cytidine-5’-diphosphoethanolamine (2.9×10⁻⁶ M). The seeding density was 1,200 cells/mm². The cells were incubated at 37°C in humidified 10% CO₂ and 90% air. For microscopic examination, RGCs on cover glasses were fixed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) for 10 min.

Müller cell culture

Rat Müller cells were cultured according to the method in our previous study [42]. In brief, eyes from SD rats 3 to 5 days of age were rapidly enucleated into DMEM, and stored overnight at room temperature in the dark. Subsequently, the retinas were mechanically dissociated into small aggregates in the DMEM supplemented with 10% fetal calf serum (FCS). The medium was left unchanged for 5 to 6 days, and then replenished every 3 to 4 days. A purified flat cell population was obtained about a week after seeding (passage 1), and then the cells were detached by 0.05% trypsin and 1 mM EDTA. The suspension was centrifuged at 800 g for 5 min, and cells were seeded in fresh DMEM+10% FCS (passage 2). The same procedure was repeated one more time, and then cultured Müller cells were seeded on cover glasses (passage 4). Müller cells are easily distinguished from other cell types morphologically. As well as RGCs, cultured Müller cells were fixed by the fixative.

Confocal microscopy

Adult Sprague-Dawley rats were anesthetized and perfused with 4% paraformaldehyde in 0.1 M PB. The eyeballs were removed, immersed in the fixative overnight at 4°C and sectioned at 10 μm thickness in a cryostat. After being treated with 10% goat serum for 30 min, cryosections or cover glasses on which Müller cells or RGCs were cultured were incubated with primary antibodies (Flk-1 antibody at 1:1000, Flt-1 antibody at 1:5000, VEGF antibody at 1:2000) overnight at 4°C. Subsequently they were incubated with biotinylated anti-rabbit Ig (Histofine kit; Nichirei, Tokyo, Japan) for 30 min at room temperature, Alexa 488 coupled streptavidin (Molecular Probes) together with propidium iodide (Molecular Probes) at 1:1000 dilution for 60 min, and then mounted with Vectashield (Vector Lab., Burlingame, CA). A confocal laser scanning microscope (Leica TCS4D, Heidelberg, Germany) was used with ×63 oil-immersion objective lens. For the control, the sections were incubated with phosphate buffered saline (PBS) instead of primary antibodies and followed the same procedures as described above.

Immunoperoxidase labeling

Cryosections at 10 µm thickness were treated with 1% Triton X-100 and 1% H₂O₂ in PBS for 30 min and with 10% goat serum for 30 min. Then, the sections were incubated with primary antibodies (the same concentration as that in the fluorescence staining) overnight at 4°C. Subsequently, the sections were incubated with Histofine kit (Nichirei) according to the manufacturer’s protocol, treated with metal enhanced DAB kit (Amersham, Buckinghamshire, UK) and 0.04% OsO₄ in PB for 10 sec. The sections were observed with a light microscope (Olympus BX50, Tokyo, Japan).

III. Results

VEGF, Flt-1 and Flk-1 expression in the retina in vivo

The present study showed that Flt-1 and Flk-1 were detected in the nerve fiber layer (NFL) in patchy patterns, while VEGF was found in the ganglion cell layer (GCL; Fig.
1a–c). Being consistent with previous studies using in situ hybridization [21, 44, 45], we identified Flk-1 positive cells on the inner side of the inner nuclear layer (INL), suggesting that they are amacrine cells (data not shown). Both Flt-1 and Flk-1 in the NFL were also found in the papilla (Fig. 1h–j), but neither receptor was detected in the adult rat optic nerves (unpublished data). Moreover, our data clearly demonstrated VEGF, Flt-1 and Flk-1 localization in the RPE immuno-histochemically (Fig. 1d–f), supporting previous studies that showed Flt-1 and Flk-1 mRNA in RPE in vitro [13] and in vivo [21]. Both VEGF receptors were not found in the endothelial cells under normal conditions in the adult rat retina, and control sections showed no positive labeling (data not shown).

Both Flt-1 and Flk-1 localization in the cytoplasm of retinal ganglion and Müller cells in vitro

To clarify whether VEGF receptors are localized in RGC processes, we examined Flt-1 and Flk-1 expression in cultured RGCs. Figures 2a and 2b showed that both receptors were expressed not only in the cell body but also in processes as a dot-like appearance. In addition, enlarged terminal processes like growth cones sometimes had positive immunoreaction of both receptors (data not shown), but no significant differences were recognized between Flt-1 and Flk-1 labeling patterns. These data are compatible with the previous study demonstrating that Flk-1 was localized in the cell body, axon and growth cone of the dorsal root ganglia and superior cervical ganglia in vitro [36]. It is well known that astrocytes are activated to secrete VEGF under hypoxic conditions [23] and that Schwann cells possess Flk-1 [36]. Our immunocytochemical data clearly showed VEGF, Flt-1 and Flk-1 in the cytoplasm of Müller cells in vitro (Fig. 2c–e), supporting previous studies about VEGF mRNA in Müller cells of the developing mouse retina [45] and adult rat retina [32]. Flk-1 was often found at the marginal edge of the Müller cell body (Fig. 2e: arrowheads), indicating that Flk-1 was incorporated in the membrane, whereas Flt-1 was not found there at all. In addition, nucleoli in some Müller cells were labeled by Flk-1 (Fig. 2e: an arrow), suggesting that Flk-1 may serve as a nuclear protein, although it is hard to exclude the possibility of the cross-reaction with unknown proteins.

