Angiogenesis in Superficial Esophageal Squamous Cell Carcinoma: Assessment of Microvessel Density Based on Immunostaining for CD34 and CD105

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Objective: The esophagus is the only organ where changes in the superficial microvasculature from normal squamous epithelium to invasive cancer are evident by magnifying endoscopy. We investigated in detail the features of angiogenesis in early-stage esophageal cancer using CD34 and CD105 immunostaining, and also the correlation between angiogenesis and mononuclear cell infiltration.

Materials and methods: Using 10 samples of normal squamous epithelium, 7 samples of low-grade intraepithelial neoplasia, and 45 samples of superficial esophageal cancer, we determined the microvessel density at hot spots showing positive staining for CD34 and CD105. We observed the histological features of CD34- and CD105-positive microvessels that corresponded to observations made by magnifying endoscopy. We then investigated the correlation between microvessel density and each histological situation or the grade of mononuclear cell infiltration.

Results: The histological features of CD34- and CD105-positive microvessels were able to explain the morphological changes in the microvasculature during cancer progression observed by magnifying endoscopy. The microvessel density for CD34 or CD105 was significantly correlated with each of the histological types \( (P < 0.001, r_S = 0.51 \text{ and } 0.76, \text{ respectively}) \). Mononuclear cell infiltration at CD105 hot spots was most frequent in M1 and M2 cancer \( (94.7\%) \). The correlation between the degree of mononuclear cell infiltration and microvessel density for CD105 staining was also significant \( (P < 0.001, r_S = 0.49) \).

Conclusions: The microvessel density based on CD34 and CD105 immunostaining can be used to corroborate observations of superficial esophageal squamous cell carcinoma made by magnifying endoscopy. Mononuclear cell infiltration may play an important role in angiogenesis at the early stage of cancer progression.

Key words: microvessel density – CD34 – CD105 – esophageal cancer – angiogenesis
INTRODUCTION

The structure and organization of blood vessels is dynamic and undergoes considerable change during the transition from normal tissue to neoplasia, and finally invasive cancer. The esophagus is the only organ where morphological changes in the superficial microvasculature from normal squamous epithelium to invasive cancer can be observed using magnifying endoscopy in vivo (2–6).

Japanese endoscopists currently apply these features for diagnosing the depth of tumor invasion, and in this context high accuracy of preoperative magnifying endoscopy has been reported (4,5). Furthermore, we (7,8) have emphasized the importance of this phenomenon from the viewpoint of angiogenesis, and studied the molecular profiles of angiogenic factors. However, to our knowledge, magnifying endoscopic observation has never been applied systematically in the context of molecular biology.

In the present study focusing on precancerous lesions and superficial esophageal squamous cell carcinomas, we investigated the features of angiogenesis in relation to histological types or depth of tumor invasion by estimation of immunostaining for CD34 and CD105.

MICROVASCULATURE OBSERVED BY MAGNIFYING ENDOSCOPY

In Vivo

Generally, in the normal esophageal mucosa, arterioles arising in the lamina propria mucosae form a subepithelial capillary network (SCN) beneath the epithelium. From the SCN, looped capillaries known as intrapapillary capillary loops (IPCLs) arise inside the epithelial papillae, and drain into the arborescent vascular network. These IPCLs can be clearly observed in vivo using magnifying endoscopy (Fig. 1a).

In M1 and M2 cancer, the microvasculature at the tumor surface retains the shape of the IPCLs, and shows obvious dilatation and elongation relative to the normal squamous epithelium. In addition, the density of IPCLs is higher than in the adjacent normal epithelium, and therefore this microvasculature is thought to consist of modified IPCLs and newly recruited IPCL-like capillaries (Fig. 1b).

At the surface of M3 or deeper cancer, the newly developed tumor vessels appear dilated and irregularly branched, with a shape that obviously differs in comparison with the IPCL-like capillaries of M1 or M2 cancer (Fig. 1c).

