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# 科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Study the antimicrobial activity of VEGF-TFEB for endothelial cells defense against group A streptococcus infection.

研究課題名（英文）Study the antimicrobial activity of VEGF-TFEB for endothelial cells defense against group A streptococcus infection.

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研究成果の概要（和文）：化膿レンサ球菌の劇症型感染症が年に増加しており、その治療法の開拓が望まれている。GASの血管内皮からの侵入経路は感染成立にとって重要であり。今回、内皮細胞において、VEGFがGASに対する抗菌作用を持つことを見出した。内皮細胞にVEGFを添加すると、mTORC1の活性が抑制された。その結果、その基質である転写因子TFEBが脱リン酸化され、リソソームやオートファジーの機能が亢進し、GASに対する殺菌能が増強することが明らかになった。マウスGAS感染モデルおよびヒトのGAS感染患者の血液中のVEGF量と重篤度の相関をもとに、GAS感染症におけるVEGFの役割を議論する。

研究成果の学術的意義や社会的意義
GAS bacteria causes life-threatening diseases. Patients are usually companion with body liquid lost and low blood pressure and shock, due to the blood vessel damage. Here, we provide a strategy for clinical therapy that VEGF may be an option for supplement with antibiotic treatment.

研究成果の概要（英文）：Group A streptococcus (GAS) is deleterious bacteria that causes life-threatening diseases including breakdown of blood vessel. Here, we explored a strategy how endothelial cells could defense against invaded GAS. Vascular endothelial growth factor (VEGF) promotes many diverse biological functions in endothelial cells. We found that supplement of VEGF significantly enhanced GAS clearance in endothelial cells. VEGF inactivated mTOR activity, which resulted in an activation of TFEB, a transcriptional factor crucial for lysosome/autophagy biogenesis. Xenophagy was also partially rescued in VEGF-treated endothelial cells. Furthermore, there is a low VEGF concentration existing in GAS-infected patient sera accompanied with serious disease symptom, such as sepsis also our animal infection model. These suggest endothelial cells are short of VEGF stimulation under GAS infectious condition. We aim to develop this finding toward a future clinical application.

研究分野：感染細胞生物

キーワード：内皮細胞　VEGF　化膿レンサ球菌

**１．研究開始当初の背景：**GAS is a deleterious human pathogen causing pharyngitis and tonsillitis in oral cavity. GAS can colonize in dental plaque and is associated to streptococcal gingivitis and periodontal disease. One serious aspect is that the severity of GAS infectious disease is increasing during the decades. No vaccine is available. GAS penetrate from local infectious site into blood circulation causing life-threatening bacteremia. The interaction between GAS and blood vessel endothelial cell plays a critical step for pathogenesis. Although xenophagy efficiently elucidates GAS in most cells, our finding showed that blood vessel endothelial cells are defective such mechanism to against bacteria, no autophagosome formation. Since autophagy is not the only one process for intracellular degradation, we are wondering whether there is existing alternative strategy for endothelial cells defense against intracellular GAS. **We focused on the role of VEGF-mediated antimicrobial activity against intracellular bacteria.**

**２．研究の目的：**As VEGF is an important supplement for endothelial cells and our preliminary results that additional VEGF treatment successfully decrease GAS number in endothelial cells. We assume that VEGF-enhanced lysosomal degradation may provide an antimicrobial activity for endothelial cells. This is the first time to point out that VEGF is involved in intracellular bacterial survive. Furthermore, VEGF increase intracellular Ca2+ concentration, which may active calcineurin to remove phosphorylation of TFEB, a final step for TFEB activation. VEGF may increase free-form TFEB nuclear translocation and then turn on lysosome/autophagy related gene expression. **The goal of this project is to clarify the mechanism of VEGF-TFEB mediated antimicrobial effects for endothelial cell defense against GAS infection.**

**３．研究の方法**:

We proposed plans into 3 specific aims: (The detail experimental is addressed in the result section.)

