Role of endothelial cell-selective adhesion molecule in hematogeneous metastasis

CANGARA MUHAMMAD HUSNI

(Degree)
博士（医学）

(Date of Degree)
2010-03-25

(Resource Type)
doctoral thesis

(Report Number)
甲5025

(URL)
https://hdl.handle.net/20.500.14094/D1005025

氏　　名	CANGARA MUHAMMAD HUSNI
博士の専攻分野の名称	博士（医学）
学 位 記 番 号	博い第 5025 号
学位授与の 要 件	学位規則第 5 条第 1 項該当
学位授与の 日 付	平成 22 年 3 月 25 日

【 学位論文題目 】

Role of endothelial cell-selective adhesion molecule in hematogeneous metastasis(血行性転移における血管内皮特異的接着分子の役割)

審 査 委 員

主 査　教 授　横崎　宏
教 授　古瀬　幹夫
准教授　平島　正則

（論文博士関係）

学 位 論 文 の 内 容 要 旨

Role of endothelial cell-selective adhesion molecule in hematogeneous metastasis

血行性転移における血管内皮特異的接着分子の役割

神戸大学大学院医学系研究科医科学専攻
感染病理学
（指導教員：林　祥剛　教授）

CANGARA MUHAMMAD HUSNI

Introduction

Angiogenesis is a phenomenon which new capillaries are generated by sprouting from pre-existing vessels. This process is not only a important for tumor growth but also a major factor for the invasion and metastatic of malignant cells. Interaction between tumor cells and vascular endothelium is important for cancer metastases and depends on the function of inter-endothelial junctions.

Endothelial cell-selective adhesion molecule (ESAM) belongs to the immunoglobulin superfamily and was found at tight junctions. It mediates homophilic interactions between endothelial cells. ESAM plays a role in the growth of primary tumor in mice, and has been shown to regulate endothelial permeability. In this study, we sought to determine the role of ESAM in the hematogeneous metastasis of malignant tumors.

Experimental procedure

Cell culture

B16F10, CHO, L, LLC and EA cells were maintained in DMEM with 10% FBS and penicillin and streptomycin.

Pulmonary hematogeneous metastasis model

B16F10 cells were injected into the tail veins of *ESAM*+/+ and *ESAM*-/- mice. After 3 weeks, the mice were sacrificed and their lungs excised. Lung metastatic nodules's

number was counted macroscopically. For histological analyses, the lungs were stained with hematoxylin and eosin.

Histological characterization of mouse and human tumors

In immunofluorescence, tumor sections were incubated with anti-mouse CD31 antibody and FITC-conjugated secondary antibody. Tumor angiogenesis and vascular morphologies were scored as vessel density, number of branch points per vessel, number of lumens formed, and length of vessels.

Western blotting and immunostaining of human tumor and normal tissues were performed using anti ESAM , anti-VE-Cadherin or anti CD31 antibody .

TUNEL assay

TUNEL assay was performed with an *in situ* apoptosis detection kit.

Cell stimulation assays

EA cells were cultured and stimulated with cancer medium, non-cancer medium, or serum-free medium for 1 day. The cells were then lysed and cell proteins were subjected to western blotting with anti-ESAM or anti-VE-Cadherin antibodies.

In vitro migration assays

Confluent EA cells on culture-disk were transfected with control- or *ESAM*-siRNA and incubated for 1 day. The endothelial wound was created by scraping the monolayer

with a pipette tip. Conditioned medium or serum-free medium was added to the well. The extent of cell migration was assessed by counting the number of cells that had migrated into a clear area.

Proliferation, apoptosis, and tube formation assays

EA cells were seeded onto a culture disk and transfected with control- or *ESAM*-siRNA. Two hours later, the culture medium was replaced with control or B16F10 medium and incubated for 3 days. Cell proliferation was measured with the WST-1 assay.

In apoptosis assay, EA cells were seeded onto culture slide, and transfected with control- or *ESAM*-siRNA. The following day, the culture medium was replaced with control or B16F10 medium and incubated for 3 days. The average number of apoptotic nuclei was calculated by counting condensed, fragmented nuclei's number after stained with Hoechst 33258 .

In capillary tube formation assay, EA cells were plated onto Matrigel-coated plates and transfected with control- or *ESAM*-siRNA. The cells were then incubated with control or B16F10 medium for 16 hours.

Transendothelial cell invasion assay

EA cells were seeded on a Matrigel-coated transwell to form a confluent monolayer and transfected with control- or *ESAM*-siRNA. B16F10 cells were placed in the upper

reservoir. The cells were incubated overnight at 37°C. Migrated cells were stained and calculated.

Statistical analysis

Statistical significance was determined by *t*-test (unpaired). Data were expressed as mean±SEM and differences were considered significant at $P < 0.05$.