PATIENTS AND METHODS

Tissue Samples

We employed 62 samples from 47 patients who underwent histological examination at Saitama Medical Center, Saitama Medical University, between 2006 and 2012. The tissue samples comprised 10 specimens of normal squamous epithelium, 7 specimens of low-grade intraepithelial neoplasia (LGIN), and 45 specimens of esophageal cancer (M1: 12 lesions, M2: 7 lesions, M3: 7 lesions, SM1: 5 lesions, SM2: 3 lesions, SM3: 11 lesions). Tissue samples were obtained by esophageal biopsy (n = 4), endoscopic resection (n = 14), or esophagectomy (n = 44). We excluded specimens from patients who had undergone radiotherapy and/or chemotherapy before lesion resection. In order to evaluate the microvessel density (MVD), we also excluded cases where it was not possible to observe the lamina propria mucosae or submucosa in biopsy samples. We selected 10 samples of normal squamous epithelium that were located distantly from the cancer lesion in esophagectomy specimens.

Pathological diagnosis was made according to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus (10th edition) (9).

Sections were cut from 3 mm-wide step-sectioned blocks of endoscopic resection specimens, or from 5 mm-wide blocks obtained from surgically resected esophagi, and stained with hematoxylin and eosin (H&E).

We divided the specimens into four different histological types, i.e. normal squamous epithelium, LGIN, M1–M2 cancer and M3 or deeper cancer, by reference to observations made by magnifying endoscopy.

The study was performed under a protocol approved by our hospital ethics committee, and was supported by a grant from Saitama Medical University.

Immunohistochemical Staining

Tissue samples fixed in 10% formalin and embedded in paraffin were cut into sections 4 μm thick and mounted on slides.

After dewaxing and dehydration, the sections were pretreated using an autoclave in EDTA buffer (pH 8.0) at 121°C for 15 min for immunostaining with anti-CD105 antibody (clone SN6h (Abcam Co., MA, USA) diluted 1:100), which was performed using a highly sensitive indirect immunoperoxidase technique (Histofine Simple stain MAX-PO, Nichirei, Tokyo, Japan) with diaminobenzidine as the chromogen, followed by hematoxylin counterstaining.

Immunohistochemical staining of CD34 was performed using the streptavidin–biotin–peroxidase method. The slides were immunostained with anti-CD34 antibody (clone My10 (Becton Dickinson Co., NJ, USA) diluted 1:10) using a Ventana Bentimark XT machine (Ventana, Tuscon, AZ, USA).

Quantification of Microvessel Density

On the basis of CD34 and CD105 immunostaining, MVD in the lamina propria mucosae was assessed in normal esophageal mucosa, LGIN and M1 and M2 cancer. For M3 and submucosal cancer, we counted the microvessels present in the cancer stroma or at the invasive front, and sections were screened according to the method described by Weidner et al. (10). Briefly, at ×100 magnification, the areas of strongest staining (hot spots) were noted. We separately assessed MVD in two hot spots for CD105 staining, and three hot spots for CD34 staining at ×200, considering the number and width of the area of hot spots. Any brown-stained
endothelial cells or endothelial cell clusters that were clearly separable from adjacent microvessels, tumor cells and other connective tissue elements were each considered a single countable microvessel; evidence of a visible vascular lumen was not required. All assessments were performed by a single pathologist (H.M.) who was blinded to the clinical data. The average MVD for each type of immunostaining was used as the final value.

Assessment of Mononuclear Cells at CD105 Hot Spots

CD105-positive structures (hot spots) tended to be located adjacent to or within sites of mononuclear cell infiltration. The vast majority of cells comprising areas of mononuclear cells infiltration were lymphoid cells, although H&E staining also revealed some macrophages. We classified mononuclear cell infiltration in the most intense area of CD105 immunostaining in each lesion according to the following criteria (Fig. 2).

Class 1: Mononuclear cell infiltration absent or weak (Fig. 2a).
Class 2: Aggregates of mononuclear cells present, but no evident follicle formation (Fig. 2b).
Class 3: Mononuclear cell infiltration with lymphoid follicle formation (Fig. 2c).
Class 4: Severe and diffuse aggregation of infiltrated mononuclear cells (Fig. 2d).

Statistical Analysis

Spearman’s rank correlation test was applied for examining correlations between variables. Differences between groups were analyzed by Kruskal–Wallis test followed by Dunn’s test. Differences at $P < 0.05$ were considered significant.

