Endoglin (CD105) is a more appropriate marker than CD31 for detecting microvessels in carotid artery plaques

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Received: 26 June 2013   Accepted: 18 August 2013   Published: 30 September 13

Abstract

Background: Microvascular proliferation is a major risk factor for plaque vulnerability in patients with carotid stenosis. There are several vascular endothelial markers such as CD31 and CD105, but it is unclear which marker is most sensitive for microvessels. This study sought to examine the correlations between CD31 and CD105 expression in microvessels on carotid plaques and clinical manifestations.

Methods: We studied 13 lesions in 12 patients. The patients underwent carotid endarterectomy and samples were stained for CD31 and CD105. The numbers of microvessels positive for these markers within a field of view were counted.

Results: The average numbers of microvessels were 5.8 ± 5.4 for CD31 and 9.2 ± 9.3 for CD105 (P = 0.04). More microvessels were positive for CD105 than there were for CD31 in patients with diabetes mellitus (P = 0.04).

Conclusion: In patients with carotid artery stenosis, CD105 is more appropriate than CD31 for detecting microvessels in carotid plaques. In patients with diabetes mellitus, CD105 is significantly more highly expressed in microvessels than CD31.

Key Words: Carotid stenosis, CD31, endoglin, microvessels

INTRODUCTION

Several studies such as the North American Symptomatic Carotid Endarterectomy Trial (NASCET)[4] and the European Carotid Surgery Trial (ECST)[24] have demonstrated that the rate of stenosis is related to the risk of cerebral infarction. However, recent vascular biology studies have indicated that the stability of carotid plaques is correlated with the risk of cerebral infarction.[3,5] Intraplaque hemorrhage makes carotid plaques unstable and is one of the minor criteria for vulnerable plaques.[19] Intraplaque hemorrhage is usually followed by neovascularization extending from the vasa vasorum of carotid artery.[22]

CD31 is a marker of vascular endothelial cells and is expressed on microvessels in atherosclerotic plaques, but is stained more weakly than normal arteries.[13] CD105 (endoglin) is a marker of tumor microvessels and is associated with tumor progression.[7] The aim of this study was to compare the expression of CD31 and CD105 in microvessels of carotid artery plaques and to assess the relationships between their expression levels and clinical characteristics.
MATERIALS AND METHODS

Patient population
Between November 2009 and March 2011, we performed 13 carotid endarterectomy operations in 12 patients at Fukui Red Cross Hospital. One patient was operated on bilaterally at different times. Digital subtraction angiography was performed for all patients, and the stenosis rate was evaluated according to NASCET criteria.[4]

Clinical features
Demographic and clinical characteristics were collected from the patients' medical records. Clinical information included symptoms, the presence of risk factors for atherosclerosis (e.g. hypertension, hyperlipidemia, and diabetes mellitus), and the stenosis rate evaluated by digital subtraction angiography.

Sample preparation
Carotid endarterectomy was performed using conventional surgical techniques. All specimens were fixed in 10% formalin overnight and embedded in paraffin the next day. The specimens were stored at room temperature. In each case, multiple and sequential 3-μm tissue sections were cut from paraffin blocks, deparaffinized in xylene, rehydrated, and prepared for immunohistochemical studies.

Immunohistochemistry
Endogenous peroxidase activity in the tissue was blocked by incubation in 3% hydrogen peroxide for 15 min at room temperature. Antigen retrieval was performed by autoclaving in citrate buffer heated to 121°C for 5 min. To reduce nonspecific binding of the secondary antibody, tissue sections were incubated for 10 min with a protein blocking agent. Slides with arterial specimens were then incubated with the specified dilution of primary antibody overnight at 4°C. The anti-CD31 (Novocastra) and anti-CD105 (Novocastra) primary antibodies were mouse monoclonal antibodies and were used at a 1:500 dilution. Sections were incubated in biotinylated goat antimouse (DAKO) secondary antibody at room temperature for 10 min. Horseradish-peroxidase-conjugated streptavidin (DAKO) was applied to sections for 10 min at room temperature. Reacted sections were visualized with 3,3'-diaminobenzidine-tetrachloride (DAB), and counterstained with Meyer’s hematoxylin for nuclear staining.

