Expression of Vascular Endothelial Growth Factor (VEGF) and Specific Receptors (Flt-1 and Flk-1) in Rat Tongue Carcinogenesis Induced by 4-Nitroquinoline 1-Oxide

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Tongue carcinoma is one of the most common malignant neoplasms in the oral cavity. To assess the role for angiogenesis in carcinogenesis of tongue carcinoma, we performed qualitative and quantitative analyses of vascular endothelial growth factor (VEGF) and two forms of the specific receptors (VEGFRs), Flt-1 and Flk-1, in rat tongue squamous cell carcinoma induced by 4-nitroquinoline 1-oxide. In situ hybridization and immunohistochemistry revealed the colocalization of VEGF and VEGFRs mRNA signals and protein immunoreactivities, respectively in the cytoplasm of the normal epithelial cells, dysplastic cells, cancer cells, and vascular endothelial cells. Both the labeling indices of VEGF and VEGFRs in immunostained sections and the immunoreactive intensities on immunoblots for VEGF and VEGFRs disclosed a duration-dependent increase along with disease progression. An increase in the VEGF labeling index positively correlated with increases in the Ki-67 labeling index and blood vessel density. The present results suggest that angiogenesis is necessary for carcinogenesis, and that VEGF has relevance to the pathomechanism of tongue carcinoma via paracrine and autocrine mechanisms.

Key words: Angiogenesis, Vascular endothelial growth factor (VEGF), VEGF receptors (VEGFRs), 4-Nitroquinoline 1-oxide (4NQO), Rat tongue carcinoma

I. Introduction

Recent investigations have suggested that the growth of solid tumors depends on angiogenesis [12]. When angiogenesis is completely blocked, tumors cannot grow beyond 1–2 mm³ in size. Angiogenesis also takes place in conditions such as wound healing, rheumatoid arthritis, diabetic retinopathy, and tumor metastasis [1, 38]. There is a symbiotic relation between cancer cells and surrounding vascular endothelial cells by the production and secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor, hepatocyte growth factor, Thymidine phosphorylase, interleukin-8 and other angiogenic factors [11, 13, 22, 30, 35, 37]. VEGF, a potent inducer of angiogenesis [5, 8], is one of the most powerful mitogens for vascular endothelial cells, and is expressed in a great variety of solid tumors including cancers of the breast, kidney, ovary, and colon, and brain tumors [7]. VEGF has also been known as a vascular permeability factor [21]. Of the VEGF families (VEGF, VEGF-B, VEGF-C, VEGF-D and VEGF-E), VEGF has been characterized most intensively. Several VEGF isoforms such as VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆ have been identified. VEGF increases the permeability of microvessels to circulating macromolecules with a potency approximately 50,000-fold as much as histamine [32], and this property is likely to account for the well-documented hyperpermeability of tumor blood vessels. It has been shown that VEGF binds to specific receptors (VEGFRs) on the surface of the vascular endothelial cells. There are two high-affinity VEGFRs containing the domain of tyrosine kinases: fms-like tyrosine kinase-1 (Flt-1) [36]
and fetal liver kinase-1 (Flk-1) [16].

Many studies have suggested the significance of the interaction of VEGF with VEGFRs in tumor angiogenesis and tumor progression. However, the localization of mRNAs and proteins of VEGF and VEGFRs is controversial. Some studies showed that VEGF mRNA and protein were localized in the cancer cells, and that VEGFRs mRNAs and proteins were localized in the vascular endothelial cells [2, 30]. Others showed that VEGF and VEGFRs proteins were localized in both the cancer cells and the vascular endothelial cells [34, 41]. Since the roles for VEGF and VEGFRs can change in different stages of carcinogenesis, it is important to identify both the expression levels and histological localization of these substances throughout the course of cancer disease. Thus, examination of an experimental cancer model is a beneficial approach for obtaining chronological data in carcinogenesis.

4-Nitroquinoline 1-oxide (4NQO) is a toxic, potent carcinogenic agent for the rat tongue [28]. Oral administration of 4NQO in rats induces tongue carcinoma, and is a useful model for squamous cell carcinoma of the human tongue. In the present study, we examined chronologically the 4NQO-induced rat tongue carcinoma in several stages, to demonstrate the localization of VEGF and VEGFRs mRNAs by in situ hybridization, the localization of VEGF and VEGFRs proteins by immunohistochemistry, and the expression levels of VEGF and VEGFRs proteins by immunoblotting. Moreover, to clarify the relation between proliferation activities of cancer cells and vascular endothelial cells, we verified and compared labeling indices of VEGF and Ki-67 with blood vessel density. This is the first report showing the qualitative and quantitative analyses of mRNAs and proteins of VEGF and VEGFRs in the processes of carcinogenesis in rat tongue carcinoma induced by 4NQO.

