Angiogenesis and Developmental Expression of Vascular Endothelial Growth Factor in Rat Lingual Papillae

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Received 30 October 2006, accepted 5 April 2007

Summary: We used an embryological approach to investigate development and microvasculature of lingual papillae, and expression of vascular endothelial growth factor (VEGF) in the rat tongue. Temporal changes in the rat tongue at each developmental stage from embryonic day 13 (E13) to postnatal day 7 (P7) were observed by intravascular injection of India ink and immunohistochemistry using a VEGF antibody. At E13, the primordium of circumvallate papilla was observed among various lingual papillae. VEGF was widely expressed at E16 on the proliferated epithelium and the connective tissue core of circumvallate papilla. Invasion by capillary sprouts forming the lingual papillae was observed at E17. The primordium of fungiform papilla was observed at E14. VEGF was strongly expressed around the basal cells of proliferated epithelial tissues of fungiform papilla at E17. At E18, blind-ended capillary sprouts invaded into connective tissue cores from subepithelial sinusoidal capillaries by sprout angiogenesis. At P1, the invading capillary sprouts formed loops by vascular remodeling. The primordium of foliate papillae was observed at E16. VEGF was slightly expressed, but uniformly at E17 on the epithelium, muscle cells, and fibroblasts of foliate papillae. At E18, vascular density was increased by angiogenesis. The primordium of filiform papilla was observed at E17. It was the last to develop among the lingual papillae. VEGF was expressed in the cytoplasm of grown epithelial cells of filiform papilla at E19, and in blind-ended capillary sprouts formed by angiogenesis in the connective tissue cores at E20. The capillary sprouts formed loops by vascular remodeling at P1. Consequently, VEGF was expressed on the papillary epithelium and connective tissue cores of papillae during development of the papillary epithelium, and invasion by capillary sprouts into each papillae was observed thereafter. These results suggest a close relationship between expression of VEGF and angiogenesis of lingual papillae in the rat.

Key words tongue, lingual papillae, angiogenesis, vascular endothelial growth factor (VEGF), immunohistochemistry

INTRODUCTION

The tongue has different functions including deglution and mastication, and it also serves as an important information processing system controlling taste sensation. Morphological characteristics of the rat tongue have been described by various studies. It was reported that 4 types of lingual papillae: circumvallate papillae, fungiform papillae, foliate papillae, and filiform papillae exist in the dorsum of the tongue, and filiform papillae showed 3 morphological characteristics depending on regions on the lingual dorsum [1]. It is histologically known that taste buds form in circumvallate papillae, fungiform papillae, and foliate papillae,
but not in the 3 types of filiform papillae which have their own specific differentiated epithelia. Although there are slight differences in the classification of the 3 types of filiform papillae depending on observers, they are classified into simple conical papillae, giant conical papillae, and branched papillae [2,3]. Many researchers reported on development of papillae [4–9], but no study has been performed to simultaneously observe development and angiogenesis of lingual papillae. A detailed study of the vascular morphology of lingual papillae in the adult rat was reported with respect to the vascular system [2,3,10], but angiogenesis of lingual papillae has not been observed except for that of filiform papillae [11].

It is generally known that angiogenesis plays an important role in the early embryonic stages of organs [12–15]. Vascular endothelial growth factor (VEGF) is a cytokine which plays a leading role in angiogenesis. VEGF was initially discovered as a vascular permeability factor (VPF) [16]. Then, a heparin-binding growth factor that acts specifically on endothelial cells was discovered, and was named as VEGF [17]. It was later clarified that VEGF and VPF were the same glycoprotein, and is currently considered as one of the most potent angiogenic factors having both a growing effect on endothelial cells and a vascular hyperpermeability effect. VEGF is secreted according to oxygen and nutritional requirements of tissues or cells [18–21]. VEGF is expressed in many organs and tissues during the embryonic period, as well as in cancerous cells showing strong proliferation [22–24]. VEGF family has been expanded by the isolation of four additional growth factors, VEGF-B, -C, -D, and -E. In this study, the expression of the VEGF namely VEGF-A which is the most powerful angiogenic factor in early development is investigated. Moreover, five isoforms of VEGF (-A) exist due to differential splicing of a single gene: VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206. Of these, VEGF121 and VEGF165 are secreted soluble glycoproteins and VEGF165 is the most abundant VEGF. In this study, we examined by using the VEGF antibody that mainly react with the VEGF165 abundant VEGF. In this study, we examined by using the VEGF antibody that mainly react with the VEGF165 abundant VEGF.