Different distribution of Flt-1 or Flk-1 in the ciliary body, iris, and cornea

In situ hybridization study demonstrated that ciliary body had VEGF, Flt-1, and Flk-1 [21]. Our immunohistochemical study was basically consistent with those findings and demonstrated cellular distribution in greater detail. VEGF appeared to be expressed in both epithelial cells of the ciliary body (Fig. 3a), and both receptors did not seem to be bounded to the cell membranes (Fig. 3b, c). Especially, Flt-1 was recognized near the nucleus, suggesting that Flt-1
is associated with Golgi apparatus (Fig. 3b inset). VEGF was found in the iris epithelium (Fig. 3d), but Flt-1 was not (Fig. 3e). Flk-1 was detected in the iris epithelium and fibroblast-like cells in the vascular layer of the iris (Fig. 3f). Although previous studies about VEGF receptor gene expression have suggested that Flt-1 mRNA was detected in the cornea [8, 21], the present study showed that only Flk-1 was recognized in the corneal endothelial cells (Fig. 3g–i). These data suggest that VEGF affects various extracellular cells via different VEGF receptors in a tissue specific manner.

IV. Discussion

In the present study, we demonstrated that VEGF and VEGF receptors, Flt-1 and Flk-1, were expressed in the various ocular tissues. Both Flt-1 and Flk-1 were mainly localized in the RGC axon and in the RPE of the adult rat retina. In vitro experiments showed that both receptors were detected in RGCs and Müller cells. Ciliary body epithelium possessed VEGF, Flt-1 and Flk-1, while iris epithelium showed VEGF and Flk-1 but not Flt-1 and corneal endothelium only demonstrated Flk-1. These data suggest that VEGF plays a role in many ocular tissues under normal conditions.

Sondell et al. [36] reported that VEGF has neurotrophic activity and stimulates axonal growth, and proliferates Schwann cells in the peripheral nervous system in vitro. In addition, our recent study showed that Flt-1 was localized in rat cerebellar Purkinje cells [41]. This study demonstrated the evidence that VEGF probably affects nervous system in the retina. Our data indicates that majority of VEGF receptors seem to be localized in the cytoplasm, although no studies about the ultrastructural localization of VEGF receptors have been reported yet. Soluble forms of VEGF receptors could explain this difference. Soluble Flt-1, a truncated form lacking the transmembrane sequence, appears to be able to inhibit the activities of VEGF [24, 25]. Actually, Aiello et al. [4] reported that soluble VEGF receptor chimeric proteins suppressed retinal neovascularization in a murine model of retinopathy of prematurity. In addition, soluble Flt-1 protein inhibits tumor growth by suppressing tumor angiogenesis in vivo [22]. Our immunohistochemical findings hypothesize that soluble Flt-1 exists under physiological conditions, and maybe soluble isoform of Flk-1 does as well, although this has not been identified biochemically yet.
Fig. 3. Photographs of ciliary bodies (a–c), iris (d–f), and cornea (g–i) labeled with VEGF (a, d, g), Flt-1 (b, e, h) or Flk-1 (c, f, i). VEGF, Flt-1 and Flk-1 are all expressed in the ciliary epithelium (a–c). Insets represent higher magnification. Iris epithelium is labeled by VEGF and Flk-1 (d and f), but not by Flt-1 (e). Fibroblast-like cells are also stained with Flk-1 in the iris (f, double arrows). Corneal endothelium is labeled with only Flk-1 (i, arrowheads). Arrows indicate immunopositive reactions. Bar=20 μm (f), 100 μm (c, i and insets).
Iris neovascularization (rubeosis iridis) often occurs with other ocular diseases, including retinal vascular occlusive disease, chronic retinal detachment and intraocular tumors such as retinoblastoma [20] or melanoma [11]. Recent studies show that VEGF level increases in a primate model of iris neovascularization [29], and that intravitreal injections of recombinant human VEGF_{165} are sufficient to produce noninflammatory iris neovascularization in a non-human primate [39]. Furthermore, prolonged exposure to VEGF_{165} can produce ectropion uveae and neovascular glaucoma [39]. In contrast, VEGF antibody injection into vitreous can produce ectropion uveae and neovascular glaucoma.

VI. References

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VI. References


VEGF and Its Receptor Localization in the Eye


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