Immunohistochemical Localization of Vascular Endothelial Growth Factor in the Endocrine Glands of the Rat*

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Summary. Vascular endothelial growth factor (VEGF) is a secreted polypeptide with specific effects on endothelial cell growth and vascular permeability. While previous studies have focused on the expression of VEGF associated with angiogenesis in tumor and embryonal tissues, little is known about the role of VEGF in normal adult tissues. In the present study, a specific antibody was used to study the immunohistochemical localization of VEGF in the entire body of normal adult rats. Intense to moderate immunoreactivities for VEGF were detected in some endocrine cell types, namely, the parafollicular cell of thyroid gland, B cell of endocrine pancreas, N cell of adrenal medulla and a minority of the thyrotrophs of the pituitary gland. A certain exocrine cell type, i.e., the surface mucous cell of stomach, was also immunoreactive for VEGF. At the ultrastructural level, VEGF immunoreactivity was localized exclusively in the secretory granules of all immunopositive endocrine and exocrine cells examined. The present study provided immunohistochemical evidence for the occurrence of VEGF in subsets of endocrine and exocrine cells of normal adult rats, suggesting that these secretory cells regulate local vascular permeability through a paracrine action of VEGF.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a secreted angiogenic mitogen isolated from a variety of normal and tumor cells of human, bovine and rodent origin. Biochemically, VEGF is a heparin-binding, disulfide-linked dimeric glycoprotein with a molecular weight of ~46 kD and about 20% structural homology with platelet-derived growth factor (PDGF) (GOSPODAROWICZ et al., 1989; KECK et al., 1989; LEUNG et al., 1989). Human VEGF has several subtypes derived from alternative splicing, whereas the rat predominantly expresses a single subtype of VEGF (CONN et al., 1990). Biologically, VEGF causes both endothelial cell proliferation (FERRARA and HENZEL, 1989) and vascular permeabilization (SENGER et al., 1983; COLLINS et al., 1993) by binding its high affinity receptors located on the endothelial cell (DEVRIES et al., 1992; TERMAN et al., 1992). The expression and localization of VEGF has been studied primarily in the embryos, tumors and some pathological tissues, where VEGF has been implicated in the neoangiogenesis (BREIER et al., 1992; JAKEMAN et al., 1993; PLATE et al., 1994; PIERCE et al., 1995). In contrast, relatively little information is available as to the occurrence of VEGF in normal adult animals. Abundant VEGF mRNA is known to be expressed in various human and rat organs, including the brain, lung, heart, liver, kidney, spleen, testis, ovary, uterus, placenta, pancreas and adrenal gland (PHILLIPS et al., 1990; MONACCI et al., 1993; SHWEIKI et al., 1993; CHRISTOFORIT et al., 1995; SIMON et al., 1995), though there have been few studies on the cellular and subcellular localization of the VEGF protein. The present study aimed to elucidate the immunohistochemical localization of VEGF in a variety of organs and tissues of normal adult rats at both light- and electron-microscopic levels.

MATERIALS AND METHODS

Animals and tissue preparation
Male and female Wistar strain rats weighing 200–350 g were sacrificed under sodium pentobarbital anesthesia by a transcardial perfusion with cold physiological saline. For immunohistochemical purposes, the animals were further fixed by perfusion with 4%
paraformaldehyde in 0.1 M phosphate buffer. The following organs and tissues were excised out: the brain, pineal body, pituitary gland, eye, submandibular gland, thyroid gland, parathyroid gland, trachea, lung, heart, lymph node, thymus, spleen, liver, bile duct, pancreas, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, adrenal gland, kidney, urinary bladder, ovary, uterus, oviduct, testis, epididymis, prostate, soleus muscle and skin. They were further fixed by immersion in the same fixative for 4 h at 4°C, and then each was divided into several specimens. The specimens for cryosectioning were rinsed in 30% sucrose/0.1 M phosphate buffer overnight at 4°C for cryo-protection. The specimens for paraffin sectioning were dehydrated in a graded series of ethanol/xylene and embedded in paraffin at 60°C. The specimens for immuno-electron microscopy were cut into small pieces, dehydrated in a graded ethanol series and embedded in LR-White resin (London Resin Co., London, UK) in the presence of 1% benzoyl peroxide. They were then polymerized for 24 h at room temperature with an ultraviolet polymerizer. Some organs of special interest were subjected to Western blot analysis. For this purpose, following sacrifice of the animals by perfusion with cold physiological saline, the kidney, liver, pituitary gland, thyroid gland, pancreas, adrenal gland and stomach were excised out, frozen immediately in liquid nitrogen and kept at −80°C until homogenization.