MATERIALS AND METHODS

Animals

Study protocols have been approved by the Committee on Animal Experimentation, Kurume University. Female Wistar rats after mating were raised at the Kurume University center as experimental animals, and the fetuses and newborn rats from maternal animals, and adult rats were used. Mating was confirmed by the presence of a vaginal plug, and embryonic stages were calculated by considering animals with a plug as the first embryonic day (E1). Tongues to be examined in this study were removed from embryos from E13 to E21, and the first postnatal day (P1) to P7, and from 8 adult rats.

Preparation of specimens

Preparation of specimens for histological observation

Heads of fetuses and newborn rats were dissected, and were immediately immersed and fixed in Bouin solution. Following fixation, specimens were prepared using the entire head of animals from E13 to E18, and the isolated tongue alone of animals from E19 to P7. Tissues were embedded in paraaffin according to conventional methods. Tongue specimens of adult rats were prepared by isolating the tongue alone. These were fixed and embedded in the same manner. Then, 8 to 15-μm serial sections were cut sagittally, and frontal and horizontal sections were used for staining.

Hematoxylin/eosin (H/E) staining: Paraffin sections were stained with H/E, using conventional methods, and morphologies of the tongue and lingual papillae of adult rats and embryonic specimens were observed by light microscopy.

Immunohistochemistry: Immunohistochemistry was performed for tongue paraffin sections of each embryonic stage using a VEGF antibody as primary antibody. Following deparaffinization, each section was incubated with 0.3% hydrogen peroxide solution to inactivate endogenous peroxidase activity, and was reacted for 60 min with 10% normal rabbit serum to block non-specific binding sites for the secondary antibody. Anti-VEGF goat polyclonal IgG (SANTA CRUZ, CALIFORNIA, USA) was used as primary antibody, and biotinylated anti-goat rabbit-IgG (NICHIREI, TOKYO, JAPAN) was used as secondary antibody. Peroxidase-conjugated streptavidin (NICHIREI, TOKYO, JAPAN) was used as the enzymatic reagent, and 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer (pH 7.6) containing 0.003% hydrogen peroxide was used for...
Visualization of the final reacting products. This anti-VEGF antibody reacts with the 165, 189 and 121 amino acid splice variants of VEGF of mouse and rat origin (SANTA CRUZ, CALIFORNIA, USA). To confirm specificity of immunocytochemical staining, the primary antiserum was replaced by phosphate buffered saline (PBS).

**Preparation of microvascular specimens**

**Injection of Indian ink:** Before injection of Indian ink, 99% diethyl ether was aspirated in rat fetuses and newborn rats, and animals were fixed in the supine position under general anesthesia. Following thoracotomy, the heart was injected with physiological saline at 37°C, and was fully perfused until blood in the tongue vessels was eliminated. Then, Indian ink solution at 37°C in 10% gelatin was immediately injected within 2 to 3 min using hand pressure of 100-120 mmHg [27]. After confirmation of black coloration of the dorsum of the tongue by Indian ink injection, the specimens were immediately cooled to coagulate gelatin, and were further fixed with 10% formaldehyde solution.

**Celloidin embedding and observations:** The tongue was isolated after fixing the specimens for 2 days in 10% formaldehyde solution, dehydrated with alcohol, and embedded with celloidin. Using prepared Indian ink-injected microvascular specimens at each stage, the tongue surface was observed using stereoscopic microscopy. Serial 60-μm thick sagittal sections were prepared, and angiogenesis of the tongue and lingual papillae at each embryonic stage was observed using frontal and horizontal sections and light microscopy.

**RESULTS**

**Morphology of the tongue and lingual papillae in the adult rat**

**Macroscopic observations**

The tongue of adult rat is divided into 3 areas: the apex which is the tip of the tongue, the body which covers the area from the apex of the tongue to the circumvallate papillae, and the root which is the posterior area of the circumvallate papillae. Situated at the center of the body of the tongue is the intermolar eminence with a side view protrusion dividing the body of the tongue into anterior and posterior regions. On the midline of the dorsum of the tongue from the apex of the tongue to the center of the body of the tongue is the median lingual sulcus.