Immunohistochemical analysis
We counted microvessels using a BX51 microscope (Olympus). After scanning an immunostained section at low magnification (×40), the areas with the greatest number of distinctly highlighted microvessels stained with anti-CD31 were selected, and microvessels were counted at higher power (×100). Microvessels stained with anti-CD105 were counted in an adjacent slice stained with anti-CD31. Vessels were confirmed as microvessels if more than half of the endothelial cells were positive for the marker used. Microvessels were identified based on the architecture, including a lumen lined by endothelial cells as confirmed by staining of an adjacent slice with hematoxylin–eosin.

Statistical analysis
Numerical data are expressed as means ± SD. The statistical significance of differences was estimated using the Wilcoxon signed rank test or Fisher's exact test. Values of P < 0.05 were considered significant. All data were analyzed statistically using R version 2.15.2 for Windows.

RESULTS
The mean age of the patients at the time of operation was 69.6 ± 7.0 years, and all patients were male. The features of onset were cerebral infarction in four patients, transient ischemic attack in two patients, and amaurosis fugax in one patient. Six patients were asymptomatic. The average stenosis rate was 78 ± 14% (50–95%) [Table 1].

The average numbers of microvessels counted in higher power fields were 5.8 ± 5.4 for CD31 and 9.2 ± 9.3 for CD105 (P = 0.04) [Figure 1].

Atherosclerosis risk factors and clinical manifestations for the entire group are shown in Table 2. Ten patients had hypertension and seven patients had diabetes mellitus. All of these patients received appropriate medical treatment [Table 2].

Atherosclerosis risk factors and the numbers of microvessels positive for CD31 and CD105 are shown in Table 3. In the patients with diabetes mellitus, significantly more microvessels were stained with CD105.
More microvessels were stained with CD105, although the difference was not significant. The number of microvessels did not differ significantly according to symptoms.

**Illustrative case**

**Case 1 (Patient 8)**

A 61-year-old male was incidentally found to have left internal carotid artery stenosis. He had hypertension, hyperlipidemia, and diabetes mellitus. Angiography revealed 80% stenosis. He underwent carotid endarterectomy, and the results of pathology are shown in Figure 2. Compared with CD31, CD105 was more strongly expressed in microvessels.

**Case 2 (Patient 1)**

A 78-year-old male presented left hemiparesis and magnetic resonance imaging (MRI) demonstrated cerebral infarction in the right hemisphere. He had hypertension and diabetes mellitus, and angiography revealed 65% right carotid artery stenosis. He underwent carotid endarterectomy, and the results of pathology are shown in Figure 3. The microvessels were faintly stained with CD31, but strongly expressed CD105 in the whole circumference.

**DISCUSSION**

In this study, we found that CD105 was more strongly expressed in microvessels on carotid plaques than CD31. CD105 is a transmembrane glucoprotein expressed on activated vascular endothelial cells\(^{[14]}\) and is an accessory protein for the transforming growth factor-β (TGF-β) receptor system.\(^{[1]}\) A number of studies have shown that CD105 is expressed on endothelial cells of both mature and immature blood vessels\(^{[8,25]}\) and that it is

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Table 1: Clinical and radiological findings and the number of microvessels (vessels/HPF) stained with CD31 or CD105

| Patients | Age (years), gender | Risk factors for atherosclerosis | Clinical manifestations | Stenosis (%) | CD31 (vessels/HPF) | CD105 (vessels/HPF) |
|----------|---------------------|---------------------------------|------------------------|--------------|-------------------|-------------------|
| 1        | 78, M               | Hypertension, diabetes mellitus | Cerebral infarction    | 65           | 18                | 31                |
| 2        | 72, M               | Hypertension, hyperlipidemia     | Cerebral infarction    | 90           | 5                 | 6                 |
| 3        | 81, M               | Diabetes mellitus               | Transient ischemic attack | 95           | 16                | 28                |
| 4        | 57, M               | Hypertension, diabetes mellitus | Cerebral infarction    | 55           | 1                 | 6                 |
| 5        | 61, M               | Hypertension, hyperlipidemia     | Amaurosis fugax        | 50           | 0                 | 3                 |
| 6        | 73, M               | None                            | Cerebral infarction    | 70           | 7                 | 8                 |
| 7        | 61, M               | Hypertension, hyperlipidemia, diabetes mellitus | Asymptom | 80           | 8                 | 12                |
| 8        | 73, M               | Hypertension, diabetes mellitus |Transient ischemic attack | 95           | 6                 | 0                 |
| 9        | 66, M               | Hypertension, diabetes mellitus | Asymptom               | 85           | 6                 | 5                 |
| 10       | 63, M               | Hypertension                      | Asymptom               | 85           | 1                 | 7                 |
| 11       | 72, M               | Hypertension                      | Asymptom               | 90           | 0                 | 0                 |
| 12       | 75, M               | None                            | Asymptom               | 80           | 3                 | 4                 |
| 13       | 73, M               | Hypertension, diabetes mellitus | Asymptom               | 70           | 5                 | 10                |