Result

ESAM is expressed in the vascular endothelium of metastatic tumors in the human lung

Immunostaining analysis showed that ESAM was expressed in the endothelium of vascular in tumors and co-localization with CD31. Western blotting disclosed that ESAM protein level was significantly higher in metastatic adenocarcinoma of the lung than in normal lung tissue.

Cancer cells induce ESAM expression

Western blotting showed a marked induction of ESAM and VE-cadherin in EA cells exposed to cancer medium, while a non-cancer or control medium did not affect ESAM levels.

ESAM deficiency attenuates lung metastasis of melanoma cells

Assessment of hematogeneous metastasis in the lung showed that the number of metastatic nodules was significantly lower in *ESAM*-/- than in *ESAM*+/+ mice.

Moreover, metastatic nodules in *ESAM*-/- mice were smaller in size than those in *ESAM*+/+ mice.

ESAM deficiency results in reduced angiogenesis and enhanced apoptosis in metastasis

Abundant blood vessels were observed in *ESAM*+/+ mice, while in *ESAM*-/- mice was significantly reduced. Closer examination revealed that blood vessels in *ESAM*-/- mice displayed some morphological changes: the tumor vascular were shorter and had fewer lumens and fewer branch points compared to those of *ESAM*+/+ mice. Furthermore, there were more apoptotic cells in *ESAM*-/- mice than in *ESAM*+/+ mice.

Endothelial migration was selectively reduced by downregulation of ESAM

In an *in vitro* wound healing assay, the B16F10 medium induced migration of control EA.hy926 cells to a greater extent than did the control medium. In contrast, when *ESAM* was downregulated, cells hardly migrated in response to either the control or B16F10 medium. No difference in cell proliferation and apoptosis between *ESAM*- and control-siRNA transfected EA.hy926 cells in response to either control or B16F10 medium.

In the presence of the B16F10 medium, control EA cells formed an tube-like structure with an extensive network, while the formation of this structure was attenuated when the control medium was used. In contrast, when *ESAM* was downregulated, the

tube-like structure was barely induced.

Endothelial ESAM expression does not affect transendothelial migration of tumor cells

Although the B16F10 cells migrated through the monolayer, there was no significant difference of migrated cells's number between the *ESAM*- and control-siRNA treatments.

Discussion

The findings of this study have furthered understanding of the effect of *ESAM* deficiency on blood vessel morphology in tumor tissues. The absence of *ESAM* was found to be associated not only with a reduction in the number of tumor vascular but also in blood vessel morphology and apoptosis of adjacent tumor cells. We were also able to characterize ESAM expression in metastatic tumors of humans. ESAM was expressed in the vascular endothelium of metastatic tumors, and its expression was increased in tumor tissues compared to that in normal tissues, which reflects the increase of blood vessels in metastatic lesions. These findings strongly imply that the ESAM level in tumor tissue would be a marker for its vascular density.

In this study, we have demonstrated that ESAM regulates some of these angiogenic processes and subsequent tumor metastatis. Cancer medium-induced endothelial migration was significantly inhibited when *ESAM* expression was

downregulated by siRNA transfection. However, this downregulation did not alter the proliferation or apoptosis of endothelial cells, suggesting that endothelial migration is selectively regulated by ESAM. Thus, cancer medium-induced endothelial migration is, at least in part, mediated by ESAM expression in endothelial cells. In view of the presence of ESAM in intercellular junctions of endothelial cells, it is reasonable to speculate that ESAM may contribute to the assembly of junctional complexes required for the association of endothelial cells in the tumor vessel wall. In support of this hypothesis, we found that ESAM expression was directly related to the formation of the tube-like network in the presence of metastatic tumors.

Our findings indicate that tumor cells may release unidentified growth factors which induce the expression of ESAM in endothelial cells. In a preliminary study, we examined whether well-known growth factors increase ESAM expression in human umbilical vein endothelial cells and found, as far as we could determine, that none of VEGF, HGF, EGF, insulin or phorbol esters affected *ESAM* expression.

We have demonstrated that ESAM does not contribute to the transendothelial migration of tumor cells. This means that, during tumor metastasis in *ESAM*-/- mice, the attenuation of angiogenic processes may overcome the increase in endothelial permeability. We therefore, speculate that, in the absence of ESAM, the reduced

vasculature in the primary lesion may prevent the tumor cells from entering the circulation, and that the attenuated angiogenesis in the metastatic lesion may inhibit tumor cells growth.

To summarize, we have managed to identify the role of ESAM in tumor angiogenesis and metastasis by using a lung metastasis model. ESAM expression appears to be induced in tumor tissues, and promotes the endothelial angiogenic processes. In particular, ESAM plays a crucial role in endothelial cell migration and tube formation, which is induced by the interaction with cancer cells. The findings of our study have provided a novel insight into the pathogenesis of tumor angiogenesis and indicate that ESAM could well be a candidate molecule for antiangiogenic therapeutic strategy for malignant tumors.