There are 4 types of lingual papillae on the dorsum of the tongue in adult rat, similarly to the human tongue: circumvallate papillae, fungiform papillae, foliate papillae, and filiform papillae. In the rat, filiform papillae are further classified into 3 subtypes (simple conical papillae, giant conical papillae, and branched papillae) with different morphologies depending on their location on the dorsum of the tongue. The simple conical papillae and the fungiform papillae are located on the anterior region of the tongue. Cone shaped simple conical papillae possess tongue-like processes at the tip, and the processes are sloped backward toward the direction of the root of the tongue, and are found in dense numbers from the apex of the tongue to the anterior region of the tongue. Low and round fungiform papillae are sporadically observed among simple conical papillae. Giant conical papillae are observed in dense numbers in the limited crescent region on the intermolar eminence. They are bigger than simple conical papillae in both thickness and height, and the tongue-like processes at the tip are slanted forward in the direction of the apex of the tongue. In the posterior region of the tongue, branched papillae, foliate papillae, and circumvallate papillae are well distributed. Branched papillae are observed in dense numbers in the posterior region of the tongue from the back of giant conical papillae to the front of circumvallate papillae, and the tip of tongue-like processes is forked into several branches, and slanted in the direction of the body of the tongue. Five to 6 foliate papillae separated by several vertical furrows are observed at the lingual side of the posterior region of tongue. One oval-shaped circumvallate papilla was observed at the most poste-
rior midline of the posterior region of the tongue, and there were no lingual papillae in the root of the tongue behind the circumvallate papilla (Figs. 1a, b).

**Light microscopical observation of lingual papillae**

Morphology of lingual papillae in adult rat was observed by light microscopy. Lingual papillae generally consisted of epithelial and connective tissues. Epithelial tissues are stratified squamous epithelium with a keratinized layer observed on the utmost surface layer. Connective tissues are abundant with fibroblasts and blood vessels, and the connective tissue core is formed by expansion. Connective tissues just under the apical papillary epithelium form a rolling shape, and secondary papillae are formed at the top of connective tissue core.

**Circumvallate papillae**: Circumvallate papillae are single, large, oval papillae with a longitudinal diameter of approximately 700 μm, and a width of approximately 600 μm. They were surrounded by a U-shaped furrow of approximately 400 μm in depth, which lacks a front portion. Epithelial tissues on the dorsum of the tongue of circumvallate papillae consist of several layers of stratified squamous epithelium. There is a thin keratinized layer on the utmost surface layer, and rolling secondary papillae form at the top of the connective tissue core. There are numerous bell-shaped taste buds in the lateral epithelium facing the depressed furrows, and serous glands are observed in connective tissues at the bottom of the furrows (Figs. 2a-c).

**Fungiform papillae**: Fungiform papillae are sea anemone-like protrusions approximately 150 μm in diameter and 180 μm in height, from the epithelial basal layer with a small depression on the top. There were numerous fungiform papillae in the anterior dorsum region of the tongue. Epithelial tissues were thin, and lack a keratinized layer, and there was one bell-shaped taste bud in the epithelium depressed at the center. Secondary papillae formed at the top of the connective tissue core (Fig. 2d).

**Foliate papillae**: On both sides of the lingual margins of the posterior region of the tongue, we observed foliate papillae which extended between several furrows of depressed epithelium. Of the 5 to 6 foliate papillae on each side, the most posterior was the biggest in size (approximately 200 μm in both width and furrow depth). Epithelial tissues of foliate papillae facing the dorsum of the tongue were made of several layers of stratified squamous epithelium, possessing a thin keratinized layer on the utmost surface, and rolling secondary papillae formed at the top of connective tissue cores. In the lateral epithelium facing the depressed furrows were a few bell-shaped taste buds, and serous glands were observed in the connective tissue just below the furrows (Fig. 2e).

**Filiform papillae**: Three types of epithelial tissues (anterior papillae (AP), posterior papillae (PP), and interpapillary epithelium (IE)) with specific differentiation patterns characterized the 3 types of filiform papillae (simple conical papillae, giant conical papillae, and branched papillae) (Figs. 2f-g). Cone shaped simple conical papillae of the anterior region of the tongue were approximately 100 μm in height and 50 μm in basal diameter, and their tips were bent backward toward the root of the tongue. The anterior part of papillae consisted of a granular layer containing numerous keratohyalin granules in the cytoplasm, which were strongly stained by eosin whereas the keratinized layer was uniformly and lightly stained by eosin. There was no keratohyalin granule in the posterior part of papillae, and layers of squamous cells with small nuclei stained by eosin were observed in the keratinized layer. Numbers of keratohyalin granules with different sizes increased from the middle to upper layer of epithelial tissues of the interpapillary epithelium. The keratinized layer was strongly stained in a rolling manner, and neither nuclei nor keratohyalin granules were observed (Fig. 2f). The shape of the giant conical papillae on the intermolar eminence was similar to that of simple conical papillae, but the former were thicker and taller than the latter, and the tongue-like processes were slanted forward compared to those in the simple conical papillae. In giant conical papillae, a granular layer was observed in the posterior part but not in the anterior part of papillae. The interpapillary epithelium was narrower than the epithelium of simple conical papillae, and fewer keratohyalin granules were present on the upper layer of epithelial tissues (Fig. 2g). Tongue-like processes of branched papillae of the posterior region of the tongue were branched to form several tips which were slanted toward the root of the tongue. Similarly to the simple conical papillae, a granular layer was observed in the anterior part, but not on the posterior part of papillae. The interpapillary epithelium was very narrow and a few small keratohyalin granules were observed (Fig. 2h).

