Immunohistochemical Evaluation of Angiogenesis with Anti-CD105 in Pancreatic Tumors

Ruizhe QIAN, Tomoko INAGAKI, Toshiaki KUNIMURA and Toshio MOROHOSHI

Abstract: Angiogenesis is an essential process in the progression of malignant tumors. Whereas pan-endothelial markers, such as CD34, are generally used in evaluation of angiogenesis, pan-endothelial antibodies react not only with developing vessels but also with normal vessels simply trapped within tumor tissues. It has been recently reported that an anti-CD105 antibody preferentially reacts with activated endothelial cells in angiogenic tissues such as tumor tissues. Thus the efficacy of anti-CD105 monoclonal antibody (mAb) in evaluating angiogenesis in pancreatic tumors was assessed. We immunohistochemically investigated 27 cases of pancreatic tumor (including 16 cases of malignant tumor, 2 cases of borderline malignancy and 9 cases of benign tumor). Intratumoral microvessel density (IMVD) was determined with an anti-CD34 mAb (CD34-IMVD) and with an anti-CD105 mAb (CD105-IMVD). More vessels were generally recognized by the anti-CD34 mAb than by the anti-CD105 mAb. The mean CD34-IMVD in malignant tumors and in borderline/benign tumors was 136.13±56.71 and 120.55±40.65, respectively, a result which exhibited no significant difference (P>0.05). In contrast, the mean CD105-IMVD in malignant tumors and in borderline/benign tumors was 19.44±16.53 and 6.36±8.03, a statistically significant difference with P<0.05. Hence anti-CD105 mAb proved to be superior to anti-CD34 mAb in evaluating angiogenesis in pancreatic tumors.

Key words: CD105, CD34, intratumoral microvessel density (IMVD), pancreatic tumor, angiogenesis.

Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vascular bed, is a complex multistep process involving endothelial cell (EC) growth, migration, and microvessel morphogenesis. These processes are controlled by positive and negative angiogenic factors which regulate one or more of these key events. Angiogenesis plays a central role in the growth and development of normal tissues and in a variety of pathologic settings. It is an essential process in the progression of malignant tumors because solid tumors cannot grow beyond 1-2 mm diameter without angiogenesis. Intratumoral microvessel density (IMVD) is a measurement of tumor angiogenesis and can be determined using antibodies against endothelial cells (ECs). It is closely correlated with tumor growth.
and prognosis. One of three different monoclonal antibodies to endothelial cell antigens is frequently used to view tumor blood vessels. These antibodies recognise CD31, CD34, and factor VIII-related antigen, or Von Willebrand factor, which are expressed heterogeneously on most endothelial capillaries and are termed pan-endothelial markers. These antibodies have been used to evaluate angiogenesis and react not only with developing vessels but also with normal vessels simply trapped within tumor tissues. Thus pan-EC antibodies may not be the ideal reagents to visualize tumor-associated blood vessels.

CD105 is a Mr 180000 homodimeric membrane glycoprotein expressed on human ECs. It binds transforming growth factor-β1 and transforming growth factor-β3 with high affinity. It has been demonstrated that anti-CD105 antibodies have greater affinity for “activated” ECs in tissues participating in angiogenesis. Therefore it is potentially a more specific marker for tumor neovascularization.

Pancreatic cancer represents the fourth leading cause of cancer death in men and the fifth in women in the world, and is associated with a very poor prognosis. Usually pan-endothelial markers are used in evaluation of angiogenesis in pancreatic tumors. However, as far as we are aware, no reports have been published which evaluate angiogenesis using anti-CD105 antibodies in pancreatic tumors.

Our hypothesis is that the use of an anti-CD105 antibody should reduce the incidence of staining of normal blood vessels entrapped within a tumor. Therefore it is likely to be a better reagent in the visualization and quantification of IMVD representing new vessel formation in the tumor. In this study, we have compared the IMVD in 27 cases of pancreatic tumor using a mAb to the pan-endothelial marker CD34 and a mAb to CD105, which is more specific for ECs of blood vessels undergoing angiogenesis.