RESULTS

Correlation Between Magnifying Endoscopic Observation and Histological Features Using CD34 and CD105 Immunostaining

In the normal squamous epithelium, thin vessels were observed inside the epithelial papillae that correspond to the IPCLs revealed by magnifying endoscopy using CD34 immunostaining. Beneath the basal layer (at the lamina propria mucosae) thin capillaries corresponding to the SCN observed using magnifying endoscopy were also evident (Fig. 3a). Almost all of these capillaries were unstained with CD105, except for rare, very weak staining. In LGIN, IPCLs and the SCN were also observed using CD34 immunostaining. In 4 of 7 LGIN cases, some CD105-positive IPCL-like capillaries were confirmed (Fig. 3b). In most cases of M1 and M2 cancer, the epithelial papillae were elongated, and thus IPCL-like capillaries inside them were also elongated. The number of CD34-positive IPCL-like capillaries was increased in comparison to the normal esophageal mucosa (Fig. 3c). In all M1 and M2 cases, CD105-positive IPCL-like capillaries and the SCN were observed. In M3 or deeper cancer, epithelial papillae were destroyed. Capillaries strongly positive for CD34 and CD 105 were observed at the cancer stroma, even at the surface of the tumor, in addition to the deeper part of the tumor or invasive front (Fig. 3d). These capillaries corresponded to the dilated and irregularly branched tumor vasculature that was observed by magnifying endoscopy.

MVD After Immunostaining for CD34 and CD105

Microvessel density (MVD) data for each histological type assessed on the basis of immunostaining for CD34 and CD105 are shown in Fig. 4. The median MVD (range) for CD34 staining in the normal esophageal mucosa, LGIN, M1–M2 cancer and M3 or deeper cancer was 24.8 (12.7–69.7), 36.0 (20.0–55.3), 47.3 (24.3–80.0) and 55.3 (23.0–115.7), respectively. MVD assessed on the basis of CD34 positivity, being lowest for normal squamous epithelium, followed in ascending order by LGIN, M1–M2 cancer and M3 or deeper cancer, and the correlation was significant but weak ($P < 0.001$, $r_S = 0.51$) (Fig. 4a).

The median MVD (range) for CD105 immunostaining in normal esophageal mucosa, LGIN, M1–M2 cancer and M3 or deeper cancer was 0.5 (0–2.5), 7.0 (0–17.5), 13.0 (5.0–19.5) and 22.0 (4.0–65.0), respectively. MVD assessed on the basis of CD105 positivity was also lowest for normal squamous epithelium, followed in ascending order by LGIN, M1–M2 cancer and M3 or deeper cancer, and the correlation was significant and strong ($P < 0.001$, $r_S = 0.76$) (Fig. 4b).
Figure 2. Typical cases to illustrate classification of mononuclear cell infiltration. (a) Class 1; weak infiltration of mononuclear cells in the lamina propria mucosae beneath M2 cancer (H&E staining, ×200). (b) Class 2; presence of aggregates of mononuclear cells, without evidence of follicle formation in the lamina propria mucosae beneath M2 cancer (H&E staining, ×200). (c) Class 3; mononuclear cell infiltration with follicle formation in the lamina propria mucosae beneath M1 cancer (H&E staining, ×100). (d) Class 4; marked and diffuse mononuclear cell infiltration in the lamina propria mucosae beneath M1 cancer (H&E staining, ×200).

Figure 3. Microvasculature of each histological grade revealed using CD34 or CD105 immunostaining. Black arrows: IPCL; dotted arrows: subepithelial capillary network; white arrows: tumor vasculature. (a) normal squamous epithelium (CD34 immunostaining, ×200), (b) Low-grade intraepithelial neoplasia (CD105 immunostaining, ×200), (c) M2 squamous cell carcinoma (CD34 immunostaining, ×200), (d) M3 squamous cell carcinoma (CD105 immunostaining, ×200).
The median (range) CD105/CD34 MVD ratio in normal esophageal mucosa, LGIN, M1–M2 cancer and M3 or deeper cancer was 0.02 (0–0.10), 0.13 (0–0.63), 0.25 (0.10–0.60) and 0.35 (0.04–0.99), respectively. The CD105/CD34 MVD ratio was lowest for normal squamous epithelium, followed in ascending order by LGIN, M1–M2 cancer and M3 or deeper cancer, and the correlation was significant ($P < 0.001$, $r_S = 0.65$).

Correlations between CD105-positive MVD and CD34-positive MVD are shown in Fig. 4c. Spearman’s rank correlation test demonstrated a significant but weak correlation ($P = 0.003$, $r_S = 0.38$).