II. Materials and Methods

Animals and tissue preparation

A total of 48 male Spraque-Dawley rats aged seven weeks old, which weighed 180 to 270 g, were purchased from Charles River Japan Inc. (Yokohama, Japan), and were housed in a well-ventilated room under controlled illumination (12 hr light/12 hr dark), temperature (22 to 23°C), and humidity (40 to 60%). Treatment with 4NQO (Nakara Tesque, Kyoto, Japan) was performed as an established method described previously [19]. In brief, 40 animals were divided into five groups with 4NQO treatment for 4, 8, 12, 16 and 20 weeks; each group was composed of eight animals. The remaining eight animals were used as untreated controls (0 week). These rats were anesthetized under ethyl alcohol, and sacrificed at the end of 0 (controls), 4, 8, 12, 16 and 20 weeks after starting of 4NQO treatment. The rat tongues in each group were grossly observed, and subsequently removed. All animal experiments were performed in accordance with the principles of laboratory animal care, and approved by the Institutional Animal Care and Use Committee of Tokyo Women’s Medical University, Tokyo, Japan. Of the eight rat tongues of each group, five were fixed in 20% buffered formalin (pH 7.6), embedded in paraffin, and used for conventional hematoxylin-eosin (H&E) staining, in situ hybridization and immunohistochemistry. The remaining rat tongues were frozen, and stored at −80°C for Western blotting.

cDNA probes

Total RNA was extracted from a normal rat kidney by the acid guanidinium thiocyanate-phenol-chloroform method [4]. For reverse transcription-polymerase chain reaction (RT-PCR), the reaction mixture containing 10 µg of the total RNA, 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 0.5 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 20 U of RNase inhibitor, 100 pM random hexamer (TaKaRa, Kyoto, Japan) and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Gaithersburg, MD, USA) in a final volume of 20 µl were incubated for 60 min at 37°C followed by heating for 5 min at 94°C to terminate the reaction, and the obtained cDNA samples were then stored at −20°C.

Oligonucleotide primer sets for the published cDNA sequences of rat VEGF, Flt-1 and Flk-1 were constructed by TaKaRa. The sequences of the rat VEGF primers were 5'-CACGACAGAAGGGGAGCAGAA-3' corresponding to bases 2198–2220 for sense nucleotide, and 5'-TGGCATCTTGAGAACAATAT-3' corresponding to bases 2778–2800 for antisense nucleotide [5]. This cDNA probe recognizes the 121, 145, 165, 189 and 206 amino acid splice variants of VEGF. The sequences of the rat Flt-1 primers were 5'-TACAGCACCAGTAGTACGTG-3' corresponding to bases 3462–3483 for sense nucleotide, and 5'-TCTCTCATCCTCTGCGCCACAG-3' corresponding to bases 456–477 for antisense nucleotide [44]. The sequences of the rat Flk-1 primers were 5'-CCTAGTCAAGCAGCCTCGT-3' corresponding to bases 98–118 for sense nucleotide, and 5'-CCAAGCCTCTGCCCACC-3' corresponding to bases 456–477 for antisense nucleotide [39]. The predicted sizes of RT-PCR amplification products of the rat VEGF, Flt-1 and Flk-1 were 369, 700 and 602 bp, respectively.

cDNA samples were diluted 1:5 in RNase-free water, and transferred to fresh tubes. For preparation of digoxigenin-labeled cDNA probes, an amplification mixture containing aliquots of 5 µl of cDNA, 200 µM sense and antisense primers, 200 mM dNTP (dATP, dCTP, dGTP and dTTP), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 10 mM MgCl₂, 2.5 U of Taq DNA polymerase (TaKaRa), and digoxigenin (Roche Diagnostics Corporation, Indianapolis, IN, USA) in a final volume of 100 µl was processed for 35 cycles in a Zymoreactor thermo-cycler (ATTO, Tokyo, Japan). The amplification profiles consisted of denaturation for 1.5 min at 94°C, annealing for 1.5 min at 56°C for Flt-1 or at 58°C for VEGF and Flk-1, and extension for 1 min at 72°C. Sequences of the RT-PCR products were verified using an ABI 377 autosequencer (Perkin Elmer Applied Biosystems,
In situ hybridization