**Specific aim 1**: to study VEGF-enhanced endosomal degradation.

**Specific aim 2**: to demonstrate whether VEGF mediate antimicrobial activity through TFEB activation.

**Specific aim 3**: to clarify whether VEGF-mediated TFEB activation rescue xenophagy in endothelial cells.

**４．研究成果:**

**(1) VEGF ameliorated GAS clearance in endothelial cells.** Endothelial cell requires specific growth factors for survive and replication, and however, we found that the ingredients of laboratory cell culture medium are different between brands, whom all claimed their medium are suitable for endothelial cell. Indeed, the morphology of endothelial cells showed no difference between different medium sets. We tested bacterial growth in different culture medium and to our surprise, GAS replication was much decreased in EGM2 set medium cultured endothelial cells. We found there are three contents in the supplement of EGM2 medium, including ascorbic acid, R3-type insulin growth factor-1 and VEGF. We removed each one of them from EGM2 set to evaluate their effects on GAS growth. We found that GAS growth was increased under absent of VEGF in EGM2 set medium. In contract, additionally supplement of VEGF in M200 medium (the other medium set without VEGF) suppressed GAS growth, while it was canceled by VEGF receptor inhibitor. Our results indicate that VEGF stimulation might enhance GAS clearance in endothelial cells. Furthermore, we found VEGF might need autophagy to suppress GAS, as GAS growth is more in VEGF-treated ATG7 KO cells. However, VEGF still mediated a dose dependent suppression on GAS growth in Atg7KO endothelial cells. <u>This indicates that VEGF enhanced GAS clearance through not only autophagy but also other pathway, endosome/lysosome pathway.</u>

**(2) VEGF enhanced lysosome function for endothelial cell against bacteria.** Since VEGF enhance GAS clearance even under the absent of autophagosome formation, we assumed VEGF may directly enhance lysosome function. First, we confirmed that VEGF protein did not affect GAS growth, nether its secreted virulence factors, SLO and SLS, which required for GAS damage endosome membrane to escape into cytosol and required for GAS intracellular survival. Next, we observed lysosome recruitment to GAS. We found VEGF enhanced the efficiency of LAMP-1 recruitment to GAS, where it is absent with galectin 3 (Gal3), a damaged endosome marker. VEGF increased lysosome recruitment, but decreased endosome damage. In addition, we found electron microscopy images showed that large numbers of lysosomes were located near GAS vacuoles, but only in VEGF-treated cells (Figure 1). This indicates that VEGF promotes lysosome fusion on GAS-containing vesicles. GAS virulence is suppressed prior to it make damage on membrane. A previous report has showed that lysosome acidification was required for suppress GAS to release hemolysin, SLO and SLS. We analyzed lysosome pH levels under GAS infection and our results showed that lysotracker positive GAS was absent with Gal3 and VEGF enhance a high percentage of lysotracker on GAS-containing vesicles. These results confirmed that VEGF might enhance vesicle acidification and lysosome function in GAS-vesicles.

**(3) VEGF actives TFEB and increase lysosomal biogenesis.** To study how does VEGF enhance lysosome function, we focused on the transcription factor EB (TFEB), which plays a pivotal role in regulation of lysosome biogenesis. As a recent report has showed that VEGF could induce TFEB activation for vessel biological function. We found that VEGF stimulation could promote TFEB nuclear translocation in endothelial cells. The expression of TFEB downstream genes, including ATPV6 and LAMP-1, have been turned on more by VEGF, and there was a higher protein production of lysosomal proteins, LAMP-1, Rab7, and TFEB. These results confirmed that VEGF could activate TFEB in endothelial cells. To investigate the molecular mechanism of how VEGF signals mediate TFEB activation, we focused on the regulation of phosphorylation on TFEB. It has been reported that mTOR is one of the major kinases to regulate TFEB phosphorylation, and calcineurin, a calcium-dependent phosphatase, is required for dephosphorylation before TFEB turns to a free form to enter nuclear. However, we found that VEGF did not (or just slightly) alternate mTOR activity. Next, we focused on Ca2+-dependent calcineurin phosphatase. Previous reports have shown that Ca2+ as an important signal molecule induced in downstream of VEGF signal transduction, and Ca2+ is mainly released from ER. Here, we also showed that VEGF induces cytosol level of Ca2+. Moreover, this release of Ca2+ is required for VEGF-mediated GAS growth suppression, as blockage of Ca2+ release from ER canceled VEGF-suppressed GAS growth. These results indicated that VEGF-mediated Ca+ signals might be the main pathway for TFEB dephosphorylation and activation.