**CORRELATION BETWEEN CD34 AND CD105 HOT SPOTS AND MONONUCLEAR CELL INFILTRATION**

Mononuclear cells were often observed at the lamina propria mucosae of LGIN and M1–M2 cancer, but at the cancer stroma or invasive front in M3 or deeper cancer. It was evident that many CD105-staining hot spots matched sites of mononuclear cell aggregation. This tendency was most apparent in M1 and M2 cancer, where the rate of such matching was 94.7%. There was a significant difference among the four groups in terms of the grading of mononuclear cell infiltration ($P < 0.001$, Kruskal–Wallis test). Post hoc Dunn’s test revealed a significant difference only between normal squamous epithelium and M1–M2 cancer, and between normal squamous epithelium and M3 or deeper cancer ($P < 0.01$) (Table 1).

The median MVD (range) based on CD105 immunostaining for each degree of mononuclear cell infiltration was 1.0 (0–35.0) for Class 1, 9.50 (4.0–45.0) for Class 2, 14.5 (3.0–45.0) for Class 3 and 17.5 (5.0–65.0) for Class 4. The CD105-positive MVD increased in the order of mononuclear cell infiltration Grade 1–4, and the correlation was significant ($P < 0.001$, $r_S = 0.49$) (Fig. 5).
The median MVD (range) based on CD34 immunostaining for each degree of mononuclear cell infiltration was 35.7 (12.7–80.3) for Class 1, 54.8 (38.3–94.3) for Class 2, 49.8 (24.3–80.0) for Class 3, and 46.0 (27.0–115.7) for Class 4. There was no significant correlation between the CD34-positive MVD and mononuclear cell infiltration ($P = 0.23$, $r_S = 0.15$).

**DISCUSSION**

Understanding the changes that occur in vascular structure and distribution during cancer progression would undoubtedly help to clarify the mechanisms of cancer development, and might reveal new targets for treatment or prevention. We have previously reviewed and reported the profiles of angiogenic factors at the early stage of progression of esophageal squamous cell cancer, and proposed a hypothesis of ‘multi-step angiogenesis’ in early-stage esophageal squamous cell carcinoma (7,8).

Many pathological studies (11–23) have revealed that MVD in esophageal squamous cell carcinoma determined after staining with anti-endothelial antibodies increases in proportion to disease progression, tumor size and stage, and depth of invasion. Most analyses have confirmed that MVD has significant prognostic value. Our present investigation employed two anti-endothelial antibodies: anti-CD34 and anti-Endoglin/CD105. Anti-CD34 antibody is a pan-endothelial marker that stains whole micro-capillaries including newly formed and preexisting vasculature (24,25). We found here that MVD estimated on the basis of CD34 staining was significantly correlated with histological type and the depth of tumor invasion. In this connection, we had previously shown that SCN and IPCLs of M1 or M2 cancer present in the lamina propria mucosae (beneath the tumor) are densely arranged in comparison with the adjacent normal squamous epithelium, based on stereoscopic microscopy observations after MICROFIL injection (4,8). This allowed us to account for the redness of superficial esophageal cancer relative to the normal esophageal mucosa, when observed by conventional endoscopy or magnifying endoscopy.

In addition, we employed anti-Endoglin/CD105 antibody. Endoglin/CD105, a member of the transforming growth factor 1 receptor complex, is well acknowledged as being the most reliable marker of endothelial cell proliferation, and is overexpressed on tumor vessels (26). Using anti-CD105 antibody, Kubota et al. (27) assessed the MVD of Lugol-unstained non-dysplastic epithelium (esophagitis), and low- and high-grade dysplasia of squamous epithelium in biopsy samples. They found that CD105-positive vessels were already present in esophagitis and low-grade dysplasia, and that CD105-positive MVD in high-grade dysplasia was significantly higher than in low-grade dysplasia. These observations are compatible with our present findings, and suggest that CD105-positive vessels were already recruited from non-cancerous lesions. In addition, the MVD of CD105-positive capillaries was strongly correlated with the depth of tumor invasion. Furthermore, the data for the CD105/CD34 MVD ratio indicated that the vasculature nourishing the tumor was gradually replaced by CD105-positive vasculature during the early stage of progression of esophageal neoplasia. This supports our hypothesis of ‘multi-step angiogenesis’ in early-stage esophageal squamous cell carcinoma (7).

