Expression of RECK in endothelial cells of glioma: comparison with CD34 and VEGF expressions

Nunung Nur Rahmah,[1] Keiichi Sakai,[1,2] Kenji Sano,[3] Kazuhiro Hongo[1]

[1]Department of Neurosurgery, Shinshu University School of Medicine, Matsumoto, Japan

[2]Department of Neurosurgery, Shinshu Ueda Medical Center, Ueda, Japan

[3]Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan

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Corresponding Author:

Keiichi Sakai, M.D.

Department of Neurosurgery, Shinshu University School of Medicine

Asahi 3-1-1, Matsumoto 390-8621, Japan

Tel. +81-263-37-2690; Fax. +81-263-37-0480

e-mail: skeiichi@shinshu-u.ac.jp

Abstract

Purpose: Angiogenesis is thought to involve in progression of glioma grades, and RECK has been said to involve in maturation of vessels. In this study, we aimed to determine whether high micro-vessel density (MVD) expressed by RECK in glioma tissue is correlated with grades of glioma. We also compared RECK expression with that of the formerly known vessel marker, CD34 and VEGF.

Methods: RECK, CD34 and VEGF immuno-reactivities of 72 glioma tissues were studied.

Results: RECK was seen in microvessels of glioma tissues. CD34 showed similar pattern to RECK, whereas VEGF showed positive staining in cytoplasm of tumor cells and endothelial cells. Average MVD with RECK was 107.6 microvessels (range: 7-290). RECK was positively correlated with grades of glioma. RECK and CD34 also showed strong correlation (P= 0.001). Higher frequency of VEGF staining was also correlated with higher grade of glioma.

Conclusions: This is the first study describing expression of RECK in glioma, and its angiogenesis-related nature may provide a potential therapeutic target for glioma treatment in the future.

Keywords: glioma, microvascular density, RECK, CD34, VEGF

Introduction

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) acts as a membrane-anchored metalloproteinase regulator[1-4] which functions as a regulator of extracellular matrix integrity in normal condition, contributing to tumor and metastasis suppressing agent. However, recent study found abundant expression of RECK in the cells associated with blood vessels undergoing rapid remodeling in the mouse implantation chambers, suggesting that RECK has a role in vascular remodeling which may involve non-sprouting mechanisms such as intussusception and pruning.[5] Positive RECK staining was also found in endothelial cells of osteosarcoma, indicating their role in angiogenesis of tumor.[6] However, controversies still remain on the function of RECK.

In glioma, angiogenesis has been told to be the cause of increased grades and malignancy. Angiogenesis markers such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-B) and vascular endothelial growth factor (VEGF) have been mentioned to play a role in this process. [7-13] However, there has not been any published report explaining the angiogenesis profile of RECK in glioma. Therefore in this study, we aimed at exploring RECK role in angiogenesis of glioma by performing immunohistochemistry study and comparing it with other well-known angiogenesis markers such as CD34 and VEGF.

Materials and methods

Patients

This study was approved by the local Ethical Committee of Shinshu University School of Medicine under registration number 1321. Seventy two patients diagnosed with glioma from Shinshu University Hospital were included in this study. Glioma was graded based on WHO classification,[14] details are explained in Table 1. Information on glioma grades was extracted from the medical records.

Immunohistochemical study

For single labeling, glioma tissues in formalin-fixed and paraffin-embedded tissue samples were stained with RECK (R&D Systems, Inc), CD34 (Beckman Coulter, Inc, Fullerton, Calif) and VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Paraffin-embedded tissue samples were washed free of fixative with phosphate-buffered saline (PBS, pH 7.4), exposed to 0.3% H2O2 in PBS for 5 minutes to inactivate endogenous peroxidase, then pretreated with trypsin solution 37°C for 30 minutes for RECK and CD34, and EDTA (microwaved) for 30 minutes for VEGF. The sections were then reacted with primary antibody overnight in a humidity chamber at 4°C. Dilution of primary antibodies was 1:400 for anti-RECK IgG, 1:100 for anti-CD34, and 1:50 for anti-VEGF IgG. Polyclonal goat IgG antibody, as the secondary antibody for RECK, polyclonal mouse IgG antibody for anti-CD34 and polyclonal mouse IgG antibody for VEGF, were applied to the sections for 30 minutes. The color was developed with 0.02% 3-3`diaminobenzidine tetrahydrochloride and 0.006% H2O2 in PBS. The slides were counterstained with Mayer's hematoxylin. Between steps, the slides were washed three times in PBS. RECK expression on human pancreatic tissue served as a positive control.