**Antibodies**

Anti-VEGF (V3) antibody, a purified IgG fraction of rabbit antiserum raised against a synthetic pentadecapeptide (SERRKHLFVQDPQTC) corresponding to amino acids 121-134 of the mature human and rat VEGF (Conn et al., 1990), was purchased from Immuno Biological Laboratories (IBL), Fujioka, Japan. For the negative control, the antibody was absorbed by the same synthetic VEGF peptide (kindly provided by IBL) at a concentration of 10 μg/ml for 8 h at 4°C. Antisera against growth hormone (GH), prolactin, thyroid stimulating hormone (TSH) and luteinizing hormone (LH) were donated by Dr. K. WAKABAYASHI, Institute for Molecular and Cellular Regulation, Gunma University. Antisera or antibodies against the following peptides were obtained from commercial sources: adrenocorticotropic hormone (ACTH), S-100 protein, calcitonin gene-related peptide (CGRP), insulin, glucagon, somatostatin and phenyl-ethanolamine-N-methyl transferase (PNMT).

**Western blot analysis**

Purified recombinant human VEGF was purchased from PeproTech, Inc, Rocky Hill, NJ. The frozen organ specimens were homogenized in ice-cold 10 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl using an Ultra-Dispenser (Yamato, Tokyo, Japan). They were then centrifuged at 3000 rpm for 10 min and the supernatants were used as homogenate samples after measurement of their protein content using BCA Protein Assay Reagent (Pierce, Rockford, Ill). The purified VEGF, 0.3 μg in amount, and the homogenate samples, 20 μg in amount, were separated under reducing conditions by electrophoresis in a 15% polyacrylamide slab gel at pH 8.8 in the presence of 0.1% SDS, according to LAEMMLI (1970). They were then transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) by the method of TOWBIN et al. (1979). The membranes were incubated overnight at room temperature with the anti-VEGF antibody at a concentration of 5 μg IgG/ml and with the same antibody preabsorbed by the VEGF peptide. After washing in PBS/0.1% Tween 20, the immunoreaction was visualized by incubating the membranes successively with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) at 1: 200 dilution for 1 h, streptavidin-conjugated horseradish peroxidase (Dakopatts, Glostrup, Denmark) at 1: 300 dilution for 1 h, and with 3′3′-diaminobenzidin tetrahydrochloride in the presence of 0.01% hydrogen peroxide in 0.05 M Tris-HCl (pH 7.5).
Fig. 2. Light micrographs of the pituitary gland immunostained with the anti-VEGF antibody (a) and anti-VEGF antibody preabsorbed with the VEGF peptide (b). a. A minority of cells in the anterior lobe are immunopositive for VEGF. b. No cells are immunostained. a, b: ×155

Fig. 3. Light micrographs of the serial paraffin sections of the pituitary gland immunostained with the anti-VEGF antibody (a) and anti-TSH antiserum (b). Note that most VEGF-immunopositive cells (arrows) also react with the anti-TSH antiserum, whereas only a part of TSH-positive cells are VEGF-positive. ×460

Fig. 4. Electron micrograph of the anterior lobe of the pituitary gland immunostained for VEGF. Gold particles are localized in most of the secretory granules of the TSH cell, whereas the growth hormone cells (GH) are immunonegative. ×18,000
**Immunohistochemistry**