The paraffin-embedded materials of the examined tongues were cut into multiple 4 μm-thick sections. In situ hybridization was performed as described previously [20]. In brief, sections were deparaffinized, rehydrated, and incubated for 16 hr at 42°C with hybridization mixture composed of 5 x standard saline citrate (SSC: 150 mM sodium chloride/15 mM sodium citrate), 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 10.5 mg/ml sonicated salmon sperm DNA, 10% dextran sulfate, and 1 μg/ml digoxigenin-labeled probes for VEGF, Flt-1 or Flk-1 mRNA. The hybridization signals were detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody (diluted 1:500; Roche) according to the manufacturer’s instructions. Nitroblue tetrazolium was used as the chromogen, and methyl green was used as the counterstain. Sections incubated with an excess amount of unlabeled cDNA served as negative reaction controls. The localization of mRNA signals were verified by comparison with histopathological findings in consecutive H&E-stained sections.

Immunohistochemistry

Multiple 4 μm-thick sections of the paraffin-embedded tongues were examined for immunohistochemistry. The primary antibodies utilized in immunohistochemistry were rabbit polyclonal antibodies specific for VEGF, Flt-1 and Flk-1 (diluted 1:1,000, 1:1,000 and 1:1,500, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal IgG anti-Ki-67 antibody (MIB-5; diluted 1:200; Immunotech, Marseille, France), and a horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibody to Factor VIII-related antigen (ready-to-use; Dako, Kyoto, Japan). The anti-VEGF antibody recognizes the 121, 165 and 189 amino acid splice variants. Ki-67 was used as a marker for cell proliferation activity, and Factor VIII-related antigen was used as a marker for endothelial cells. Prior to immunostaining for VEGF, Flt-1 and Flk-1, sections were processed for microwaving for 23 min in 10 mM citrate buffer (pH 6.0). Sections were deparaffinized, rehydrated, quenched for 20 min at 4°C with 3% hydrogen peroxide for inhibiting endogenous peroxidase activity, rinsed in 150 mM phosphate-buffered saline (pH 7.6), pretreated with nonimmune animal serum for blocking nonspecific antibody binding, and then incubated overnight at 4°C with the primary antibodies. Antibody binding for Factor VIII-related antigen was detected by the direct immunoperoxidase method. Immunoreaction product deposits with the other primary antibodies were visualized by the labeled streptavidin-biotin (LSAB) immunoperoxidase method using an LSAB kit (Dako). 3,3'-Diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan) solution was used as the chromogen, and hematoxylin was used as the counterstain. Sections from which the primary antibodies were omitted served as negative reaction controls. The localization of immunoreactivities for VEGF, Flt-1, Flk-1 and Ki-67 was identified by comparison with findings in serial sections stained for H&E, and Factor VIII-related antigen.

Evaluation of labeling indices for VEGF, VEGFRs and Ki-67 and blood vessel density

The labeling indices for VEGF, Flt-1, Flk-1 and Ki-67 were evaluated...

Table 1. Histopathological changes of examined rat tongues

<table>
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<tr>
<th>Histopathological features</th>
<th>0 (control)</th>
<th>4</th>
<th>8</th>
<th>12</th>
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<td>1</td>
<td>0</td>
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<td>4</td>
<td>5</td>
<td>3</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>2</td>
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* Weeks after starting the 4NQO treatment

Fig. 1. Gross findings of rat tongues. The control tongues showed the normal “torus linguae” structure (arrow) on the oral surface of the posterior portion (A). After 4NQO treatment for 12 weeks, the tongues showed the rough appearance restricted to the torus linguae (arrow) (B). After 4NQO treatment for 20 weeks, the tongues showed the flat-elevated (C, D), nodular (E) or ulcerative (F) changes (arrows) located at the posterior portion including the torus linguae.
proteins were determined by calculating the percentage of immunoreactive cells in more than 500 epithelial cells in five high power fields (0.196 mm², each) at 400× magnification. Blood vessels were identified by the presence of Factor VIII-related antigen immunoreactivity in the endothelial cells or the presence of erythrocytes in the lumens in the stroma adjacent to the tongue epithelium and cancer nest. Blood vessel density was defined by counting the blood vessels using the ‘hot spots method’ as described previously [39]. Briefly, sections immunostained for Factor VIII-related antigen were screened at 40× magnification with a light microscope. Blood vessels, with diameters less than 30 μm, were counted in five high power fields (0.785 mm², each) at 200× magnification. Blood vessel density was defined as the average number of blood vessels per area of one mm².