To determine more specifically co-expression of RECK and CD34 in endothelial cells of glioma, paraffin-embedded tissue samples and frozen-section tissue samples were double stained. Paraffin-embedded double staining was performed using EnVision™ G|2 Doublestain System (Dako, Kyoto, Japan) with 2 different Chromogens: DAB^{+} Chromogen (brown color) for RECK and Permanent Red Chromogen (red color) for CD34. Section incubated without the primary antibody served as negative control. Frozen-section double staining was performed using Rhodamine-labeled anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) for RECK and Fluorescein isothiocyanate (FITC-labeled) anti-mouse IgG (Jackson ImmunoResearch) for CD34.

Frequency of the tumor cell stained was used to calculate VEGF expressions (percentage of stained tumor cell per total number of visible cells) in 400x high power field. RECK and CD34 staining was counted as microvascular density (MVD), which was obtained by manually counting the positive foci for slides counterstained with hematoxylin.

MVD was scored as the number of vessels found in the field, and the final score was the average of 3 most-vascularized areas on high power field (200x).

Statical Analysis

SPSS 18 for Mac (SPSS, Chicago, IL, USA) was used for the statistical analysis. Significance of correlations among RECK, CD34 and VEGF was assessed using Pearson`s correlation. Mean difference of expression based on histological grade of glioma was assessed using unpaired student t-test. Probability values of less than 0.05 were considered significant.

Results

Characteristics of the expression in glioma

In total 72 gliomas were included in the study. They consisted of 26 samples of grade II, 25 of grade III, and 21 of grade IV. Observers were blinded to the grades during counting. Interestingly, RECK disclosed strong staining of the endothelial cells, appearing dark brown with DAB coloring. However, there was no staining of nuclear, cytoplasm or membrane of tumor cells. RECK showed similar pattern to CD34 staining (Figure 1), whereas VEGF showed mild to moderate staining of nuclear and cytoplasm of tumor cells, and endothelial cells (Figure 2).

Correlation of RECK, CD 34 and VEGF with histological grade

MVD count using RECK gave an average score of 107.6 (range: 7-290), and CD34 was 111.6 (range: 34.7-413). CD34 is a well-known marker for endothelial cells, and in this study RECK showed similar pattern to CD34 staining, showing that RECK did stain endothelial cells of glioma tissues. RECK and CD34 expression in grade IV showed significant mean difference between grade II and grade III (P= 0.01, 0.03, 0.002, 0.013, respectively; Figure 3). Positive correlation was also observed between RECK and CD34 (Pearson`s coefficient correlation= 0.56, P= 0.001) (Table 2). In order to confirm the co-expression of RECK and CD34, double staining was performed in this study.

Paraffin-embedded double staining and forezen-section immunofluorescent double staining disclosed the similar location of expression between RECK and CD34 (Figure 4).

VEGF frequency of staining had an average score of 50.8% (range: 0-90), and higher VEGF staining correlated with higher grade of glioma (P= 0.001, 0.001, respectively; Figure 3). VEGF showed various staining patterns, such as aggregated and diffuse staining of the cytoplasm. RECK and VEGF showed different pattern of staining in glioma, and statistical analysis disclosed no correlation between the two (P= 0.369, Table 2).

Discussion

Angiogenesis and the production of angiogenic factors are fundamental for tumor progression in the form of growth, invasion and metastasis.[15] There are two known types of angiogenesis: 1) the so called sprouting angiogenesis, which is characterized by the proliferation and migration of endothelial cells into avascular sites; and 2) the `nonsprouting` angiogenesis or intussusceptive microvascular growth.[16] In glioma, as with malignant tumors elsewhere in the body, angiogenesis is necessary for the growth and progression.[17] Several growth factors are said to involve in glioma angiogenesis, notably platelet-derived growth factor (PDGF),[7] basic fibroblast growth factor (bFGF),[8] epidermal growth factor (EGF),[9] transforming growth factor beta (TGF-B),[18] and vascular endothelial growth factor (VEGF).[10-13]

Among angiogenic agents, VEGF is the most studied one, and its role in driving the angiogenic process in glioma is well known.[10-13] As mentioned by the previous authors, our study also proved the correlation of VEGF expression and glioma grades. In glioblastoma group, the expression of VEGF was at least 70%. Chaudhry et al. in their study of 41 glioma cases disclosed at least 86% immunoreactivity in glioblastoma group,[17] showing the important role of VEGF in progression of glioma. Nico et al. even explained the involvement of VEGF in intussusceptive microvascular growth in glioblastoma.[19] CD34 is well known as endothelial marker, and it gives positive staining in physiologic and pathologic vessels.[20-22] CD34 is also considered an optimum marker for microvascular density studies because of its good immunoreactivity.[22] Netto et al., in

their oligodendroglioma study, found a strong expression of CD34 in the endothelial cells without any significant correlation to the grades of oligodendroglioma.[20] However, in this study, we found a strong expression of CD34 and high grade gliomas showed higher expression of CD34, which means that high grade gliomas have higher density of vessels.