Materials and Methods

Patients

The study included 27 randomly chosen patients with pancreatic tumors. Of these, 16 patients had malignant tumours including 12 with ductal carcinoma, two with mucinous carcinoma, one with invasive carcinoma derived from intraductal papillary-mucinous carcinoma and one with intraductal papillary-mucinous carcinoma. Two patients had solid pseudopapillary tumors of borderline malignancy. The remaining nine patients had benign tumors and these included five serous cystic tumors, one mucinous tumor and three intraductal papillary-mucinous adenoma which had undergone resection. These resected samples were obtained and histopathological diagnosis was confirmed at the First Department of Pathology, Showa University, between 1998 and 2001. The average age at surgery was 63.3 ± 10.9 years (range 46–81 years). The male-to-female ratio was 12:15. The tumors were classified according to the International Union against Cancer criteria (UICC).

CD34 and CD105 immunohistochemistry

Samples were fixed in 10% formaldehyde, embedded in paraffin, and cut into consecutive 3 µm thick sections. Paraffin sections were dewaxed in xylene and rehydrated through a graded alcohol series. We examined immunohistochemically a representative tissue section for each case. For CD34, deparaffinized sections were autoclaved (120°C, 2 atm., 20 minutes) in 20 mmol/L citrate buffer. For CD105, deparaffinized sections were digested
with Proteinase K (S3004, DAKO, Kyoto, Japan 10 mg/ml, 6 minutes). The specimens were incubated with 3% hydrogen peroxide for 6 minutes to quench endogenous peroxidase activity, then incubated for 5 minutes with a protein block to suppress nonspecific binding of subsequent reagents. Activated vessels were visualized by immunostaining with monoclonal antibody against CD34 (M7165, DAKO, Kyoto, Japan) or CD105 (SN6h, M3527, DAKO, Kyoto, Japan). For CD34, sections were incubated for 30 minutes with the primary antibody diluted 1:50 in Tris-buffered saline (TBS). For CD105, sections were incubated for 20 hours at 4°C with the primary antibody diluted 1:10 in TBS. Then sections were stained using the ENVISION technique (K5027, DAKO, Kyoto, Japan). Visualization of the antibody complex was achieved with a diaminobenzidine (DAB) reaction (K3466, DAKO, Kyoto, Japan), resulting in brown staining of activated endothelial cell membranes. Sections were counterstained by hematoxylin 2%.

Vessel counting

Vessel counts within the tumor were assessed by light microscopy after staining for CD34 and CD105. Based on the criteria of Weidner and others12-15), the three areas with the highest number of discrete microvessels were identified by scanning tumor sections at low power (×100). After the areas of highest neovascularisation were identified, microvessel counting was performed at ×200 magnification (×20 objective and ×10 ocular, 0.739mm² per field), and the average counts of three areas were recorded. Any brown stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective-tissue elements was considered a single, countable microvessel12-17).

Statistical analysis

Statistical significance was evaluated by means of the t-test, with P<0.05 as the criterion of statistical significance.

Result

As demonstrated in Fig. 1 A and B, more vessels were generally recognized by the anti-CD34 mAb than by the anti-CD105 mAb in all cases. The CD34-IMVD in malignant tumors ranged from 59 to 245 per 200× high-power field (hpf) with the mean±SD being 136.13±56.71/hpf. The CD34-IMVD in borderline and benign tumors ranged from 74 to 188 at a 200× hpf with the mean±SD being 120.55±40.65/hpf. No significant difference in CD34-IMVD was observed between malignant tumors and borderline/benign tumors (P>0.05). In contrast, the CD105-IMVD in malignant tumors ranged from 3 to 68 per 200× hpf with the mean±SD being 19.44±16.53/hpf. The CD105-IMVD in borderline and benign tumors ranged from 0 to 23 per 200× hpf with the mean±SD being 6.36±8.03/hpf. A significant difference in CD105-IMVD was observed between malignant tumors and borderline/benign tumors (P<0.05) (Fig.2 A and B). No significant difference was observed between the IMVD (CD34-IMVD or CD105-IMVD) and age, gender, tumor location, lymph node metastasis, distant metastasis, pT (UICC) or stage (UICC) in 16 cases of pancreatic cancer (Table 1).
Fig. 1. Immunohistochemical detection of CD34 expression (A) and CD105 expression (B) in ductal adenocarcinoma highlights intratumoral microvessels. In this case the IMVD-CD34 was 245/hpf and the IMVD-CD105 was 68/hpf (original magnification 50×). More vessels were recognized with the anti-CD34 mAb than with the anti-CD105 mAb.