**(4) TFEB activation is required for defense against GAS infection.** Although TFEB is well known as lysosome biogenesis regulator, it is still unclear whether its activation is involved in intracellular bacterial clearance or not. Here, we showed that GAS infection induced TFEB nuclear translocation in epithelial cells, a well-used *in vitro* infectious cell model, imply TFEB may be involved in bacterial infection. Compare to epithelial cells, however, the efficiency of TFEB in nuclear translocation is lower in endothelial cells than in epithelial cells, which could efficiently suppress intracellular GAS. Here we show that intracellular bacterial infection can induce TFEB activation through negative regulation of mTOR function. However, how mTOR lost its activity is still unclear.

**(5) VEGF-enhanced TFEB activation required for GAS clearance in endothelial cells.** Since VEGF stimulation could promote TFEB activation, we assumed VEGF may rescue the short of TFEB function in

GAS-infected endothelial cells. As results, under GAS infection, VEGF increased TFEB nuclear translocation in endothelial cells, and the efficiency is as well as comparable with it in epithelial cells. VEGF also increased the TFEB downstream gene, ATPV6 and LAMP-1, expression under GAS infection. To study whether the VEGF-mediated GAS clearance is TFEB dependent or not, we evaluated the relationship between GAS killing and the TFEB protein level. As results, both VEGF and overexpression of TFEB could suppress GAS growth, while VEGF treatment in TFEB overexpressed cells did not showed further effects. Furthermore, VEGF-mediated GAS clearance was canceled under TFEB knockdown condition. This indicates that VEGF suppressed bacterial growth is through the TFEB pathway.

**(6) VEGF promoted Xenophagy defense against GAS.** The role of TFEB is not only on regulation of lysosome biogenesis but also on autophagy-related gene expression. It has also been shown that enhancement of TFEB could increase autophagy function. To investigate whether VEGF-promoted TFEB activation also rescue xenophagy in GAS-infected endothelial cells, we observed autophagy marker recruitment on GAS. We knew Gal3 was lower in VEGF treated cells, but however, there was a higher efficiency of LC3 recruitment. Since previous report showed that LC3 marker could not correctly represent as a successful autophagosome surround bacteria in endothelial cells. Conventional transmission electron microscopy also exhibited clear double membrane structures representing isolation membranes (IMs), which are precursors of autophagosomes, in the VEGF-treated group, while only single-membrane structures were observed in non-treated cells (Figure 1). To further identify the truly rescued autophagy function, we observed other autophagic core protein, FIP200, which is included in autophagic complex I critical for isolation membrane formation. The result showed that LC3 surrounded GAS was positive with FIP200 under VEGF treatment. Furthermore, the efficiency of FIP200 recruit to GAS was much enhanced by VEGF, compare to none treatment. Taken together, VEGF-mediated TFEB activation could also promote xenophagy function against bacteria in endothelial cells (Figure 2).

**(7) VEGF signaling is correlated with the severity of GAS infection in vivo.** We examined the relationship between VEGF and GAS infection in vivo. We studied changes in VEGF levels in GAS-infected mice. In a local skin infection model, GAS was inoculated into subcutaneous tissue within an air pouch. After 24-h of GAS infection, when we confirmed that bacteremia did not occur yet in this early stage, air pouch extracts were collected to analyze VEGF levels. We found VEGF significantly increased at the local infection site. These results indicate that VEGF levels are increased at the local infection site. SpeB is a cysteine protease virulence factor, which plays a critical role in the pathogenesis of local infection disease, and SpeB-deficient GAS further increased VEGF levels in local infection site than wild-type GAS. However, it seems not due to a lack of direct degradation of VEGF protein by SpeB proteolytic activity, because SpeB in GAS culture supernatant hardly degrades VEGF. We next measured serum VEGF levels in a mouse model of GAS-induced sepsis. We found that VEGF levels were increased following infection with low-dose GAS through tail vein injection, which was expected due to the immune inflammatory response. However, once GAS reached lethal doses, VEGF levels decreased. A similar trend was also observed in human patients infected with GAS. Patients were divided into non-invasive and invasive disease groups according to their symptomatology. Patients with severe symptoms indicative of bacterial invasion, such as bacteremia, sepsis, and necrotizing fasciitis, exhibited lower serum VEGF levels than those with symptoms not characterized by invasion. The low VEGF levels in the invasive disease group were not associated with patient age. A previous report showed that non-surviving patients with severe sepsis and multiple organ failure had low VEGF levels and higher soluble VEGF receptor 1 (sVEGFR1)