On the other hand, RECK has been widely published as an MMP inhibitor. In normal condition RECK is understood to have a function of regulating extracellular matrix integrity, contributing to inhibition of tumor and metastasis progression. Lack of RECK expression has been noticed in several cancers, and it is correlated with malignancy and poorer prognosis.[23] Our previous study in skull base chordoma also showed concordance result where lack of RECK expression correlated with poorer prognosis of the patients.[24] However, recent studies have disclosed RECK involvement in angiogenesis, where RECK showed positive staining in endothelial cells of neuroblastoma[25] and osteosarcoma.[6] RECK was also found in maternal and embryonic vessels in mice showing its importance in maturation process of vessels.[26]

In this study, we found out that RECK was not expressed in tumor cells, and was the same findings through out all grades. It was found in endothelial cells with increasing number in accordance to its grade. RECK expression in endothelial cells was also proved by its co-expression with CD34. There are controversies of RECK function in tumor. RECK has been said to play a role against progression of tumor, however a recent study found that treatment with an angiogenic factor, or co-culture with hypervascular tumor-derived cells markedly induced RECK expression in human umbilical vein endothelial cells (HUVEC), which indicates that RECK may possess proangiogenic function.[25] Our finding is in discordance with the results from previously published articles, however, a recent study by Miki et al. perhaps can explain the reasons. Cell lines with higher RECK-inducing activity tend to express endogenous RECK, implicating that RECK could be induced in such tumors by an autocrine mechanism. However, depletion of endogenously expressed RECK in one of these RECK-positive tumor cell lines did not result in detectable changes in cell behavior, including proliferation, cell death, migration, and invasion.[25] Therefore, in glioma, instead of acting as inhibitor, RECK maybe

involved in a similar manner, and supplying vessels to tumor cells, thus facilitating tumor growth. Further study is needed to answer this issue. Another interesting point is that in our study, we did not find any significant relationship between RECK and VEGF, even though both play roles in angiogenesis. Perhaps they employ a different path in influencing angiogenesis of tumor, as explained by Song et al. in their study of gastric cancer.[27]. Mechanistic study in order to prove the true angiogenesis function of RECK will be the next focus of our study.

Conclusion

Our immunohistochemistry study demonstrated that RECK was identified in endothelial cells of glioma and tended to increase toward higher grades. This preliminary study also identified expression of VEGF in tumor cells, and that higher expression of VEGF also correlated with grades of glioma. However, RECK and VEGF did not show any significant correlation. Further study is necessary to elucidate its role in angiogenesis of glioma, and its potentials as target therapy of glioma in the future.

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Conflict of Interest

All authors have no financial, commercial, legal, or professional relationship with other organizations or with the people working with us that may exert an influence on this research. Therefore, we have disclosed any conflict of interest in the making of this paper.

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indicator in gastric cancer. Eur J Cancer 42: 101-108, 2006

Figure Legends

Figure 1. Various expressions of RECK and CD 34 in glioma

RECK (A) and CD 34 (B) are expressed in endothelial cells in glioma, showing a highly dense tumor vessels found in high grade glioma (20x).

Figure 2. Expressions of RECK, CD 34 and VEGF based on glioma grades

RECK (A, D, G), CD 34 (B, E, H) and VEGF (C, F, I) are expressed in glioma and were grouped into grades (40x). A,B,C) are showing their expressions in grade II glioma. D,E,F) are showing the expressions in grade III glioma. G,H,I) are showing their expressions in grade IV glioma.

Figure 3. Distribution of MVD and frequency of expressions based on glioma grades

A) is showing MVD distribution of RECK expressions in different grades of glioma. B) is showing MVD distribution of CD 34 expressions in different grades of glioma. C) is showing distribution of VEGF expressions in different grades of glioma.

Figure 4. Double staining expression of glioma

CD34 (A, D), RECK (C, F) and double stained (B, E) expression in a glioblastoma case, showing a positive permanent red expression for CD34 (A) and DAB for RECK (C) in a paraffin-embedded sample (20x). Frozen-section immunofluorescent staining is showing positive FITC staining of the endothelial cells for CD34 (D) and Rhodamine staining of RECK (F) (40x). Colocalization of both staining is proven both in paraffin-embedded and frozen-section samples (B, E).