**Western blotting**

The primary antibodies utilized in Western blotting were a mouse monoclonal IgG antibody specific for VEGF, and rabbit polyclonal antibodies specific for Flt-1 and Flk-1; all of these antibodies were purchased from Santa Cruz Biotechnology, and used at a working dilution of 1:2,000. Total protein was extracted from the examined rat tongues using radioimmunoprecipitation assay (RIPA) buffer composed of 20 mM Tris-HCl buffer (pH 7.4), 150 mM sodium chloride, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (0.1 M phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). The protein concentra-
tion was measured by the Lowry method [25]. Aliquots of 100 μg of protein were electrophoresed in an SDS-polyacrylamide gel (10% for VEGF; 7.5% for Flt-1 and Flk-1), and transferred onto a polyvinylidene fluoride (PVDF) membrane (Clear Blot P membrane; ATTO). The blots were rinsed in 10 mM Tris-HCl buffer (pH 8.3) containing 0.1% Triton X-100, pretreated overnight at 4°C nonimmune animal serum, and incubated for 2 hr at room temperature with the primary antibodies, followed by reaction with the appropriate secondary antibodies conjugated with HRP (diluted 1:2,000; Dako) for 1 hr at room temperature. Immunoreaction product deposits were visualized with the chemiluminescence method using the ECL-detection-kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Fig. 3. Photomicrographs of rat tongue sections processed with in situ hybridization for VEGF (A, D, G, J), Flt-1 (B, E, H, I) and Flk-1 (C, F, I, L) mRNAs. In the control rats, signals of these mRNAs were localized in the normal epithelial cells and vascular endothelial cells (A–C). In the 4NQO-treated rats for 12 weeks, these mRNA signals were localized in the dysplastic epithelial cells and adjacent vascular endothelial cells (D–F). In the 4NQO-treated rats for 20 weeks, the signals were localized in carcinoma cells (asterisks) and adjacent vascular endothelial cells (arrows) (G–L). Magnification: ×100 (A–F); ×200 (G–I); ×400 (J–L).
Immunoreactive densities for VEGF, Flt-1 and Flk-1 were quantified using NIH Image Software.

**Statistical analysis**

The relation between two of the VEGF labeling indices, the Ki-67 labeling index and blood vessel density was statistically evaluated by the Spearman’s rank correlation test.
III. Results

Gross and histopathological observations and immunohistochemical localization of Ki-67 and Factor VIII-related antigen

The results of gross and histopathological examination and immunohistochemical staining of Ki-67 and Factor VIII-related antigen in the rat tongues are summarized in Table 1 and shown in Figs. 1 and 2. Before starting the 4NQO treatment, the tongues showed grossly the normal “torus linguae” structure on the oral surface of the posterior portion (Fig. 1A), and the squamous epithelium consisted histopathologically of regularly arranged keratinized, granular, spinous cell and basal cell layers (Fig. 2A). The Ki-67 immunoreactivity was localized in the nucleus of some of the basal cells (Fig. 2B), whereas Factor VIII-related antigen immunoreactivity was localized in the cytoplasm of almost all of the vascular endothelial cells (Fig. 2C). After 4NQO treatment for four weeks, the epithelium lining displayed hyperkeratosis, acanthosis, papillomatosis, and dysplasia. In the present study, dysplasia was defined by the proliferation of hyperchromatic basaloid cells in the epithelium without stromal invasion. After 4NQO treatment for eight weeks, dysplasia occurred mainly in hyperkeratotic areas of the epithelium. After 4NQO treatment for 12 weeks, the tongues demonstrated grossly the rough appearance restricted to the torus linguae (Fig. 1B), and histopathologically the dysplasia in most areas of the epithelium (Fig. 2D). Ki-67 immunoreactivity was seen in hyperchromatic cells and basal cells of the dysplastic epithelium (Fig. 2E). The vascular endothelial cells increased in number, and appeared in the upper layer of the subepithelial areas adjacent to the dysplastic epithelium (Fig. 2F). After 4NQO treatment for 16 weeks, the majority of animals exhibited dysplasia and initial invasive squamous cell carcinoma of the tongues. After 4NQO treatment for 20 weeks, the tongues showed grossly the flat-elevated (Fig. 1C, D), nodular (Fig. 1E) or ulcerative (Fig. 1F) changes located at the posterior portion including the torus linguae, and histopathologically the invasive well-differentiated squamous cell carcinoma, which occupied the dysplastic areas. The tumor formed various-sized cancer nests having cancer pearls, and invaded the submucosal and muscular layers (Fig. 2G), but did not show any metastases to distant organs or lymph nodes, as described previously [6, 14]. Ki-67-immunoreactive cells were distributed at the margins of the cancer nests (Fig. 2H). Various-sized blood vessels collected around the cancer nests, especially in spreading zone, and lumens of some of the immature vessels were lined by the vascular endothelial cells that were identified by the presence of
Factor VIII-related antigen (Fig. 2I).

