論 文 要 旨

B cell-derived VEGF-A promotes lymph node remodeling and modulates ensuing immune response

免疫 B 細胞発現 VEGF-A によるリンパ節リモデリングと免疫環境の機能的制御

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【序論および目的】

Lymphatic vessels are not simply inert drainage ducts; rather, they are actively involved in many physiologic and pathologic processes. For example, remodeling of lymphatic vessels by tumor-derived lymphangiogenic factors actively promotes cancer metastasis. Lymphatic vessels are also remodeled in various inflammatory conditions and these remodeled vessels promote inflammation. Recent studies have revealed that lymphatic vessel growth (lymphangiogenesis) is regulated by VEGF-C and -D via their receptor, VEGFR-3. In addition, VEGF-A and its receptor, VEGFR-2, also play an important role in lymphangiogenesis, especially in the enlargement of lymphatic vessels.

During inflammatory conditions, remodeling of lymphatic vessels occurs not only in inflamed peripheral tissues, but also in the regional LNs. Expansion of lymphatic vessels within LNs is important because it enhances the mobilization of DCs to the draining LNs. Expansion of lymphatic vessels within LNs can be locally controlled by lymphangiogenic factors released within the LNs, or remotely controlled by factors released in the peripheral tissues. In the former case, this process depends upon the presence of B cells within the LNs. B cells in inflamed LNs express VEGF-A, and can be stimulated to secrete VEGF-A *in vitro*, suggesting the involvement of B cell-derived VEGF-A in lymphangiogenesis and DC mobilization. However, the exact role of B cell-derived VEGF-A *in vivo* is still unknown.

In this study, we investigated the effect of B cell-derived VEGF-A *in vivo* using transgenic mice that express hVEGF-A specifically in B cells. We found that these mice had enlarged LNs, with expanded lymphatic vessels and increased high endothelial venules (HEVs), even when they were not immunized. To the best of our knowledge, this is the first study describing the effect of B cell-derived VEGF-A *in vivo*.

【材料および方法】

Mice of C57BL/6N background were used to generate the transgenic mice that specifically overexpressed hVEGF-A in B cells. Successful recombination was confirmed by PCR. Histopathological examination and immunohistochemical staining were used for the macroscopic analysis of tissues of the mice. Overexpression of hVEGF-A in transgenic mice was confirmed by RT-PCR analysis, ELISA of serum, tissue homogenates and isolated B cells. Flow cytometry was used to analyze various cell numbers in spleen and lymph nodes. Immunological functions of the mice were analyzed by cytokines measurement after LPS challenge and ovalbumin sensitization and challenge followed by the determination of serum Ab concentration.

【結 果】

Transgenic mice overexpressing hVEGF-A specifically in the B cells were successfully generated. Total amount of VEGF-A (hVEGF-A + mVEGF-A) in spleen homogenates was 3- to 4-fold higher in transgenic mice. We found that the hVEGF-A produced by B cells not only induced lymphangiogenesis in LNs, but also induced the expansion of LNs and the development of high endothelial venules (HEVs). Increased vascularization was also observed around the LNs. We also observed accumulation of mast cells in the LNs of transgenic mice. Enlargement of the spleen was also observed in the transgenic mice. Histological analysis of the spleens from transgenic mice revealed severe distortion of the microscopic structure and sinusoidal dilatations.

Contrary to our expectation, we observed a significant decrease in the antigen-specific antibody production after immunization with ovalbumin (OVA) and in the proinflammatory cytokine production after inoculation with lipopolysaccharide (LPS) in these mice. Although the distribution of T cells and B cells in the spleen of transgenic mice and control mice were similar, we observed that the number of $CD8^+$ T cells in spleen was decreased in transgenic mice To analyze the mechanism by which B cell-derived VEGF-A suppresses the immune response, we analyzed PD-1 expression in the $CD8^+$ cells. These $CD8^+$ T cells expressed high levels of programmed death 1 (PD-1), a negative regulator of $CD8^+$ T cells which we propose as one of the reason for the suppression of immune function in the transgenic mice.

【結論及び考察】

Our findings suggest immunomodulatory effects of VEGF-A: B cell-derived VEGF-A promotes both lymphangiogenesis and angiogenesis within LNs, but then suppresses certain aspects of the ensuing immune responses.

Besides being an angiogenic factor, VEGF-A has recently been identified as a pivotal mediator of inflammation-induced LN lymphangiogenesis. However, the precise role of VEGF-A-induced inflammatory lymphangiogenesis in the modulation of immune function remains unclear. Hosts utilize various components of the immune system to carefully maintain the delicate balance between promoting a proper immune response to invading pathogens and preventing an excessive immune response that can lead to immunopathology. In this study, we have shown that B cell-derived VEGF-A might play a role in maintaining the balanced immune responses, by orchestrating many aspects of the immune responses, including the expansion of lymphatic networks and the suppression of antibody production.