**Morphogenesis of the tongue and lingual papillae, and expression of VEGF**

**Lingual tissue differentiation and development of lingual papillae**

Mandibular processes and lateral lingual swellings were not clearly distinguished at E13, and the epithelial transition was smooth (Fig. 3a). At this stage, flat
Fig. 2. H/E staining of each lingual papilla in adult rat. **a:** Frontal section of the posterior region of circumvallate papilla. Many taste buds (\*\*) exist on the lateral epithelium. **b:** Horizontal section of middle part of circumvallate papilla. **c:** Mid sagittal section of circumvallate papilla. **d:** Sagittal section of fungiform papillae. **e:** Horizontal section of foliate papillae. **f-h:** Sagittal sections of three kinds of filiform papillae. **f:** Simple conical papillae. Apparent three types of epithelial tissues are distinguished. **g:** Giant conical papillae. **h:** Branched papillae. **AP:** Anterior papillae. **IE:** Interpapillary epithelium. **PP:** Posterior papillae. **\*:** Taste bud. (\_\_ = 100 μm)
endothelial cells were observed in the mesenchymal tissue at the bottom of the root of the tongue, whereas aggregates of cells linked together were observed in the subepithelium of the anterior tongue. Oval cells were observed in peripheral aggregates, whereas nucleated round cells were observed in central aggregates (Fig. 3f). The primordium of circumvallate papillae with a slightly depressed lingual surface was observed on the anterior root of the tongue. The epithelium of circumvallate papillae was slightly proliferated, and showed a smooth transition to the surrounding epithelium (Fig. 3a).

At E14, basic morphology of the tongue was established, and the mandible and the tongue were distinguishable (Fig. 3b). The primordium of fungiform papillae with a slight extension at the basement membrane side of the epithelium was observed on the dorsum of the anterior tongue, from the apex to the body of the tongue (Fig. 3g). Subepithelial endothelial cells still showed an immature oval shape, and blood cells were nucleated and undifferentiated.

At E15, mandibular processes were further developed, and Meckel’s cartilage was observed within them. Mesenchymal lingual tissues became spindle or star shaped, and accumulated at the center of the tongue to show a regular myoid arrangement (Fig. 3c). There were no significant changes in thickness of epithelial tissues in the dorsum of the tongue, but epithelial cells had proliferated even on the surface layer, and were not in contact with the basement membrane. The epithelium of circumvallate papillae was still slightly depressed, and had proliferated giving rise to 5 to 6 layers (Fig. 3h). Flat endothelial cells were observed subepithelially, but blood cells were still nucleated and undifferentiated.

At E16, the nasal and oral cavities were separated by lateral palatine processes, and the tongue moved down. Differentiation of mesenchymal tissues into the transverse lingual muscle, superior longitudinal lingual muscle, and vertical lingual muscle was observed, and specific arrangements of muscle tissues became notable. Morphology of the tongue resembled that of the adult rat, and the intermolar eminence was observed (Fig. 3d). Epithelial tissues depressed under connective tissues at the lingual margin of the posterior region of the tongue, and the primordium of foliate papillae could be seen (Fig. 3i). Subepithelial endothelial cells had proliferated, differentiation of blood cells was advanced, and numerous denucleated blood cells were observed.

At E17, epithelial tissues, muscle tissues, and vascular systems were further differentiated, and their morphologies resembled those in adult rat (Fig. 3e). Numerous primordia of filiform papillae were observed on the basement membrane of epithelial tissues which had proliferated to reach 4 to 5 layers on the dorsum of the tongue from the apex of the tongue to the body of the tongue (Fig. 3j). All 4 types of lingual papillae were observed after development of filiform papillae, but epithelial keratinization characteristic of lingual papillae or taste buds was still not observed. Mesenchymal cells accumulated around blood vessels running between muscle tissues, and were ready to differentiate into mural cells.

From E18 to E19, epithelial tissues of the dorsum of the tongue rapidly grew to form 5 to 6 layers. Smooth muscles surrounding blood vessels running between muscle tissues, and differentiation of mural cells were observed. At E20, epithelial tissues on the dorsum of the tongue were keratinized, and blood vessels surrounding muscle tissues matured with an expanding vascular lumen. At E21, morphology of the tongue mostly resembled that in adult rat.