For light-microscopic immunohistochemistry, the cryoprotected organ specimens were frozen and cut into 15 μm sections using a cryostat, and the paraffin-embedded tissue blocks were cut into 3 μm sections using a microtome. Both sections were mounted onto gelatin-coated glass slides, and the paraffin sections were deparaffinized with xylene/graded ethanol series. They were then subjected to the routine light immunohistochemical procedure as described previously (Yamamoto et al., 1995). Briefly, the sections were first treated successively with 0.3% Tween 20 in PBS, 0.3% hydrogen peroxide/methanol and normal swine serum (Dakopatts) in 1:20 dilution. Subsequently, the sections were incubated overnight at room temperature with the anti-VEGF antibody at concentrations of 2.5–5 μg/ml. The site of immunoreaction was then visualized with the same procedure as in Western blotting. To identify VEGF-immunoreactive cell types, serial paraffin sections were alternately immunostained with the antibodies to VEGF and various marker peptides.

For electron-microscopic immunocytochemistry, ultrathin sections were cut from the LR-White-embedded tissue blocks and mounted onto nickel grids. They were first treated with 1% bovine serum albumin in PBS for 15 min and then incubated with the anti-VEGF antibody (5 μg/ml) overnight at room temperature. After washing with PBS, the sections were subjected to the reaction with colloidal gold (20 nm)-conjugated anti-rabbit IgG (British BioCell, Cardiff, UK) at 1:20 dilution in PBS containing 0.1% BSA for 2 h at room temperature. They were then contrasted with uranyl acetate and lead citrate, and examined with a Hitachi H-700 electron microscope.

**RESULTS**

**Western blot analysis**

The recombinant VEGF reacted with the anti-VEGF antibody to form a clear immunopositive band with an approximate size of 23 kD, which corresponded to the molecular weight of the VEGF monomer in a reducing condition (Ferrara and Henzel, 1989) (Fig. 1A). The immunopositive band with the same size was also detected in the homogenates from major endocrine glands. The intensity of immunoreaction decreased in order from the thyroid gland, pancreas, pituitary body to the adrenal gland. The stomach also showed the immunoreactivity. In contrast, only a faint reactivity in the kidney and scarcely any reactivity in the liver were detected. Control reactions with the anti-VEGF antibody preabsorbed by the VEGF peptide produced no immunopositive 23 kD band either in purified VEGF or in any organ homogenate (Fig. 1B). These results confirmed the specificity of the present anti-VEGF antibody and also demonstrated the presence of VEGF immunoreactivity in some endocrine glands.

**Immunohistochemistry**

First, the cryosections from all organs and tissues were examined with light-microscopic immunohistochemistry for VEGF. Strong to moderate immunoreactivities were found in the pituitary gland, thyroid gland, pancreas, adrenal gland and stomach. A weak immunoreactivity was also found in the kidney, trachea, intestines and ovary. In all these sites, the specificity of the immunoreaction was confirmed using the control reaction with preabsorbed antibody. The rest of the organs and tissues showed no specific immunoreactivity for VEGF. Subsequently, identification of VEGF-positive cell types using consecutive paraffin sections as well as subcellular location of the immunoreactivity using electron microscopy were performed, and the following results were obtained.