**Localization of VEGF and VEGFRs mRNAs**

The results of in situ hybridization for VEGF and VEGFRs mRNAs in the rat tongues are shown in Fig. 3. In the normal epithelium, mRNA signals for VEGF and VEGFRs were expressed in the cytoplasm of all of the basal cells and spinous cells, and vascular endothelial cells (Fig. 3A–C). In the dysplastic epithelium, the VEGF and VEGFRs mRNA signals were localized in the cytoplasm of all of the epithelial cells and vascular endothelial cells (Fig. 3D–F). In the well-differentiated squamous cell carcinoma, the mRNA signals were detected in the cytoplasm of cancer cells at the margins of the cancer nests and vascular endothelial cells (Fig. 3G–I). The intensities of these signals were higher in the dysplastic epithelium and cancer nests than in the normal epithelium.

**Localization of VEGF and VEGFRs proteins**

The results of immunohistochemistry for VEGF and VEGFRs proteins in the rat tongues are shown in Fig. 4. In the normal epithelium, immunoreactivities for VEGF and VEGFRs proteins were expressed in the cytoplasm of all of the epithelial cells, and vascular endothelial cells (Fig. 4A–C). In the hyperplastic and dysplastic epithelium, the VEGF and VEGFRs immunoreactivities were localized in the cytoplasm of all of the epithelial cells and vascular endothelial cells (Fig. 4D–F). In the well-differentiated squamous cell carcinoma, the immunoreactivities were detected in the cytoplasm of the cancer cells and vascular endothelial cells (Fig. 4G–I). Thus, the distribution of the immunoreactivities for VEGF and VEGFRs proteins was similar to that of the signals for VEGF and VEGFRs mRNAs.

**Statistical analysis of VEGF, VEGFRs and Ki-67 labeling indices and blood vessel density**

The labeling indices for VEGF, VEGFRs and Ki-67 and blood vessel density in the rat tongues are shown in Fig. 5. All these indices and the density were gradually increased along with the disease progression, and reached a peak at 20 weeks. The VEGF and VEGFRs labeling indices were significantly increased from 12 to 20 weeks \((p<0.05)\), and the VEGF and Flk-1 labeling indices were significantly increased from control to 20 weeks \((p<0.01)\). The Ki-67 labeling index was significantly increased from control to 12 weeks and from 12 to 20 weeks \((p<0.05)\), and from control to 20 weeks \((p<0.01)\). The blood vessel density was significantly increased from control to 20 weeks and from 12 to 20 weeks \((p<0.01)\).

The relation between two of the VEGF labeling indices, the Ki-67 labeling index and blood vessel density is shown in Fig. 6. An increase in the VEGF labeling index was positively correlated with increases in the Ki-67 labeling index \((p<0.0001, r=0.837643)\) and blood vessel density \((p<0.0001, r=0.789084)\). An increase in the Ki-67 labeling index positively correlated with an increase in blood vessel density \((p<0.0001, r=0.768754)\).

**Western blot analysis**

The results of Western blotting and the quantitative analysis in the rat tongues are shown in Fig. 7. Western blots of the tongues with the antibodies against VEGF, Flt-1 and Flk-1 showed 25 kDa, 155 kDa and 160 kDa immunoreactive bands, respectively. The VEGF and Flk-1 determinants were detected in the rat tongues at the 0, 4, 8, 12, 16 and 20 weeks. The Flt-1 determinant was detectable in the rat tongues at 4, 8, 12, 16 and 20 weeks, but undetectable in the controls. The densitometry revealed that the immunoreactiv-
ities for VEGF and VEGFRs were the strongest in the 4NQO treated rats at 20 weeks during the examined periods and in contrast the lowest in the untreated rats.