**Morphogenesis of each lingual papilla: expression of VEGF and angiogenesis**

**Circumvallate papillae:** Following development of the primordium of circumvallate papilla with a slightly depressed epithelium at E13, epithelial tissues thickened further at E15 (Figs. 3a, h). At E16, epithelial tissues at the posterior part of circumvallate papilla were further depressed in connective tissues, and the connective tissue core at the anterior part of circumvallate papilla became thickened, and expanded. Fibroblast proliferation was observed on the expanding connective tissue core, and VEGF was widely expressed on the thickened epithelium and expanded connective tissue core (Figs. 4a, b). There was no capillary sprout invading the expanding connective tissue core at this stage (Fig. 4c). At E17, the connective tissue core was extended at the anterior part of circumvallate papilla, capillary sprouts invaded into the connective tissue core (Figs. 4d, e). From E19 to E21, furrows made by depressed epithelium became more evident, and morphology of circumvallate papilla was similar to that in adult rat. At P1, taste buds were confirmed in the lateral epithelial tissues of the furrows, and blood vessels developed (Figs. 4f, g). At P7, differentiation of circumvallate papillary epithelium had progressed, with increasing number and size of taste buds observed in the lateral epithelium of furrows, and blood vessels that formed papilla were further grown (Figs. 4h, i).

**Fungiform papillae:** Following development of the
Fig. 3. Morphogenesis of the tongue and lingual papillae at E13, E14, E15, E16, and E17. a-e: Sagittal section through oral cavity at each stage. a-e: Magnified views of a-c (□). a: Tongue and mandibular processes are not clearly distinguished. Primordium of circumvallate papilla (▼) was observed. b: Tongue (T) and mandibular process (M) are distinguishable. c: Primordium of fungiform papillae ( ○ ) on the dorsum of the tongue at E14. d: Meckel’s cartilage ( △ ) was observed within developed mandibular processes, and a regular myoid arrangement ( △ ) was developed in the tongue. e: Epithelium of circumvallate papilla proliferated. d: Intermolar eminence ( ▼ ) is observed in developed tongue. An additional right upper photomicrograph is horizontal section of rat head. f: Side margin of the posterior region of the tongue. Primordiums of foliate papillae ( ▼ ) are distinguished. e: Well developed tongue like as in adult. j: Anterior part of the dorsum of the tongue at E17. Some primordiums of filiform papillae (▽) are observed. (  = 30 μm,  = 300 μm).
Fig. 4. Photomicrographs of circumvallate papilla at E16, E17, P1, and P7. a-c: Sagittal sections of circumvallate papilla at E16. a: Primordium of circumvallate papilla is shown as ○. H/E staining. b: VEGF immunoreactivity was widely expressed on the thickened epithelium and expanded connective tissue core. c: No capillary sprouts observe in the expanding connective tissue core. India ink injection. d and e: Sagittal sections of circumvallate papilla at E17. d: Circumvallate papilla slightly grows. H/E staining. e: Capillary sprouts invade into the connective tissue core. India ink injection. f and g: Frontal sections of circumvallate papilla at P1. f: Taste buds (▼) are differentiated in the lateral epithelial tissues of the circumvallate papilla. H/E staining. g: India ink injection. h and i: Sagittal sections of circumvallate papilla at P7. h: Circumvallate papilla is developed like an adult. H/E staining. i: Well developed blood vessels observe. India ink injection. ( — = 100 μm)
Fig. 5. Photomicrographs of sagittal sections of fungiform papillae at E17, E18 and P1. a and b: Anterior region of the tongue at E17. a: Lingual muscle are differentiated. H/E staining. b: Blood vessels between muscle tissues develop. India ink injection. c-e: Fungiform papillae at E17. c: Primordium of fungiform papilla is shown as ○. H/E staining. d: VEGF immunoreactivities are demonstrated around the basal cells of proliferated epithelial tissues. e: No capillary sprouts observe in the connective tissue core of fungiform papillae primordium. India ink injection. f and g: Fungiform papilla at E18. f: Fungiform papilla is growing. H/E staining. g: Blind-ended capillary sprout invades in the connective tissue core. India ink injection. h and i: Fungiform papilla at P1. h: Fungiform papilla form like an adult. A taste bud (▼) is differentiated at the top of the fungiform papilla. H/E staining. i: Capillary sprout grows to reveal a loop shape. India ink injection. ( = 20 μm,  = 200 μm)
Fig. 6. Photomicrographs of horizontal sections of foliate papillae at E17 (a-c), E18 (d-f), E20 (g and h), P1 (i and j), and P7 (k and l). a: Slightly developed primordiums of foliate papillae. H/E staining. b: No apparent VEGF immunoreactivities. c: Linear and thin blood vessels are observed. India ink injection. d: Proper mucous membrane of foliate papillae become thicker. H/E staining. e: VEGF immunoreactivity appear in the vicinity of the epithelial basement membrane. f: Blood vessels protrude toward the epithelium in a loop shape. India ink injection. g: Epithelium and secondary papillae are developed. H/E staining. h: Blood vessels are widely observed. India ink injection. i: Taste buds (*) were observed in the lateral epithelium. H/E staining. j: Blood vessels increase density in the connective tissue core. India ink injection. k: Folate papillae forms like an adult. H/E staining. l: Well developed blood vessels in the foliate papillae. India ink injection. ( = 30 μm,  = 150 μm)
Fig. 7. Photomicrographs of sagittal sections of filiform papillae at E19 (a-c), E20 (d and e), P1 (f and g), and P7 (h and i). a: Slightly developed primordiums of filiform papillae. H/E staining. b: VEGF Immunoreactivities observe in the epithelial layer. c: No capillary sprouts invade into connective tissue cores. India ink injection. d: Slightly differentiated filiform papillae. A keratinized layer stained with eosin differentiates on the surface of epithelial layer. H/E staining. e: Blind-ended capillary sprouts are observed. India ink injection. f: Three types of epithelia anterior papillae (AP), posterior papillae (PP), and interpapillary epithelium (IE) are differentiated. H/E staining. g: Capillary sprouts become loop shaped. India ink injection. h: Well developed filiform papillae. H/E staining. i: Well developed blood vessels in the filiform papillae. India ink injection. ( = 30 μm, = 300 μm)
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Fig. 8. Lingual development, in particular, angiogenesis and expression of VEGF from E13 to P7.
primordium of fungiform papillae at E14 (Fig. 3g), a dome shaped expansion became pronounced at E17. At this stage, each lingual muscle was differentiated, and a specific arrangement with regards to lingual muscles became evident. Blood vessels between muscle tissues developed rapidly, and we observed dense sinusoidal capillaries in the subepithelium (Figs. 5a, b). A distinct dome shaped expansion of fungiform papillae was seen with epithelial proliferation and expansion of the connective tissue core (Fig. 5c). VEGF was expressed around the basal cells of proliferated epithelial tissues (Fig. 5d). Capillary sprouts that had invaded into the connective tissue core from the subepithelial sinusoidal capillaries were not observed at E17 (Fig. 5e). At E18, fungiform papillae were observed as bigger dome shaped eminences, and invasion by blind-ended capillary sprouts and protrusion of the connective tissue core were observed in fungiform papillae (Figs. 5f, g). From E19 to E21, fungiform papillae were observed as low, flat, dome-shaped eminences scattered among filiform papillae, while one taste bud was confirmed at the center of the epithelium facing the dorsum of the tongue at P1, and the capillary sprouts of fungiform papillae grew to reveal a loop shape (Figs. 5h, i).