levels than surviving patients. sVEGFR1 functions as a competitive inhibitor by binding with VEGF to reduce the interaction of VEGF-VEGFR1 on endothelial cells. Our sepsis mouse model also showed the same pattern, where low VEGF levels in mice infected with lethal doses of GAS had higher sVEGFR1 levels than those infected with nonlethal doses. Thus, the severity of GAS infection symptoms seems to be correlated with the strength of VEGF signaling. To determine whether higher levels of VEGF signaling protect the host from GAS infection in vivo, we analyzed mortality associated with different levels of VEGF signaling in a sepsis model involving lethal doses of GAS. After systemically infecting mice with GAS at $5\times10^7$ colony-forming units (CFU), we intravenously injected VEGF on days 1, 7, 14, and 21. VEGF treatment significantly increased the mouse survival rate. A similar effect was observed with a higher number of GAS organisms ($1\times10^8$ CFU). Furthermore, mouse mortality was increased when the VEGF signaling pathway was blocked by the oral administration of axitinib, a VEGF receptor inhibitor, at a dose that did not affect mouse survival in the absence of GAS infection. These data suggest that stronger VEGF signaling may help GAS-infected mice overcome acute infection.

Figure 1

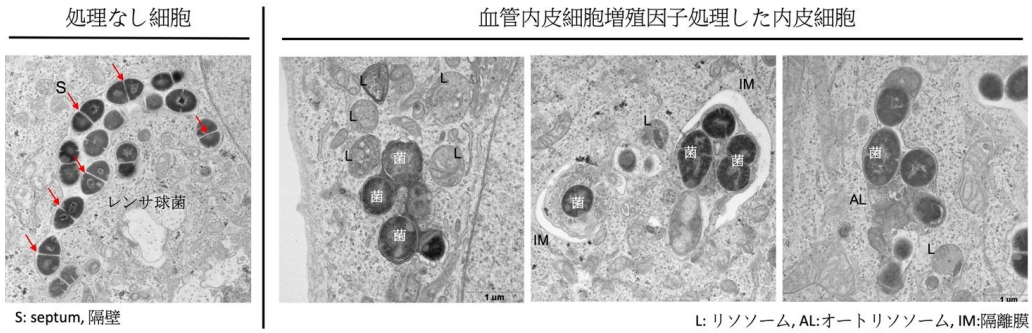
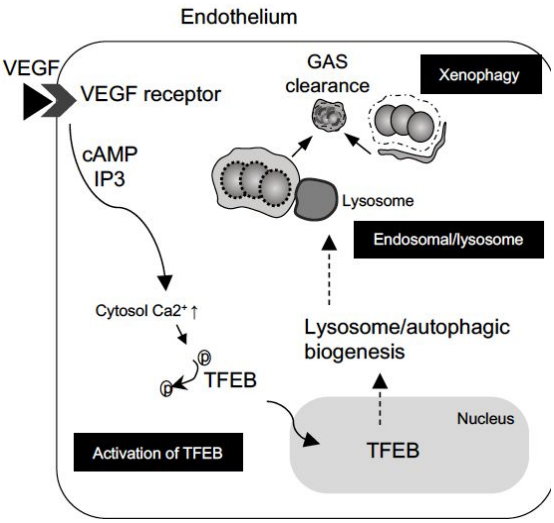



Figure 2




This work was published in *mBio* in 5th of July 2022. The title is "VEGF-Mediated Augmentation of Autophagic and Lysosomal Activity in Endothelial Cells Defends against Intracellular Streptococcus pyogenes".
https://doi.org/10.1128/mbio.01233-22