Magnifying endoscopic observation of esophageal squamous cell carcinoma has suggested that IPCLs inside mucosal cancer lesions dilate and elongate, and that their numerical density increases relative to the adjacent normal esophageal mucosa, implying that mucosal cancer induces IPCLs like newly developed vessels (2–8,28). The histology of the microvasculature revealed using CD34 and CD105 immunostaining matched the changes in morphological characteristics of the microvasculature during the transition from normal...
squamous epithelium to invasive cancer observed by magnifying endoscopy. In all cases, we were able to observe CD105-positive capillaries within epithelial papillae in histological sections of mucosal cancer. These newly induced capillaries are similar in shape to preexisting modified IPCLs evident using magnifying endoscopy in vivo. Therefore, in this context, investigation of MVD using CD105 immunostaining could be used to corroborate the presence of any increase of IPCL-like capillaries in mucosal cancer. However, we were unable to find any correlation or difference for CD34 and CD105 MVD with regard to the lymphatic invasion or venous invasion. Indeed, we were unable to recognize the features of lymphatic invasion or venous invasion endoscopically from observation of surface microvasculature morphology.

Furthermore, we were aware that many CD105-positive hot spots were located adjacent to or within sites of mononuclear cell infiltration. Our grading of mononuclear cell infiltration revealed a significant correlation with MVD in terms of CD105 staining. These CD105 hot spots with mononuclear cell infiltration were seen most frequently in the M1–M2 cancers, where mononuclear cells may play an important role in the induction of neovascularature. In this connection, Kuwano et al. (18) investigated the relationship between lymphocyte infiltration in the lamina propria mucosae and MVD based on immunostaining for factor VIII-related antigen in carcinoma in situ and micro-invasive cancer. However, they were unable to find any significant correlation between them. In our investigation, we also found no significant correlation between mononuclear cell infiltration and CD34 MVD. Thus it seemed important to focus on the relationship between CD105-positive newly induced capillaries and mononuclear cell infiltration.

The vast majority of the mononuclear cells we observed were lymphoid cells, but some macrophages were also present. Several kinds of mononuclear cells are known to induce neo-vascularure. Tumor-associated macrophages (TAMs) are a type of stromal cell releasing several angiogenic factors and cytokines, and have been shown to play important roles in tumor growth, invasion and metastasis (29–31). Koide et al. (29) reported significant correlations of TP (thymidine phosphorylase) expression in stromal cells (TAMs) and cancer cells with venous invasion, distant metastasis or MVD based on immunostaining for factor VIII-related antigen. Therefore, in this study, we confirmed that the early stage of esophageal cancer progression.

More recently, IL-17-producing T helper cells (Th17) have been reported to induce angiogenesis (32–34). Effective anti-tumor immunity depends primarily on T cells, but Th17 cells can also promote tumor growth by inducing angiogenesis, as well as playing a role in tumor regression. Lin and colleagues (34) detected Th17 cells in human esophageal squamous cell carcinoma and observed that their levels were inversely correlated with the depth of primary tumor invasion, indicating that a high concentration of IL-17-producing cells in the tumor microenvironment may inhibit tumor invasion. We suggest that CD105-positive vessels may be induced by these kinds of mononuclear cells (i.e. TAMs and Th17 cells) in addition to the influence of angiogenic factors released from the tumor cells. In the present study, however, we did not evaluate CD68-positive cells (TAMs) or IL-17 cells, and consequently this was a study limitation. Further investigations of this issue are warranted.

Considering these findings, we conclude that investigation of MVD using CD34 and CD105 immunostaining could be used to corroborate the presence of superficial squamous cell carcinoma demonstrated by magnifying endoscopy. Infiltration of mononuclear cells such as TAMs or Th17 cells in the lamina propria mucosae may play an important role in angiogenesis at the early stage of esophageal cancer progression.

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Conflict of interest statement
None declared.

References


**APPENDIX**

The depth of invasion of superficial esophageal carcinoma is expressed in accordance with the sub-classification criteria of the Japan Esophageal Society (Guidelines for clinical and pathologic studies on carcinoma of the esophagus) (1).

M1: Carcinoma in situ.

M2: Tumor invasion to the lamina propria mucosae.

M3: Tumor invasion to the muscularis mucosa.

SM1: Tumor invasion to the upper third of the submucosal layer.

SM2: Tumor invasion to the middle third of the submucosal layer.

SM3: Tumor invasion to the lower third of the submucosal layer.

MVD: microvessel density.

LGIN: low-grade intraepithelial neoplasia (borderline malignancy).

IPCL: intra-papillary capillary loop.

SCN: sub-epithelial capillary network.

TP: thymidine phosphorylase.

TAM: Tumor-associated macrophage.