**Pituitary gland**

VEGF immunoreactivity with moderate intensity was present in a small number of cells scattered throughout the anterior lobe, whereas no reactivity was observed in the intermediate and posterior lobes (Fig. 5).
Figs. 5-7. Legends on the opposite page.
This immunoreactivity was abolished when the primary antibody was absorbed by the VEGF peptide (Fig. 2b). The immunopositive cells appeared angular in shape and middle in size (Fig. 3a). To identify the type of VEGF-positive cell population, serial sections were alternately immunostained with antibodies against VEGF and one of the pituitary-specific hormones (GH, prolactin, TSH, ACTH and LH) and S-100 protein, a marker of the folliculo-stellate cell. The results demonstrated that the VEGF-positive cell population was essentially coincident with the TSH-positive cell population, although the former was smaller than the latter (Fig. 3b). In three pairs of sections from different pituitaries, an average of 92% of the total VEGF-positive cells were simultaneously positive for TSH. On the other hand, only 31% of all TSH-positive cells were positive for VEGF. No other types of cells coincided with VEGF-positive cells (data not shown). By immunoelectron microscopy, the gold particles representing VEGF immunoreactivity were localized strictly to the secretory granules in all immunopositive TSH cells, which were characterized by well-developed rough endoplasmic reticulum and small round secretory granules 120-140 nm in diameter (Kurosumi, 1968) (Fig. 4).

**Thyroid gland**

Numerous cells with very strong VEGF immunoreactivity were observed throughout the gland (Fig. 5a, b). They were scattered in the follicular epithelium and also present in groups in the interfollicular space. In serial sections, this VEGF-positive cell population was identified as the parafollicular cell with its simultaneous immunoreactivity for CGRP, a marker of calcitonin-producing cells (Kameda, 1987) (Fig. 6a, b). Virtually all parafollicular cells identified with anti-CGRP antibody appeared immunopositive for VEGF. Electron microscopy demonstrated the immunoreaction to be localized exclusively in the secretory granules of parafollicular cells (Fig. 7). No VEGF immunoreactivity was detected in the follicular epithelial cells at either light- or electron-microscopic levels.

**Pancreas**

Intense VEGF immunoreactivity was localized to the endocrine pancreas, i.e., the islet of Langerhans, where the immunopositive cells occupied a majority of the area (Fig. 8a, b). In contrast, the exocrine pancreas was free of the immunoreaction both in acinar and ductal areas. Consecutive sections incubated with anti-VEGF and anti-insulin antibodies clearly demonstrated the staining of identical cell populations (Fig. 9a, b), whereas the immunoreactivity for glucagon or somatostatin did not coincide with VEGF immunoreactivity (data not shown). These results indicated that the VEGF-positive cell type is the B cell of the endocrine pancreas. In electron microscopy, the immunoreactivity was confined to the secretory granules characteristic of B cells (Erlandsen, 1980) (Fig. 10).

**Adrenal gland**

VEGF immunoreactivity with moderate intensity was restricted to the medulla, where the immunopositive cells scattered in clusters constituted the minor cell population (Fig. 11a, b). In contrast, much weaker reactivity was recognized in the major cell population of the medulla, with no reactivity in the cortex. When two consecutive sections were immunostained for VEGF and PNMT, a marker enzyme specific for the adrenaline (A) cell, the distribution patterns of the two immunopositive cell populations were completely opposite (Fig. 12a, b). These results indicated that VEGF immunoreactivity occurs preferentially in the noradrenaline (N) cell. Electron microscopy further demonstrated the localization of immunoreactivity to the secretory granules characteristic of N cells, with slightly larger sizes and darker cores as compared with those of A cells (Coupland and Kobayashi, 1976) (Fig. 13).

**Stomach**

Moderate VEGF immunoreactivity was localized to

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**Fig. 8.** Light micrographs of the pancreas immunostained with the anti-VEGF antibody (a) and anti-VEGF antibody preabsorbed with the VEGF peptide (b). a. An intense immunoreactivity for VEGF is present in most cells of the pancreatic islet (I), whereas the exocrine pancreas (E) is immunonegative. b. No cells are immunostained. ×83

**Fig. 9.** Light micrographs of the serial paraffin sections of the pancreas immunostained with the anti-VEGF antibody (a) and anti-insulin antiserum (b). The identical cells in the islet are immunostained. ×165

**Fig. 10.** Electron micrograph of the pancreatic islet immunostained for VEGF. Abundant gold particles are localized in many of the secretory granules of the B cell (a), whereas those of the A cell (b) and D cell (c) are immunonegative. E exocrine cell. ×26700
Figs. 8-10. Legends on the opposite page.
Figs. 11-15. Legends on the opposite page.
the surface mucous cells in all areas of the stomach, with slightly higher intensity in the pyloric region, whereas the cells of mucosal glands, including the mucous neck cells, were free of the immunoreaction (Fig. 14a, b). Electron microscopy further confirmed the localization of VEGF immunoreactivity in the apical secretory granules of surface mucous cells (Fig. 15).

