Immunohistochemical Localization of Vascular Endothelial Growth Factor in Esophageal Cancer

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Summary: We have studied the expression of vascular endothelial growth factor (VEGF) in esophageal cancer using immunohistochemistry. A total of 101 specimens of esophageal cancer tissue were fixed by formalin, embedded in paraffin wax, and examined in 3 μ m sections by avidin-biotin peroxidase complex method. VEGF was noted in the cytoplasm of normal esophageal glandular cells, monocyte-macrophages, squamous carcinoma cells and of the vascular endothelial cells themselves. VEGF expression by monocyte-macrophages was observed in all cases, in contrast the incidence of VEGF expression in the tumor cells was relatively low at 26.7% of all specimens. However, in the cases where the tumor cells were positive for VEGF, it was discovered that the main source of the VEGF production was the tumor cells themselves. In the cases with proper mucosal invasion the incidence of VEGF expression by the tumor cells was quite low at 7.6%. However, when the tumor invaded the submucosal layer the expression increased to 33.3%. There was also a significant correlation in those with the submucosal invasion between the expression of VEGF in the tumor cells and the density of microvessels at the tumor invasive edge. These results suggest that VEGF may play an important role in tumor progression and in the angiogenesis via auto-crine and para-crine mechanisms in esophageal cancer.

Key words: vascular endothelial growth factor (VEGF) — immunohistochemistry — esophageal cancer — histopathological factors — vessel count at tumor invasive edge — angiogenesis

Introduction

Previous experimental studies (Liotta et al. 1974; Folkman, 1986) have demonstrated that tumor growth and metastasis are dependent upon angiogenesis. Increased vascularity may allow not only

for an increase in tumor growth but also for hematogenous tumor embolization. Weidner et al. (1991) reported a statistically significant correlation between the incidence of metastasis and the density of microvessels in invasive breast cancer. This finding has been confirmed in

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numerous studies of breast cancer (Bosari et al. 1992; Guidi et al. 1994) as well as cervical cancer (Smith-McCune and Weidner, 1994), and other tumors (Weidner et al. 1993).

VEGF are known to induce angiogenesis in rodent tumor models (Leung et al. 1989; Ferrara et al. 1992). It is also well known that VEGF has a specific potential for proliferating endothelial cells (Keck et al. 1989; Leung et al. 1989; Conn et al. 1990). Further identification of the specific angiogenic factor may provide a target for anti-angiogenic therapy in the neoplasm.

In this study, we report the localization of VEGF and a correlation between the expression of VEGF in tumor cells and the density of vessels in esophageal cancer.

Materials and Methods

Patients

Formalin-fixed and paraffin-embedded specimens of esophageal cancer were studied from 101 randomly chosen patients who had undergone surgery in the 1st Department of Surgery, Kurume University School of Medicine between 1989 and 1994. The cancer specimens involved of 13 either *in situ* (ep) or invading the muscularis mucosa (mm), 24 invading the submucosa (sm), 15 pT2, 31 pT3 cancers, and 18 pT4 (according to TMN classification). After an initial review of all available hematoxylin and eosin-stained (H.E.) slides of the surgical specimens, we selected one paraffin block in which the tumor invasive edge and the viable tumor were shown from each case for study. Consecutive 3 μ m

sections were recut from each block. One section from each case was stained by H.E., a second was stained by elastica-vanginson stain (EVG), and a third was immunostained for VEGF.

Immunohistochemical staining

Immunohistochemical staining was performed by avidin-biotin peroxidase complex method. Anti-human VEGF mouse monoclonal IgG (R&D SYSTEM, Minneapolis) was used at 1:200 dilution as a primary antibody. Negative controls used all reagents except the primary antibody.

Evaluation of immunostaining of VEGF and vessel counting

We have evaluated positive staining of cell types for VEGF. The intensity of staining for VEGF in tumor cells was graded on a scale of 0 (representing no detectable stain or only a few positive cells), 1+ (representing a cluster of positive cells, but less than 50%), and 2+ (representing more than 50% positive). In 24 cases of sm invasion, vessel counts were made at the invasive edge by light microscopy after EVG staining.

Statistical analysis

Correlation between the vessel counts and the intensity of VEGF was investigated by standard chi-square test. The p-value level of significance was set at 0.05.