Foliate papillae: Following development of the primordium at E16 (Fig. 3i), epithelial tissues on upper papillae became thin with 1 to 2 layers at E17, and epithelial depressions of 3 to 4 layers were observed in connective tissues. However, localization of VEGF immunoreactivity was not obvious at this stage (Figs. 6a, b). The proper mucous membrane between the epithelium and muscle layers was thin, with 7 to 8 layers, but linear and thin blood vessels were observed in a horizontal direction (Fig. 6c). At E18, the basal epithelium was further depressed, and an epithelial cell layer of 5 to 6 layers was observed. The proper mucous membrane which was equivalent to connective tissues also became thicker. VEGF localization was not evident, and it was expressed weakly and uniformly on the epithelial cell layer, muscle cells, and fibroblasts. Strong VEGF expression was observed only in the vicinity of the epithelial basement membrane (Figs. 6d, e). Blood vessels of the proper mucous membrane protruded toward the epithelium in a loop shape (Fig. 6f). At E20, epithelial cells of foliate papillae had grown, M-shaped secondary papillae had developed on the basal layer, and a thin keratinized layer had developed on the utmost surface of the epithelium. Epithelial tissues were further depressed toward connective tissues (Fig. 6g). Blood vessels were widely observed in the proper mucous membrane from the subepithelium to the muscle layer (Fig. 6h). At P1, serous gland cells densely stained by hematoxylin were observed in connective tissues under the bottom furrows. Taste buds were observed in the lateral epithelium of furrows, and blood vessels observed in the connective tissue core had developed with increasing density (Figs. 6i, j). At P7, differentiation of the papillary epithelium consisting of foliate papillae was advanced, and numerous taste buds in the lateral epithelium and enlarged vascular lumens in the connective tissue core were observed (Figs. 6k, l).