Other organs and tissues
No VEGF immunoreactivity was recognized in the peptide-amine endocrine cells other than those described above, including the neurons of the hypothalamus, chief cells of pineal body and parathyroid gland, as well as various enteroendocrine cells. Weak VEGF immunoreactivity was demonstrated in the glomerular epithelial cell (podocyte) of the kidney, luteal cell of the ovary and a certain subset of mesenchymal cells in the trachea and digestive tract. Further analysis of these cell populations was not performed in the present study.

DISCUSSION
Unlike other growth factors, which target a wide range of different cell types, VEGF is thought to act selectively on endothelial cells through binding its specific receptors, flt-1 and KDR/flk-1 (DEVRIES et al., 1992; TERMAN et al., 1992). Also, VEGF immunoreactivity was demonstrated in the glomerular epithelial cell (podocyte) of the kidney, luteal cell of the ovary and a certain subset of mesenchymal cells in the trachea and digestive tract. Further analysis of these cell populations was not performed in the present study.

In regard to the occurrence of VEGF in normal adult organs and tissues, there is significant inconsistency between the mRNA expression revealed by previous studies and the immunoreactivity revealed by the present study. The abundant expression of VEGF mRNA has been reported for a variety of cell types in organs rich in vasculature, namely, the neurons of the brain, hepatocytes of the liver, alveolar cells of the lung, podocytes of the kidney, cortical cells of the adrenal gland, Leydig cells of the testis, luteal cells of the ovary and endometrial cells of the uterus (PHILLIPS et al., 1990; MONACCI et al., 1993; SHWEIKI et al., 1993; SIMON et al., 1995). However, the present study shows only weak VEGF immunoreactivity in the podocyte and luteal cell, and no reactivity in the rest of above cell populations. This discrepancy may be interpreted as follows: the cells described in the previous studies lack secretory granules and release VEGF rapidly in a constitutive manner resulting in low intracellular levels of VEGF, whereas the cells described in the present study accumulate VEGF in their secretory granules and release it in a regulated manner. This notion is supported by the present Western blot analysis demonstrating the presence of VEGF immunoreactivity in the endocrine organs in question and its absence in the liver, an organ known
to express abundant VEGF mRNA.

The biological significance of the occurrence of VEGF in distinct endocrine cell types should be discussed in terms of their relation with the blood vascular system. Since neoangiogenesis in normal adult animals is restricted to certain areas such as the ovarian corpora, the role of VEGF released by the present endocrine cell types is most likely vascular permeabilization rather than angiogenesis. An abundant supply of blood vessels containing fenestrated capillary endothelium is a characteristic of most endocrine glands. The permeability of local microvessels is considered crucial in endocrine cells for their release of hormone products into the blood stream. Furthermore, all of the VEGF-positive endocrine glands, i.e., pituitary gland, thyroid gland, endocrine pancreas and adrenal gland, are composed of more than two types of endocrine cells. Taken together, the present endocrine cell types, by secreting VEGF in a regulated manner, may increase the permeabilization of local microvessels and thereby facilitate their own endocrine functions and also those of adjacent endocrine cell types.