Results

Localization of VEGF in esophageal cancer tissue

VEGF staining was noted in the cytoplasm of normal esophageal glandular

Fig. 1. Immunohistochemical staining for VEGF in esophageal cancer tissue. Normal squamous epithelial cells are negative for VEGF (a: $\times 50$); Supra nuclear staining of VEGF is seen in esophageal glandular cells adjacent the tumor (b: $\times 100$); Minute granular or diffuse cytoplasmic staining of VEGF by monocytes is observed in all cases (c: $\times 200$); Endothelial cells of vessel at tumor invasive edge or tumor stroma express VEGF (d: $\times 200$); Minute granules of VEGF are noted in esophageal squamous carcinoma cells (e: $\times 100$, f: $\times 200$).

cells, monocyte-macrophages, neutrophils, fibroblast-like cells, squamous carcinoma cells, and of endothelial cells themselves, in esophageal cancer tissue. Supra nuclear staining of VEGF was observed in esophageal glandular cells (Fig. 1b), but minute granular or diffuse staining was seen in monocyte-macrophages (Fig. 1c), endothelial cells (Fig. 1d) and tumor cells (Fig. 1e, f). The tumor cells along with the endothelial cells of adjacent small blood vessels in the tumor stroma were stained strongly. In contrast, the normal distal squamous epithelium was stained very weakly or not at all (Fig. 1a), and the endothelial cells of small blood vessels in the normal distal epithelium were not stained. Smooth muscle cells of an artery or vein were sometimes weakly stained. VEGF expression by monocyte-macrophages was observed in all cases and tended to be intensive at the tumor invasive edge. By contrast, the incidence of VEGF expression in the tumor cells (1+ or 2+) was observed in only 26.7% of

cases (1+: 22.7%, 2+: 4.0%). However, in all cases where tumor cells were positive for VEGF, the tumor cells themselves were considered as the main source of the VEGF production.

Relationship between the expression of VEGF and pathological factors

As shown Table 1, the incidence of positive cases for VEGF in the tumor cells was low at 7.7% (1 of 13) in ep or mm cancer and was 33% (8 of 24) in sm cancer. There was no relationship between the expression of VEGF and histopathological factors such as lymph node involvement, histological tumor differentiation, lymphatic permeation or venous invasion (data not shown).

Correlation between vessel counts at the tumor invasive edge and VEGF expression

Among the 24 cases of sm cancer, 7 cases were associated with a small number of vessels (0-4), and 17 cases were associated with a large number of vessels (more than 5) at the tumor invasive edge. All the cases positive for VEGF in the tumor cells were associated with a large number of vessels. There

TABLE 1.
The expression of VEGF and tumor depth

VEGF expression	Tumor depth				
	ep, mm	sm	pT2	pT3	pT4
(-)	12	16	11	21	14
(+)	1	8	4	10	4

Values are number of cases; VEGF (+) includes 1+ and 2+ described in Materials and Methods; ep, mm, and sm mean superficial carcinoma. ep and mm are limited to mucosa. ep: carcinoma *in situ*, mm: tumor invades lamina muscularis mucosa, sm: tumor invades submucosa; pT2, pT3, and pT4 are defined according to TMN classification.

TABLE 2.
Relationship between the expression of VEGF and vessel counts at the tumor invasive edge (in the cases of sm cancer)

VEGF expression	Vessel counts	
	0-4	5≤
(-)	7	9
(+)	0	8

Values are number of cases; VEGF (+) includes 1+ and 2+ described in Materials and Methods, $p < 0.05 (\chi^2 = 4.941)$.

was a significant correlation between the VEGF expression in the tumor cells and the vessel counts at the invasive edge (Table 2).

Discussion

We have determined the immuno-histochemical localization of VEGF in esophageal squamous cell carcinoma tissues. VEGF was noted in tumor cells, normal esophageal glandular cells, inflammatory cells, fibroblasts and in endothelial cells of the blood vessels themselves. We found a statistically significant correlation between the expression of VEGF in the tumor cells and the density of vessels at the invasive edge. Recent studies (Takahashi et al. 1995; Warren et al. 1995) have demonstrated that KDR/Flk-1, as a receptor for VEGF, was expressed specifically in the endothelium of colon cancer tissues. These evidences suggested that VEGF might play a role in angiogenesis even in cancer tissue via either an auto-crine and/or a para-crine mechanism. However, from our immunohistochemical study, the main cell source of VEGF production appeared to be the tumor cells, in all cases where the tumor cells were positive for VEGF. Also the expression of VEGF by stromal cells and normal esophageal glandular cells tended to be associated with the expression of this protein by tumor cells. In addition, Brown et al. (1993) demonstrated a higher expression of mRNA for VEGF using *in situ* hybridization in malignant colonic epithelium as compared to benign colon epithelial tumors.