Filiform papillae: After development of the primordium at E17 (Fig. 3j) on filiform papillae, the connective tissue core of filiform papillae was clearly observed with a rapid thickening of epithelial tissues from E18 to E19 (Fig. 7a). VEGF was expressed in the cytoplasm of growing epithelial cells at E19, but capillary sprouts invaded into connective tissue cores were not observed (Figs. 7b, c). At E20, a keratinized layer uniformly stained with eosin was observed on the surface layer of epithelial tissues. Although differentiation specific to epithelial tissues of filiform papillae was not observed yet, a cell layer with keratohyalin granules was observed under the keratinized layer, and mitotic epithelial cells were observed only in the basal layer (Fig. 7d). Invasion by blind-ended capillary sprouts was observed from the subepithelial sinusoidal capillaries into the connective tissue core of filiform papillae (Fig. 7e). At P1, epithelial differentiation to the three types of epithelia (anterior papillae, posterior papillae, and interpapillary epithelium) was observed in epithelial tissues of filiform papillae, and the basic morphology of filiform papillae was completed (Fig. 7f). With epithelial differentiation of filiform papillae, capillary sprouts of the connective tissue core became loop shaped (Fig. 7g). At P7, epithelial tissues had further matured and thickened, and the interpapillary epithelium between filiform papillae was expanded. With maturation of muscle and fat tissues, blood vessels running between connective tissues became denser, and the vascular lumen was more expanded (Figs. 7h, i).

Morphogenesis of the rat tongue, development of each lingual papillae, expression of VEGF and angiogenesis in lingual papillae are summarized to Fig. 8.

DISCUSSION

This is the first paper reporting angiogenesis and expression of VEGF during morphogenesis of lingual papillae in rats. As previously reported, with respect to morphology of each papilla in adult rat, circumvallate
papillae and foliate papillae contain taste buds in the lateral papillary epithelium, and serous glands are observed at the bottom of furrows. Fungiform papillae are dome shaped eminences having one taste bud in the dorsum of the tongue [7,28,29]. It was reported that there were 3 types of filiform papillae in adult rat depending on the region of the dorsum of the tongue, and epithelial tissues showed 3 types of differentiation and keratinized layers having different characteristics [1,5,8]. The same morphology of papillae in the adult rat reported by previous authors was observed in our study.

**Morphogenesis of the rat tongue and lingual papillae**

Morphogenesis of the rat tongue and lingual papillae are summarized to Fig. 8. The primordium of the rat tongue develops at E13 by fusion of lateral lingual swellings and hypobranchial eminence [28,29]. In our study, we observed the lingual primordium at E13, the mandible and tongue were clearly distinguished at E14, and basic morphology of the tongue was confirmed. Theories on development of lingual papillae slightly differ depending on observers, but it is accepted that the primordium of circumvallate papillae is the first stage in lingual development, followed by developments of the primordium of fungiform papillae, foliate papillae, and filiform papillae, respectively [4,5,30,31]. The primordium of circumvallate papillae was also initially observed in our study at E13 together with development of the lingual primordium, followed by developments of the primordium of fungiform papillae, foliate papillae, and filiform papillae at E14, E16, and E17, respectively. Development of lingual papillae was observed as local changes in epithelial tissues on the basement membrane. In other words, primordia of circumvallate papillae and foliate papillae were confirmed by local proliferation of epithelial tissues, whereas the primordia of fungiform papillae and filiform papillae were confirmed by meandering of epithelial tissues on the basement membrane. Considering that Sonic hedgehog is closely involved in morphogenesis of lingual papillae, and from data of E13 to E16 [36], the vascular system is completed when differentiation of mural cells is observed (vascular myogenesis) [13,14,20]. With regards to the embryonic vascular system in rat, endothelial cells form the lumen of the dorsal aorta which develops at an earliest stage at E12, and mesenchymal cells differentiate to mural cells from E13 to E16 [36]. For angiogenesis of lingual papillae, it is reported that blind-ended neovascularity occurs at E20 in filiform papillae, at which time epithelial tissues are specifically differentiated, and a loop shaped vascular network is observed at P1 [11].

In this study, aggregates of cells suggested to correspond to the blood island were observed subepithelially on the tongue primordium at E13. These aggregates consisted of peripheral cells, suggestive of oval angioblasts and hemopoietic cells in the center of the aggregates, as seen in a blood island. Subepithelial endothelial cells became flat throughout development of keratinized layer of each papilla was also observed at E20 in our study, and development of taste buds was confirmed on circumvallate papillae, fungiform papillae, and foliate papillae at P1. At this stage, differentiation of the 3 specific epithelial tissues were observed in filiform papillae, and basic morphology of each papilla was confirmed. Observations in this study suggested that specific morphology of each papilla accompanied by development of a taste bud takes place relatively late from E20 to P1. Timing of development of human taste buds greatly varies from fetal day 8 to week 28 depending on different studies, due to difficulties in collecting samples [33,34], but differences with the rat lie in the fact that human taste buds develop relatively early during the fetal period.