神戸大学大学院医学系研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第2106号	氏名	CANGARA MUHAMMAD HUSNI
論文題目 Title of Dissertation	Role of endothelial cell-selective adhesion molecule in hematogeneous metastasis 血行性転移における血管内皮特異的接着分子の役割		
審査委員 Examiner	主査 Chief Examiner 横崎 宏 副査 Vice-examiner 古瀬幹夫 副査 Vice-examiner 平島正則		

（要旨は１，０００字～２，０００字程度）

Angiogenesis は、新しい毛細血管が既存の血管から発芽するように発生する現象である。この過程は、悪性腫瘍の成長だけでなく、悪性細胞の転移、浸潤にも重要である。腫瘍細胞と血管内皮細胞間の相互作用は、悪性細胞の転移にとって重要で、特に内皮細胞間の接着機能と強く関係している。Endothelial cell-selective adhesion molecule (ESAM)は、イムノグロブリンスーパーファミィリーに属し、血管内皮細胞のtight junction 部位で発見された。ESAMは、マウスで腫瘍の成長と血管透過性の調節に関わっていることが明らかになっている。本研究は悪性腫瘍の転移におけるESAMの役割の解明を目的とし、ESAMノックアウトマウス（ESAM -/-マウス）尾静脈から癌培養細胞を注入し、コントロールマウスと肺転移巣の数と大きさ、その転移巣における腫瘍血管の形成や癌細胞のアポトーシス誘導を病理学的検索で比較した。また、EA細胞（血管内皮細胞由来の培養細胞）にESAM-siRNAを導入し、ESAM発現を減じることにより、増殖能、管状形成能、遊走能、血管透過性の変化について、ESAMの機能をin vitro で解明した。ESAM -/-マウスでは、肺への癌細胞の血行転移巣の大きさ、数ともに明らかに減少し、ESAM -/-マウス転移巣の腫瘍血管数が減少し、癌細胞のアポトーシスがより誘導されていた。ESAM -/-マウスにおいては、腫瘍血管の形態も変化し、腫瘍血管が短く、内腔が狭くなり、分枝が少なくなっていた。また、癌細胞の培養液はEA細胞のESAM発現を誘導した。ESAM-siRNA細胞を使ったin vitroの実験では、細胞の遊走能は、明らかに減少するが、細胞増殖やアポトーシス誘導には、コントロール細胞と差異を認めなかった。

腫瘍血管の形態に対するESAMの影響を検討すると、ESAMが欠乏すると腫瘍血管の数が減少し、癌細胞にアポトーシスを誘導した。また、腫瘍組織のESAMの発現レベルを知ることにより腫瘍血管の発達の程度を推測できることが示された。さらに、ヒトの転移結節の解析においても、ESAMは腫瘍血管で発現が認められ、腫瘍部分で発現が亢進していたことから、ESAMレベルが腫瘍血管の密度を反映していると考えられ、ESAMが腫瘍の血管新生に積極的に関わっている可能性が示唆された。一方、siRNAでESAM発現を抑えるとin vitroで内皮細胞の遊走能が抑えられたが、内皮細胞の増生は抑えられなかった。

以上、本研究により、ESAMが血管の管状形成に関わり、ESAMが直接的に血管形態形成に関わっていることが示唆された。なお、VEGF、HGF、EGF、insulin、phorbol estersはESAM発現を誘導せず、腫瘍血管の内皮細胞がどのような分子機序でESAM発現亢進をしているのかは不明のままである。ESAM -/-マウスにおいて転移結節の数、大きさともに減少するのは、ESAM抑制による血管新生の減少によるものと推察された。本研究では、腫瘍血管の新生におけるESAMの働きをESAM -/-マウスを用いた肺癌転移モデルによりin vivo で解析し、培養細胞へのsiRNA導入によるESAM発現抑制により in vitro でも詳細に解明した。ESAM発現が、腫瘍組織で亢進し、腫瘍血管新生を促進することを明らかにした。特にESAMは、血管細胞の遊走と血管の管状形成に関わっており、それらは、癌細胞との関連で非常に重要であることを示した。

本研究は、癌の腫瘍血管形成におけるESAMの意義を、ESAM −/−マウスと培養細胞へのESAM siRNA導入によるESAM発現抑制実験を用いて研究したものであるが、ESAMが今後、腫瘍血管のモニタリングに応用できる可能性を示し、分子標的治療の標的となりうる可能性を示した価値ある集積であると認める。よって、本研究者は、博士（医学）の学位を得る資格があると認める。