The mechanisms by which B cell-derived VEGF-A suppresses the immune responses have not been fully characterized, however, several explanations can be considered. First, B cell-derived VEGF-A can suppress T cells in spleen. The number of $CD8^+$ T cells in spleen was decreased in transgenic mice, and these $CD8^+$ T cells expressed high levels of programmed death 1 (PD-1), a negative regulator of $CD8^+$ T cells. Second, B cell-derived VEGF-A can suppress the immune responses through the accumulation of mast cells within LNs. Recent studies have shown that angiogenic factors, such as VEGF-A, recruit mast cells at picomolar concentrations, and that mast cells have immunomodulatory functions that can decrease the magnitude or duration of the immune response. In this study, we observed the accumulation of mast cells in LNs of transgenic mice, suggesting the possibility that mast cells might be involved in the immunosuppression observed in $CD19^{Cre}$/hVEGF-A^{fl} mice. VEGF-A can mediate negative as well as positive immunomodulatory roles, and we propose that VEGF-A can stimulate and later suppress certain features of the immune responses.

論文審査の要旨

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B cell-derived VEGF-A promotes lymph node remodeling and modulates ensuing immune response

（免疫 B 細胞発現 VEGF-A によるリンパ節リモデリングと免疫環境の機能的制御）

VEGF-A は血管新生作用を有するとともに、リンパ管新生を促進する。炎症反応に伴うリンパ管新生（炎症性リンパ管新生）は自然免疫から獲得免疫への移行における初期の構造的適応であり、特にリンパ節においては免疫環境最適化へのインフラ的役割をもつと考えられる。近年、リンパ節における炎症性リンパ管新生に関して B 細胞の発現する VEGF-A の中心的役割を示した論文が報告された（Angeli et al. Immunity, 2006）。そしてこの VEGF-A を軸として展開される炎症性リンパ管新生がその後の獲得免疫環境の方向性をどのように修飾しているのかについては検討されるべき課題として残されていた。そこで学位申請者らは CD19 プロモーターによる Cre/LoxP recombination system を用いた B 細胞特異的 VEGF-A トランスジェニックマウス（以下 B-VEGF tg）を作成して VEGF-A によるリンパ節腫脹の構造解析およびマウス個体の LPS への反応性について検討した。

その結果、本研究にて主に以下の知見が明らかにされた。

1）B-VEGF tg のリンパ節においてはリンパ管新生と高内皮小静脈の形成が促されるとともに、リンパ節へのマスト細胞の遊走が観察され、VEGF-A のリンパ節組織における特異的作用が確認された。

2）LPS の腹腔内投与による炎症性サイトカインの発現プロフィールは B-VEGF tg が LPS tolerance を獲得していることを示すものであった。

3）B-VEGF tg は ovalbumin（OVA）感作に対する OVA 特異的 IgG1 の産生を有意に抑制していた。

マスト細胞は近年、自然免疫および獲得免疫に対する免疫制御性細胞として注目されている。今回の知見より B 細胞 VEGF-A は炎症性リンパ節腫脹の中心的役割を果たし、特にリンパ節に特化した脈管機能・構造をもつ高内皮小静脈の形成を制御している可能性と VEGF-A にドライブされるリンパ節腫脹が円滑な獲得免疫反応のプロセスに加えて炎症収束の機転までも包含する機能的構造適応である可能性を示した。

本研究は B 細胞特異的 VEGF-A 過剰発現マウスの作成によりリンパ節腫脹を無菌的に再現し、リンパ節腫脹の構造的変化に伴うマウス個体の免疫環境の機能的適応に新しい知見を加えるとともに、VEGF-A のリンパ節特異的な作用を証明した点において興味深い。よって本研究は学位論文として十分な価値を有するものと判定した。

最終試験の結果の要旨

報告番号	総研第 116 号		学位申請者	スレスタ ビニタ
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主査および副査の５名は、平成 23 年 01 月 19 日、学位申請者 スレスタ ビニタ 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

質問１） Is there any data reporting signal transduction caused by human VEGF-A after binding with mouse VEGFR-2?
（回答） There are several *in vitro* and *in vivo* studies that have indicated that there is little, if any, species-specificity in the effects of VEGF-A (*Endocr Rev*, 2004, 25, 581-611). Also in another study (*Nat. Med.*, 2004, 10: 1095-1103), transgenic mice that overexpressed human $VEGF_{165}$ in the lung showed increased angiogenesis in the lung. Thus, we can say that human VEGF-A can bind with mouse VEGFR-2 and cause signal transduction for the development of blood vessels.