Table 1. Distribution of glioma cases based on WHO classification

WHO Grading	Type	Frequency
II	Pilomyxoid astrocytoma	9
	Diffuse astrocytoma	10
	Oligoastrocytoma	7
III	Anaplastic astrocytoma	25
IV	Glioblastoma	21
Total		72

Table 2. Correlation among markers (RECK, CD34 and VEGF)

Expression	Pearson Correlation & P Value		
	RECK	CD34	VEGF
RECK		0.56 P= 0.001	0.11 P= 0.39
CD34			0.106 P= 0.416
VEGF			

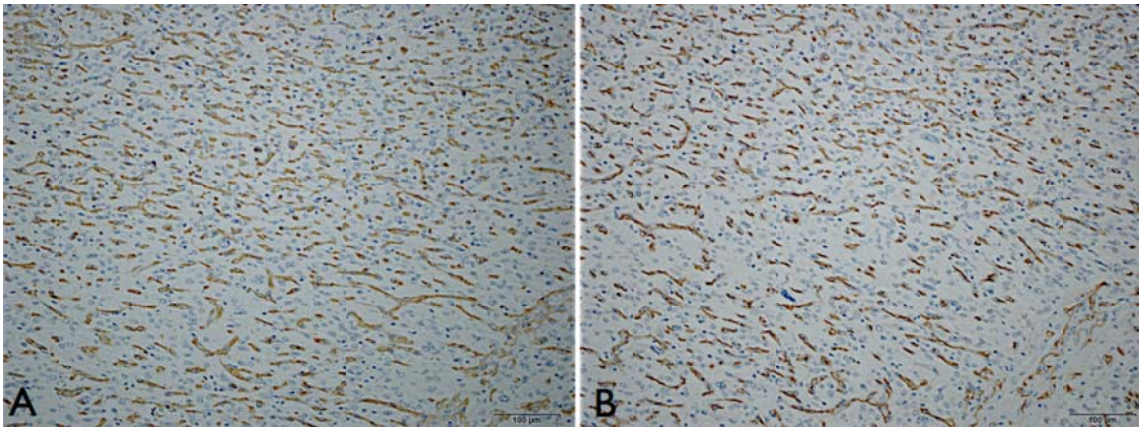

Figure 1.