Therefore, the existence of tumor cells may act as a trigger for VEGF-mediated angiogenesis in cancer tissues.

With regard to direct regulating factors of VEGF production, it has been reported that epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and/or platelet derived growth factor (PDGF) in human malignant glioma cell lines, and transforming growth factor- β (TGF- β) in mouse embryo-derived cell line and human lung adenocarcinoma cell line induce VEGF production *in vitro* (Pertovaara et al. 1994; Tsai et al. 1995). VEGF mRNA expression is known to be stimulated by phorbol ester and cAMP analogs, indicating that both protein kinase C- and protein kinase A-mediated pathways are involved in the regulation of VEGF gene expression (Claffey et al. 1992).

In cancer tissue, various stimulatory factors for VEGF are thought to be produced by the interaction of tumor cells and host cells. These factors are thought to be active in a wide range of tumor phenotypes resulting in the various concentration of VEGF expression in cancer tissue. Our studies have shown that there was no correlation with histopathological factors other than with tumor depth. Because the *fms*-like tyrosine kinase 1 (Flt-1) and the KDR/Flk-1 receptor for VEGF have been shown to be expressed specifically in endothelial cells, we have to evaluate not only VEGF expression but also the receptors in cancer tissue to estimate true VEGF activity. Moreover, because several variant forms of VEGF generated by alternative mRNA splicing have different activities as a mitogen for

endothelial cells or vascular permeability factor (DeVries et al. 1992; Terman et al. 1992; Millauer et al. 1993), it is important to evaluate individually the expression of the variant forms in cancer tissue. We expect that VEGF activity may have a strong correlation with tumor progression and metastatic spread, and become a useful clinical marker. VEGF may be a specific angiogenic factor which could potentially provide a target for anti-angiogenic therapy for cancer.

References

- Bosari S, Lee AKC, Dlellis RA, Wiley BD, Heatley GJ et al. Microvessel quantitation and prognosis in invasive breast carcinoma. *Hum Pathol* 1992; 23:755-761.
- Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinoma of the gastrointestinal tract. *Cancer Res* 1993; 53:4727-4735.
- Claffey KP, Wilkison WO, and Spiegelman BM. Vascular endothelial growth factor. *J Biol Chem* 1992; 267:16313-16322.
- Conn G, Bayne ML, Soderman DD, Kwok PW, Sullivan KA et al. Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc Natl Acad Sci U.S.A.* 1990; 87:2628-2632.
- DeVries C, Escobedo JA, Ueno H, Houck K, Ferrara N et al. The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992; 255:989-991.
- Ferrara N, Houck K, Jakeman L, and Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of protein. *Endocr Rev* 1992; 13:18-32.
- Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? G. H. A. Clowes Memorial Award Lecture. *Cancer Res* 1986; 46:467-473.
- Guidi AJ, Fischer L, Harris JR, and Schnitt SJ. Microvessel density and distribution in ductal carcinoma *in situ* of the breast. *J Natl Cancer Inst* 1994; 86:614-619.
- Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 1989; 246:1309-1312.
- Leung DW, Cachianes G, Kuang WJ, Goeddel DV, and Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Washington DC)* 1989; 246:1306.
- Liotta L, Kleinerman J, and Sadel G. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Canver Res* 1974; 34:997-1004.
- Millauer B, Wizigmann-Voos S, Schnürch H, Martinez R, Møller NH et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993; 72:835-846.
- Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N et al. Vascular endothelial growth factor is induced in response to transforming growth factor- β in fibroblastic and epithelial cells. *J Biol Chem* 1994; 269:6271-6274.
- Smith-McCune KK, and Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. *Cancer Res* 1994; 54:800-804.
- Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, and Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; 55:3964-3968.
- Terman BI, Dougher-Vermazen M, Carrion EM, Dimitrov D, Armellino DC et al. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun* 1992; 187:1579-1586.

- Tsai JC, Goldman CK, and Gillespie GY. Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. *J Neurosurg* 1995; 82: 864-873.
- Warren RS, Yuan H, Matli MR, Gillett NA, and Ferrara N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995; 95:1789-1797.
- Weidner N, Carroll PR, Flax J, Blumenfeld W, and Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993; 143:401-409.
- Weidner N, Semple JP, Welch WR, and Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991; 324:1-8.