質問２） Production of IgG1 depends on TH2 cells whereas production of IgG2a depends on TH1 cells. In your antibody response experiment, why did you just measure IgG1 level? What might be the TH1 response for the production of IgG2a?
（回答） To evaluate the complete immune response of transgenic mice, we should have measured TH1 cells response (IgG2a) as well as TH2 cell response (IgG1), i.e. measurement of OVA-specific IgG2a and OVA-specific IgG1. But VEGF-A has been reported to enhance TH2 mediated sensitization (*Nat. Med.*, 2004, 10: 1095-1103). So, this time we measured just TH2 response.

質問３） PD-1 is expressed not only in CD8 T cells but also in CD4 T cells. Did you measure PD-1 expression in CD4 T cells?
（回答） The protein PD-1 is expressed by activated T, B, and myeloid cells. Previous studies have shown that increased PD-1 expression was a mechanism by which CD8 T cells became functionally impaired during chronic viral infection(*Nature*, 2006, 439: 682-687) and HIV infection (*Nature*, 2006, 443: 350-354). As the upregulation of PD-1 expression and decreased function of CD8 T cells have been studied in detail, we measured PD-1 expression in CD8 T cells this time. But it should be interesting to measure PD-1 expression in CD4 T cells.

質問４） Spleen size is significantly increased in Tg mice however the cell numbers are not much increased. Is the increase in spleen size in Tg mice due to hyperpermeability?
（回答） In case of spleen of Tg mice, size might be increased due to hyperpermeability as hVEGF-A is over expressed in the B cells of Tg mice and VEGF-A increases permeability of the endothelial cells. However, we have not measured the fluid content.

質問 5. Are there ascites or edematous condition present on histological examination of other tissues in the Tg mice?
（回答） Among the tissues we examined histologically such as spleen, LNs, liver, lung, pancreas and heart, we did not notice edematous condition. As for the presence of ascites, during dissection of Tg and Cre mice, we observed more fluid in the abdominal cavity of Tg mice. But we have not measured the fluid volume.

質問 6. LNs size is increased in Tg mice. Does number of cells in LNs depend upon the size of LNs? Did relative cell number increase in the LNs?
（回答） Based on the following data, we speculate that the relative cell number decreased in Tg mouse LNs. The average LN weight of Tg mice is 2.55 times heavier than that of the Cre mice. The total numbers of CD19 cells are 1.89 times higher; CD8 cells are 1.61 times higher; CD4 cells are 1.76 times higher; CD11b cells are 1.51 times higher and 33D1 cells are 1.23 times higher in Tg mice. So, the number of cells per gram of LN is lower in Tg mice.

質問 7. In ovalbumin sensitization and challenge experiment, why is spleen size increased in Tg mice challenged with ovalbumin but not in the Cre mice challenged with ovalbumin?
（回答） Spleen size is not usually increased by ovalbumin sensitization in normal mice as reported in previous study (*International Immunology*, 2005, 17:705-712). However, in case of our Tg mice, spleen size is significantly increased. The mechanism of this observation is strongly suggested to be due to the overexpression of human VEGF-A in B cells, however the detail mechanism has not been analyzed.

質問 8. In ovalbumin experiment, is the increase in spleen size in Tg mice due to increased cell numbers or is it due to hyperpermeability?
（回答） We have not checked it yet because we have used spleen samples for measuring IgG levels. But it must be interesting to check whether the number of B cells and plasma cells in spleen is higher in OVA-challenged Tg mice.

最終試験の結果の要旨

質問 9. In histological staining, how did you evaluate the mast cell by toluidine blue staining?
（回答）We stained mast cell with toluidine blue in acidic condition. Toluidine blue is blue in color. Mast cell should stain red-purple (metachromatic staining) and the background stain blue (orthochromatic staining). As the staining was according to the stated condition, we confirmed that staining of mast cell was successful.

質問 10. What do you mean by the distortion of spleen structure?
（回答）In our paper, we referred to the disorganized structure of red pulp area and white pulp area and the increase of sinusoidal dilatation as distortion of spleen structure.