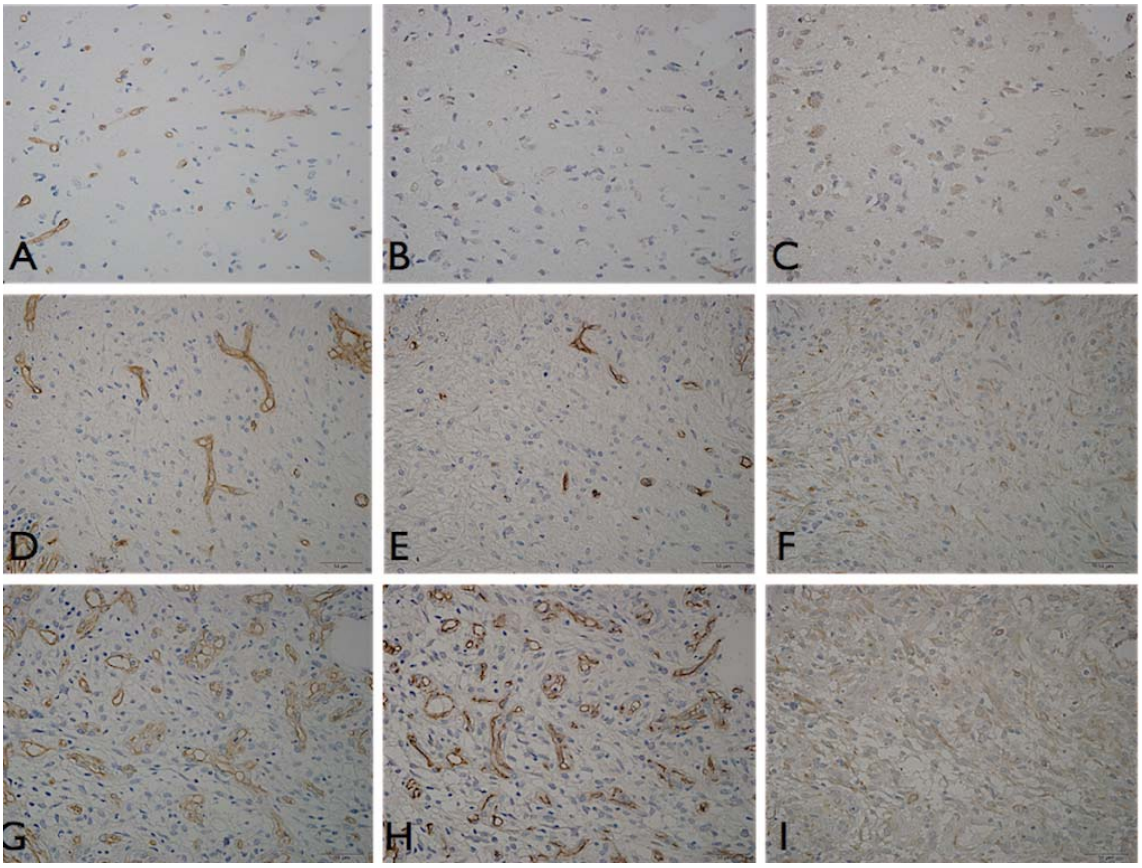

Figure 2.

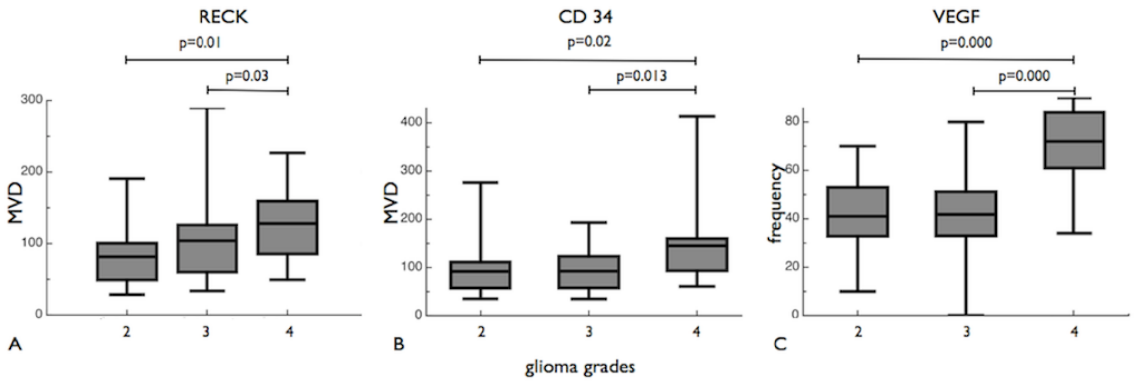

Figure 3.

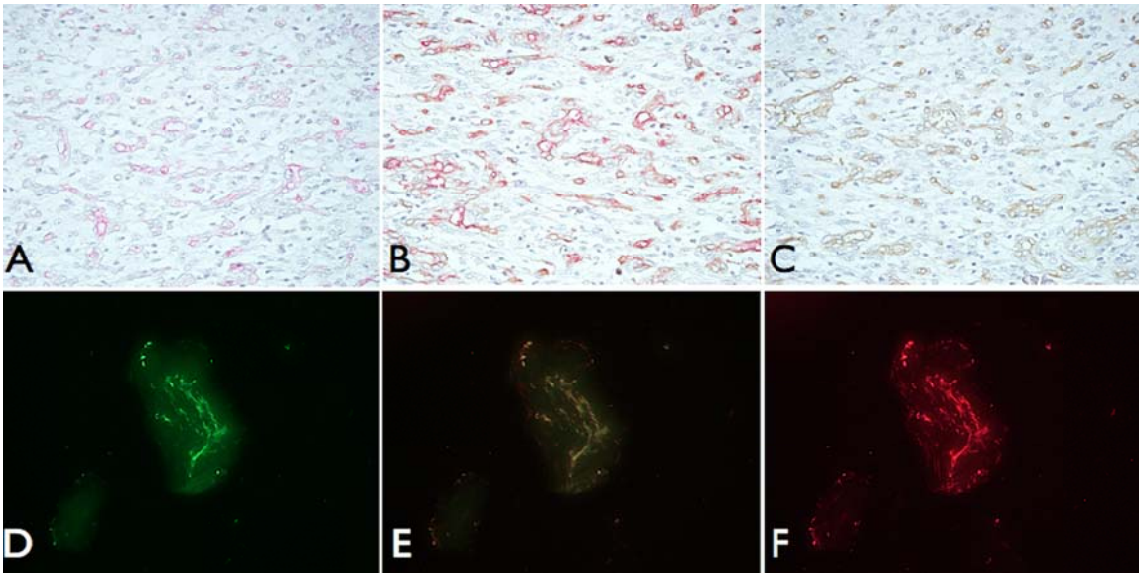

Figure 4.