M: Male, HPF: High-power field, CD: Cluster of differentiation

**Table 2: Summary of clinical cases**

| Clinical or radiological feature | No. (%) |
|---------------------------------|---------|
| Age (years)                     | 69.6±7.0|
| Male                            | 13 (100)|
| Atherosclerosis risk factors    |         |
| Hypertension                    | 10 (77) |
| Hyperlipidemia                  | 3 (23)  |
| Diabetes mellitus               | 7 (54)  |
| Clinical manifestations         |         |
| Cerebral infarction             | 4 (31)  |
| Transient ischemic attack       | 2 (15)  |
| Amaurosis fugax                 | 1 (7)   |
| Asymptom                        | 6 (46)  |
| Stenosis (%)                    | 77±14   |

**Table 3: Clinical data and microvessels (vessels/HPF) stained with CD31 or CD105**

|                      | CD31     | CD105    | P value |
|----------------------|----------|----------|---------|
| Symptom              | 7.6±6.4  | 11.7±11.5| 0.13    |
| Yes                  | 3.8±2.8  | 6.3±3.9  | 0.10    |
| Hypertension         | 5.0±5.1  | 8.0±8.5  | 0.11    |
| Yes                  | 8.7±5.4  | 13.3±10.5| 0.10    |
| No                   | 4.3±3.3  | 7.0±3.7  | 0.25    |
| Hyperlipidemia       | 6.3±5.8  | 9.9±10.3 | 0.10    |
| Yes                  | 8.6±5.7  | 13.1±11.0| 0.18    |
| No                   | 3.8±2.6  | 4.7±2.7  | 0.04*   |

*Significant, HPF: High-power field, CD: Cluster of differentiation
overexpressed in vascular endothelial tissues undergoing angiogenesis.[6,17,25] CD105 is the most suitable marker available to quantify tumor angiogenesis,[8,25] and the density of microvessels stained strongly with CD105 correlates with prognosis in cancer patients.[8,11,20,25] In patients with atherosclerosis, CD105 is a better marker than CD31 to assess the vulnerability of plaques on the coronary artery.[15]

The components of carotid artery plaques have a large impact on the risk of cerebral infarction.[3,5] Naghavi et al.[19] described some of the criteria of vulnerable plaques. These criteria included a large lipid core, a thin fibrous cap, outward remodeling, and intraplaque hemorrhage. An MRI study showed that carotid artery stenoses with intraplaque hemorrhages were associated with a higher stroke recurrence rate than those without intraplaque hemorrhage.[12]

A pathological study revealed a microvascular network (the vasa vasorum) extending from the adventitia through the media and into the thickened intima, and found that nonatherosclerotic vessels rarely had a vasa vasorum.[10] Intraplaque hemorrhage is believed to arise from the disruption of thin-walled microvessels that are lined by a discontinuous endothelium without supporting smooth-muscle cells.[23] Several studies have suggested that intraplaque hemorrhage and rupture of the fibrous cap are associated with an increased density of microvessels.[2,16,18] Therefore, it is important to find a useful marker for detecting fragile microvessels.

CD105, CD31, and CD34 are all used as vascular endothelial markers. CD31 and CD34 are expressed in various tumor microvessels, but they can react with both normal vessels and activated vessels, whereas CD105 has a greater affinity for activated endothelial cells.[21] In the coronary artery, CD31 is more strongly expressed in vascular endothelial cells of normal arteries than in those of arteries with atherosclerosis.[13] These findings suggest that CD105 is a useful marker to examine genuine fragile vessels on carotid artery plaques.

In the present study, the difference in the numbers of microvessels in patients with hypertension and hyperlipidemia was not significant. The difference was only significant for patients with diabetes mellitus. A recent study indicated that, in diabetes patients, angiogenesis is induced and arteriogenesis is impaired in atherosclerotic plaques,[9] so it is suggested that more immature and fragile microvessels are present on carotid atherosclerotic plaques in patients with diabetes mellitus.

CONCLUSION

In patients with carotid artery stenosis, CD105 is more suitable than CD31 for detecting microvessels in carotid plaques. In patients with diabetes mellitus, CD105 is significantly more highly expressed in microvessels than CD31.
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