質問 11. What is the number of macrophages in lymph nodes and spleen?
（回答）We did not use macrophage-specific antibody but used CD14 antibody which detects cells such as monocytes, Kupffer cells in addition to macrophage. $CD14^+$ cell numbers were as follows: Lymph node Tg: 133583 Cre: 141750; Spleen Tg: 1999365 Cre: 1170614

質問 12. What is lymph node remodeling? What are the histological criteria of the lymph node remodeling?
（回答）In previous studies (*Immunity*, 2006, 24:129-131; *Am J Pathol*, 2010, 176: 1525-41; *PLoS pathogens*, 2009, 5:1-12), increase in the size of LNs and increase in the lymphatic vessels within the LNs is used to be referred to remodeling of the nodal lymphatic architecture. In our study, we observed the increase in LN size as well as increase in lymphatic vessels and high endothelial venules in Tg mice, so we referred it as lymph node remodeling.

質問 13. C57BL/6N mouse is LPS resistant mouse. Why did you use this mouse for LPS challenge experiment?
（回答）When we generated transgenic mice, we did not consider LPS challenge experiment. However as both Tg and Cre mice are of C57BL/6N background, we suppose that the results can be compared in these two groups.

質問 14. Is lymphangiogenesis in Tg mice caused directly by VEGF-A or by the enhanced production of VEGF-C?
（回答）VEGF-A can directly promote lymphatic vessel enlargement via VEGFR-2 signaling (*J. Exp. Med.*, 2007, 204:1431-1440). Besides it, inflammatory macrophages, in response to stimulation with VEGF-A, release VEGF-C/-D that contributes to lymphangiogenesis (*J. Clin. Invest.*, 2004, 113: 1040-1050). We observed that the levels of VEGF-C mRNA (but not of VEGF-D mRNA) increased in Tg mice. So, we suggest that VEGF-A promotes lymphangiogenesis in Tg mice, either directly or via the up-regulation of VEGF-C.

質問 15. In PD-1 protein level, fluorescence intensity is very low. There might be no difference between Tg, Cre or LoxP mice. What is the positive control used?
（回答）We should have used positive control cells that obviously express high amount of PD-1 protein level. But in this experiment we have not used any positive control so we think that we need to confirm this result once again.

質問 16. In ovalbumin experiment (Figure 5C), why is there no difference between the size of spleen in Tg mice and Cre mice in control group though it is shown that spleen size is increased in Tg mice normally in figure 4A?
（回答）Spleen size increased in Tg mice after age of 14 weeks. But in case of ovalbumin experiment we used young mice of age about 10-12 weeks. So the spleen size in the control group of Tg and Cre mice was similar.

質問 17. In your mice, B cells express human VEGF-A. In physiological condition what type of cells can produce VEGF-A?
（回答）VEGF-A is expressed by vascular smooth muscle cells, folliculostellate cells, monocytes and macrophages in physiological condition (*Science*, 1989, 246:1306-1309). VEGF-A is also expressed by podocytes (*N Engl J Med*, 2008, 358: 1129-1136), megakaryocytes and platelets (*Proc Natl Acad Sci USA*, 1997, 94: 663-668) and various types of cells such as in epithelial and mesenchymal cells and B cells (*Immunity*, 2006, 24: 203-215; *Leuk Lymphoma.* 2000, 38:387-94; *Br J Haematol.*, 1999, 104:482-5).

質問 18. Why is hVEGF-A over-expressed in mice instead of mVEGF-A?
（回答）Because it is easy to detect the expression level of hVEGF-A which helps us to know how much of the hVEGF-A is overexpressed. Also if we wish to suppress the effect of hVEGF-A overexpression and see the effect afterwards then it is possible to block the function of overexpressed hVEGF-A using antibody against hVEGF-A that does not recognize mouse VEGF-A. This will not affect the normal function of mouse VEGF-A.

質問 19. If HEVs are increased in LNs, what kind of condition occurs?
（回答）Increase in HEVs causes increased lymphocyte entry into the LNs, this will increase immune response (Immunity, 2001, 15:237-247).

質問 20. In LPS challenge experiment, productions of proinflammatory cytokines are reduced. LPS binds with TLR-4 which is expressed on macrophages. Do you think these cytokines source are macrophages?
（回答）Macrophages express TLR-4, and are important especially in the initial phase of LPS response. Activated macrophages release cytokines which then activate other types of cells leading to further cytokine release. So, we cannot say macrophages are the sources of the cytokines we measured. But, macrophages should be important in the initial reaction.

質問 21. During ovalbumin experiment, mice were challenged with ovalbumin on day 21, 22 and 23 and samples collected on day 25. Do you get maximum response during this short period?
（回答）We referred to *Nature*, 2008, 453:1122-1126 for our experiment of ovalbumin sensitization and challenge. In another paper (*Nat Med*, 2004, 10:1095-1103), mice were challenged with ovalbumin for 10 days and samples collected after a week of challenge. Although we might not have got maximum response in this experiment, the result is sufficient to compare antibody production in Tg and Cre mice.

以上の結果から、５名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。