Discussion

Our study demonstrated that more vessels were generally recognized by the anti-CD34 mAb than by the anti-CD105 mAb in all the pancreatic tumors studied which included benign, borderline and malignant tumors. There was no significant difference in CD34-IMVD observed between malignant tumors and borderline/benign tumors ($P > 0.05$). But a significant difference in CD105-IMVD was observed between malignant tumors and borderline/benign tumors ($P < 0.05$). The finding that emerges from this study is that anti-CD105 mAb is more specifically reactive with ECs in pancreatic malignant tumors undergoing angiogenesis.

Studies have demonstrated that anti-CD105 antibodies preferentially react with activated ECs in tissues participating in angiogenesis, such as tumors, healing wounds, psoriasis, embryonic tissue, stroke tissue, and diabetic retinopathy$^{[16-21]}$. Also shown was that pan-endothelial antibodies such as anti-CD34 antibodies and anti-CD31 antibodies react with
normal vessels as well as activated vessels. Tanaka et al\textsuperscript{18} demonstrated that anti-CD105 mAb proved to be superior to anti-CD34 mAb in evaluation of angiogenesis in non-small cell lung cancer. Our result was consistent with this hypothesis.

Kumar et al\textsuperscript{16} and Tanaka et al\textsuperscript{18} demonstrated that the CD105-IMVD is an independent prognostic factor in breast carcinoma and non-small cell lung cancer, whereas the CD34-IMVD was not. They evaluated angiogenesis using an anti-CD34 mAb and an anti-CD105 mAb and reported that the CD105-IMVD showed a statistical correlation with postoperative survival, whereas the CD34-IMVD did not. These results demonstrating the superiority of the CD105-IMVD over the CD34-IMVD as a prognostic factor also support the validity of CD105-IMVD in evaluation of angiogenesis. To confirm the prognostic significance of the CD105-IMVD, further studies need to be conducted. Another study also demonstrated that CD105 expression is a marker of high metastatic risk and poor outcome in breast carcinomas\textsuperscript{22}. However in the present study, we found that there were no significant differences between the IMVD (CD34-IMVD or CD105-IMVD) and age, gender, tumor location, lymph node metastasis, distant metastasis, pT (UICC) or stage (UICC) in 16 cases of pancreatic cancer. The results may suggest that IMVD (CD34-IMVD or
Table 1. Relationship between clinicopathological features and CD34-IMVD or CD105-IMVD in 16 cases of pancreatic cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of patients</th>
<th>IMVD-CD105</th>
<th>P</th>
<th>IMVD-CD34</th>
<th>P</th>
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<tr>
<td>Age (years)*</td>
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<tr>
<td>&lt; mean age</td>
<td>6</td>
<td>14.5±11.3</td>
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<td>141.0±63.0</td>
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<tr>
<td>&gt; mean age</td>
<td>10</td>
<td>22.4±18.9</td>
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<td>133.2±57.7</td>
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<tr>
<td>Gender</td>
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<td></td>
<td></td>
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<tr>
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<td>20.0±11.5</td>
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<td>117.2±50.3</td>
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<tr>
<td>Female</td>
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<td>19.0±20.7</td>
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<td>152.2±61.4</td>
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<tr>
<td>Tumor location**</td>
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<tr>
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<td>21.0±17.8</td>
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<td>Absent</td>
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<td>20.7±23.8</td>
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<td>131.3±76.6</td>
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<td>143.4±45.9</td>
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<td>Distant metastasis</td>
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<td>Stage (UICC)</td>
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<td>I</td>
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<td>135.4±47.7</td>
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</table>

NS, no significant difference observed; S, significant difference observed

*mean age±SD is 63.7±11.3

**one case is head + body

CD105-IMVD) is not useful for predicting the risk of metastasis in pancreatic cancer. More studies are necessary to confirm this and will be performed in the near future.

In conclusion, CD105 proved to be superior to CD34 as a marker for evaluation of angiogenesis in pancreatic tumors. The relationship between the CD105-IMVD and the expression of positive and negative angiogenic factors can be confirmed.

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References

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