**Tissue differentiation and angiogenesis of the tongue epithelium**

Expression of VEGF and angiogenesis in lingual papillae are summarized to Fig. 8. Dense sinusoidal capillaries develop subepithelially on the dorsum of the tongue from the lingual artery that runs deep in the lingual muscle in the tongue of adult rat, and a loop shaped vascular network extends from sinusoidal capillaries to the lingual papillae to feed lingual papillary epithelial tissues [2,3,10,35]. It is known that blood vessels develop by either vasculogenesis or angiogenesis. In vasculogenesis, the primary capillary plexus is considered to develop from a blood island, inducing hemangioblasts in the mesoderm [12,15]. On the contrary, angiogenesis involves sprouting and intussusception, and blood vessels develop by repeating fusion, branching, and pruning in response to organ morphogenesis (vascular remodeling). The mature vascular system is completed when differentiation of mural cells is observed (vascular myogenesis) [13,14,20]. With regards to the embryonic vascular system in rat, endothelial cells form the lumen of the dorsal aorta which develops at an earliest stage at E12, and mesenchymal cells differentiate to mural cells from E13 to E16 [36]. For angiogenesis of lingual papillae, it is reported that blind-ended neovascularity occurs at E20 in filiform papillae, at which time epithelial tissues are specifically differentiated, and a loop shaped vascular network is observed at P1 [11].

In this study, aggregates of cells suggested to correspond to the blood island were observed subepithelially on the tongue primordium at E13. These aggregates consisted of peripheral cells, suggestive of oval angioblasts and hemopoietic cells in the center of the aggregates, as seen in a blood island. Subepithelial endothelial cells became flat throughout development of
Angiogenesis was not observed in lingual papillae at early stages of development, and this may be because oxygen and nutritional requirements of tissues were fully satisfied by diffusion from subepithelial sinusoidal capillaries when the epithelial tissue was undifferentiated and expansion of the connective tissue core was slow. However, in circumvallate papillae, fungiform papillae, and filiform papillae in which thickening of epithelial tissues and expansion of the connective tissue core became prominent, VEGF was expressed around the fibroblasts of the expanded connective tissue core and basement membranes of epithelial tissues, and angiogenesis was confirmed on the following days. Blind-ended neovascularity of sprouting was observed especially in fungiform papillae and filiform papillae. It is considered that acceleration of angiogenesis by VEGF is associated with increased requirements of oxygen and nutrients by morphogenesis of lingual papillae. In foliate papillae which develop by epithelial depression, VEGF was observed in small amounts around basement membranes of epithelial tissues, and angiogenesis of intussusception was observed where the existing thin, linear blood vessels became dense in a plexus state by proliferation. Our observations showed that VEGF was locally expressed in lingual papillae prior to angiogenesis, suggesting a close involvement of VEGF in angiogenesis of lingual papillae. When timings of angiogenesis of blood vessels and vascular network were compared between the 4 types of lingual papillae, absolute amount of the connective tissue core was minimal in filiform papillae lacking taste buds, and angiogenesis into the connective tissue papillae was also delayed. Therefore, oxygen and nutritional requirements are considered to be relatively lower to other lingual papillae containing taste buds. The loop shaped vascular network is established in each subepithelial papilla by birth, and timing is very close to differentiation of the primordium of taste buds and epithelial tissues of filiform papillae, suggesting a close relationship between higher differentiation of epithelial tissues and development of the vascular system.

Differentiation of taste buds was reported by several researchers, and a close relationship between development of taste buds and that of the nervous system has been demonstrated [37,38]. During early developmental stages, invasion of nerve fibers into circumvallate papillae and fungiform papillae was particularly reported at E16 [29,30,32]. Although an integrated report on vascular and nervous systems related to morphogenesis of lingual papillae has not been published yet, our study suggested that subpapillary invasion took place at an earlier age in the nervous system compared to the vascular system.

We reported that VEGF expression was closely involved in morphogenesis of lingual papillae and angiogenesis of lingual papillae, and a close relationship existed between higher differentiation of lingual papillary epithelial tissues and development of the respective vascular system.

ACKNOWLEDGMENTS: We would like to thank the late professor Mitsuaki Yoshizuka, our mentor, for his kind and unlimited guidance.

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Kurume Medical Journal Vol. 54, No. 1, 2, 2007