Also, this work was invited for a punctum article published in *Autophagy reports* in 24th of Oct 2022. The title is "VEGF (vascular endothelial growth factor) provides antimicrobial effects via autophagy and lysosomal empowerment in endothelial cells. https://doi.org/10.1080/27694127.2022.2137755

**Keywords of VEGF, endothelial cells, autophagy, GAS, TFEB, lysosome**

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　1件／うちオープンアクセス　2件）

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|---|---|
| １．著者名<br>Lu Shiou-Ling、Omori Hiroko、Zhou Yi、Lin Yee-Shin、Liu Ching-Chuan、Wu Jiunn-Jong、Noda Takeshi | ４．巻<br>13 |
| ２．論文標題<br>VEGF-Mediated Augmentation of Autophagic and Lysosomal Activity in Endothelial Cells Defends against Intracellular Streptococcus pyogenes | ５．発行年<br>2022年 |
| ３．雑誌名<br>mBio | ６．最初と最後の頁<br>e01233-22 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1128/mbio.01233-22 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスとしている（また、その予定である） | 国際共著<br>該当する |

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| １．著者名<br>Lu Shiou-Ling、Noda Takeshi | ４．巻<br>1 |
| ２．論文標題<br>VEGF (vascular endothelial growth factor) provides antimicrobial effects via autophagy and lysosomal empowerment in endothelial cells | ５．発行年<br>2022年 |
| ３．雑誌名<br>Autophagy Reports | ６．最初と最後の頁<br>555～558 |
| 掲載論文のDOI（デジタルオブジェクト識別子）<br>10.1080/27694127.2022.2137755 | 査読の有無<br>有 |
| オープンアクセス<br>オープンアクセスとしている（また、その予定である） | 国際共著<br>－ |

〔学会発表〕　計5件（うち招待講演　2件／うち国際学会　2件）

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| １．発表者名<br>Shiou-Ling Lu, Hiroko Omori, Chao-Ping Liao, Yee-Shin Lin, Ching-Chuan Liu, Takeshi NODA |
| ２．発表標題<br>化膿レンサ球菌感染におけるVEGFに依存した抗菌作用の解明 |
| ３．学会等名<br>日本分子生物学会 福岡（招待講演） |
| ４．発表年<br>2019年 |

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| １．発表者名<br>Shiou-Ling Lu, Hiroko Omori, Chao-Ping Liao, Yee-Shin Lin, Ching-Chuan Liu, Takeshi Noda. |
| ２．発表標題<br>VEGF activates endo-lysosomal activity through mTORC1-TFEB pathway that suppresses Group A streptococcus infection in endothelial cells. |
| ３．学会等名<br>8th International Symposium on Autophagy (ISA), Taipei, Taiwan（国際学会） |
| ４．発表年<br>2019年 |

１．発表者名
Shiou-Ling Lu, Hiroko Omori, Chao-Ping Liao, Yee-Shin Lin, Ching-Chuan Liu, Takeshi Noda.

２．発表標題
The mechanism of VEGF-mediated antimicrobial effects for endothelial cell defense against GAS infection.

３．学会等名
International Symposium of Infectious Disease, Tainan, Taiwan.（国際学会）

４．発表年
2019年

１．発表者名
Shiou-Ling Lu and Takeshi Noda

２．発表標題
Study the mechanism of VEGF-TFEB mediated antimicrobial effects for endothelial cell defense against GAS infection.

３．学会等名
第11回オートファジー研究会（招待講演）

４．発表年
2018年

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Shiou-Ling Lu and Takeshi Noda

２．発表標題
Study the mechanism of VEGF-TFEB mediated antimicrobial effects for endothelial cell defense against GAS infection.

３．学会等名
分子生物学会 横浜

４．発表年
2018年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

| | 氏名<br>（ローマ字氏名）<br>（研究者番号） | 所属研究機関・部局・職<br>（機関番号） | 備考 |
|---|---|---|---|

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

| 共同研究相手国 | 相手方研究機関 | | | |
|---|---|---|---|---|
| その他の国・地域（台湾） | Department of Microbiology&Immunology | College of Medicine | National Cheng Kung University | |