IV. Discussion

There is increasing evidence for the mechanism of carcinogenicity of 4NQO. 4NQO, artificially administrated, is reduced in the cytosol to 4-hydroxyaminoquinoline 1-oxide (4HAQO), which passes through the nucleic membrane and binds to purine bases to form 4HAQO-DNA adducts [23, 33]. When these abnormal DNA sites are not repaired completely, DNA errors occur and initiate carcinogenesis [17, 23]. Oral administration of 4NQO in rats has the advantage of minimal chemical stress without mechanical stress to the animals. Thus, it is likely that this experimental cancer model may provide pathological processes similar to those of spontaneous carcinogenesis [33]. In general, 4NQO-induced rat tongue carcinoma typically shows usually local invasion but rare distant metastasis [6, 14, 33].

In the present study, the morphological analyses by in situ hybridization and immunohistochemistry demonstrated that mRNAs and proteins of VEGF and the specific receptors, Flt-1 and Flk-1, were colocalized in the cytoplasm of the normal epithelial cells, dysplastic cells, cancer cells and vascular endothelial cells, and that the distribution of VEGF and VEGFRs proteins was similar to that of VEGF and VEGFRs mRNAs. The VEGF labeling index in rat tongue carcinoma induced by 4NQO gradually increased in the processes of carcinogenesis and tumor progression. In contrast to these findings, some previous studies suggested that VEGF mRNA and protein were restricted to the cancer cells, and that VEGFRs mRNAs and proteins were restricted to proliferating vascular endothelial cells [2, 27, 30]. Thus, the diversity of the localization of these substances in cancers and adjacent stroma among the different studies should be considered with the caveat that the results of morphological studies can be influenced by a variety of methodologies and materials. The present results suggest that the VEGF/VEGFRs system in 4NQO-induced rat tongue carcinoma is mediated by paracrine and autocrine mechanisms.

The current study showed statistical significance in the labeling indices of immunohistochemical markers, blood vessel density and length of disease course. The labeling indices of VEGF, VEGFRs and Ki-67 and blood vessel density increased along with disease progression of this experimental cancer model. Blood vessel density was higher at the stroma adjacent to the spreading zone of cancer nests than at the stroma adjacent to dysplastic and normal epithelium, as described in previous studies [18]. The increase in VEGF labeling index positively correlated with the increases in Ki-67 labeling index and blood vessel density. Moreover, the increase in the Ki-67 labeling index positively correlated with the increase in the blood vessel density. Similar findings have been shown in a recent study describing that both the blood vessel density and proliferating cell nuclear antigen (PCNA) labeling index were higher in rat tongue carcinomas induced by 4NQO than in control rat tongues, and that the increase in blood vessel density positively correlated with the increase in PCNA labeling index during the disease processes [18]. These observations suggest that angiogenesis is closely related to carcinogenesis and cell proliferation activity in 4NQO-induced carcinoma.

We also quantified the expression levels of VEGF and VEGFRs on immunoblots. The molecular sizes of VEGF, Flt-1 and Flk-1 were 25 kDa, 155 kDa and 160 kDa, respectively. These findings are consistent with previous studies showing the molecular sizes of VEGF (18–40 kDa) [2, 26], Flt-1 (165–180 kDa) [42, 43], and Flk-1 (170–200 kDa) [42–44]. The immunoreactive densities of VEGF and VEGFRs in rat tongues began to increase at the dysplastic
stage, and peaked at the advanced stage of cancer. The present quantitative data by Western blot analysis support our semiquantitative data by statistical analysis of the labeling indices of VEGF and VEGFRs as mentioned above.

In conclusion, the present study demonstrated the colocalization of mRNAs and proteins of VEGF and VEGFRs in the cytoplasm of both cancer cells and vascular endothelial cells in rat tongue carcinoma induced by 4NQO, suggesting that the binding of VEGF to VEGFRs is mediated by paracrine and autocrine mechanisms. The duration-dependent increase in both the labeling indices of VEGF and VEGFRs in immunostained sections and the immunoreactive densities of these proteins on immunoblotted membranes could suggest that angiogenesis is implicated in carcinogenic transformation and tumor progression. In addition, VEGF has been shown to be activated or reactivated by some mediators including heparanase [15]. VEGF is synthesized in certain cells, and released into the extracellular matrix. Heparanase liberates VEGF conjugated to the extracellular matrix, resulting in binding of free form of VEGF to VEGF receptors. Thus, heparanase mediates angiogenesis and tumor progression [31]. However, it remains to be determined whether heparanase may regulate VEGF activity in our rat tongue cancer model. Finally, it is important to clarify whether VEGF may directly induce the proliferation of malignant cells, and have any other properties. Elucidation of these questions will provide beneficial information for understanding of the pathomechanism of tongue carcinoma and exploiting therapeutic strategies for this disease.

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VI. References