It should be mentioned that VEGF was originally isolated from the cultured folliculo-stellate (FS) cell of the bovine pituitary (Gospodarowicz et al., 1989; Ferrara and Henzel, 1989), whereas the present study shows the absence of VEGF immunoreactivity in FS cells. Two explanations are possible for this discrepancy. One is that FS cells do not synthesize VEGF in vivo but become capable to do so after being grown in a culture system. Alternatively, FS cells, without secretory granules, synthesize VEGF but secrete it constitutively, as hepatocytes do, so that the routine immunohistochemical procedure is unable to detect VEGF in the FS cell cytoplasm. Also, it is of interest that, unlike other VEGF-immunoreactive endocrine cell types found in this study, which are immunopositive in almost all of the population, only a part of the total TSH cell population was immunopositive for VEGF. Whether this result indicates that VEGF-positive TSH cells constitute a distinct subtype of TSH cells or they represent a different functional state of the same TSH cell population will require further investigation.

In the thyroid gland, networks of numerous anastomosing capillaries tightly surround individual follicles like baskets (Fujita and Murakami, 1974). Parafollicular cells are situated in the periphery of the follicles and also in the interfollicular connective tissue, and hence in the close vicinity of the microvessels. The present study raises the possibility that the parafollicular cell, besides producing calcitonin, plays an important role in regulating vascular permeability through the action of VEGF, and thereby facilitates the endocrine functions of both parafollicular and follicular epithelial cells.

In the endocrine pancreas, the expression and localization of VEGF in islet cells—more accurately the B cells—has previously been reported by other investigators (Kuroda et al., 1995, Christofori et al., 1995), although the subcellular localization of VEGF is not mentioned by these authors. We have demonstrated with immuno-electron microscopy that VEGF is localized exclusively in the secretory granules of B cells, suggesting that this cell type produces and secretes VEGF into the interstitial space in a regulated manner. The unique characteristic of islet blood flow is that some of the arteries entering the pancreatic lobule first terminate in the capillary network of the islet, where the blood receives the islet hormones and then carries them into another network of capillaries in the acinar tissue (Fujita, 1973). The previous and present results suggest that the B cell regulates the vascular permeability in islets through VEGF, and thereby influences the functions not only of endocrine cells but also of exocrine cells.

In the adrenal medulla, two chromaffin cell populations, i.e., A and N cells, have been distinguished by a variety of criteria at the light-and electron-microscopic levels (Wood, 1963; Coupland and Kobayashi, 1976), including PNMT as a marker of A cell (Wurtman and Axelrod, 1966). The present study indicates VEGF to be a histochemical marker of the N cell. Also, chromaffin cells have been known to localize not only monoamines but also peptides such as enkephalin in their secretory granules (Kobayashi et al., 1983). The present study provides evidence that VEGF is also secreted by chromaffin cells together with monoamines in a regulated pathway.

Finally, the surface mucous cell of stomach is exceptional among the present VEGF-immunopositive cell types in that it belongs to the exocrine cell population. VEGF, located in the secretory granules of surface mucous cells, is most likely released into the gastric lumen together with mucus. The recent observation of VEGF mRNA expression in the epithelia of prostates and the seminal vesicle, as well as the occurrence of VEGF protein in semen, is also indicative of VEGF production by exocrine cells (Brown et al., 1995). The pathway by which gastric luminal VEGF reaches mucosal blood vessels is not clear. Possibly, VEGF penetrates into the lamina propria through some discontinuity in the tight junctions between the epithelial cells. In connection with this, the microvessels just beneath the surface epithelium are known to carry not only oxygen and nutrients but also parietal cell-derived bicarbonate ions, which...
may neutralize the back diffusion of luminal gastric acid to the mucosal surface (GANNON et al., 1982). It is tempting to assume that VEGF secreted by surface mucous cells takes part in the gastric mucosal protection by increasing the permeability of subepithelial microvessels. Alternatively, luminal VEGF may act directly on the mucosal epithelium. Such a possibility is suggested by recent evidence of the occurrence of flk-1, a VEGF receptor, on the pancreatic ductal epithelium (ROOMAN et al., 1997). Further attempts to search for VEGF receptors on the epithelial cells of gastrointestinal tract will be necessary.

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