

学位論文の内容の要旨

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論文題目	Repeated antigen painting and sublingual immunotherapy in mice convert sublingual dendritic cell subsets

(論文内容の要旨)

The sublingual mucosa (SLM) is utilized as the site for sublingual immunotherapy (SLIT) to induce tolerance against allergens. The contribution of SLM-dendritic cells (SLM-DCs) has not been clarified. The aim of this study was to examine the dynamics and phenotype of SLM-DCs after topical antigen painting and SLIT. SLM-DCs were histologically evaluated after FITC painting. A novel murine Japanese cedar pollinosis (JCP) model was generated and change in SLM-DCs after SLIT was examined. The density of SLM-DCs was clearly lower compared with buccal mucosa and dorsal surface of the tongue. Topical FITC painting on the SLM induced maximal recruitment of submucosal DCs (smDCs) at 6 h, but most smDCs had vanished at 24 h. Repeated painting on the SLM induced exhaustion and conversion of the smDC phenotype. CD206^{high}CD11c^{low} round-type smDCs with fewer dendrites became dominant. In the murine model of JCP, SLIT efficiently inhibited clinical symptoms and allergen-mediated immunological responses. SLIT markedly reduced the number of SLM-DCs, converted to the round-type dominant phenotype and inhibited the activation of regional lymph node DCs. Topical antigen painting on the SLM induced rapid exhaustion and conversion of smDCs. The unique dynamics of SLM-DCs may contribute to tolerance induction in SLIT.

<Introduction>

The oral mucosa is a type II mucosal surface covered by a stratified squamous epithelium, which share many features with the skin and serves as a convenient route of low-molecular-weight drug delivery due to its high permeability and easy accessibility. The oral mucosa also represents a natural barrier and sensor against microbial pathogens. The sublingual mucosa (SLM) has long been used as a route for sublingual immunotherapy (SLIT), which induces tolerance against allergens.

Dendritic cells (DCs) are the most potent initiators of innate and adaptive immune responses and regulate immunity and tolerance by integrating various signals. DC

populations are highly heterogeneous and local microenvironments greatly affect their differentiation, maturation, and function. Oral mucosal DCs (OMDCs) play a pivotal role in induction of immune responses in concert with epithelial cells. In the steady state, at least two types of DCs, Langerhans cells (LCs) and interstitial/submucosal DCs (smDCs), exist as resident DCs. However, little is known about the physiological features of OMDCs after antigen (Ag) exposure. We previously examined the local distribution and dynamics of buccal mucosa (BM)-DCs after Ag application. After FITC painting, MHC class II (MHC II)⁺CD207⁻ DC numbers in the submucosal layer increased markedly, peaking at 6 h. In the regional LNs (RLNs), four Ag-captured migrating DC subsets were identified: resident CD11b^{hi} smDCs, newly recruited blood-derived smDCs, resident CD207⁺ smDC, and resident LCs.

Japanese cedar (*Cryptomeria japonica*) pollinosis (JCP) is the most common allergic disease in Japan and is considered a national affliction. Clinical trials showed that SLIT ameliorated JCP symptoms and improved QOL scores. However, the immunological mechanisms of SLIT and the contribution of SLM-DCs remain unclear.

To understand biological features of SLM-DCs, we focused on their distribution and dynamics after topical Ag application and examined SLM-DCs histologically in comparison to BM-DCs. We further examined SLM-DC status after SLIT in a newly established murine JCP model.

<Methods>

Mice

Female BALB/c mice were purchased from Japan SLC (Hamamatsu). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Topical painting on oral mucosae and immunohistochemistry

Five μ l of 3% FITC was applied onto the right side of the BM or the ventral side of the posterior tongue (SLM). Painted regions of mucosal tissues were surgically dissected, embedded in Tissue-Tek, frozen, and stored at -80°C until use. Serial cryostat sections were fixed in cold absolute acetone and subjected to enzymatic immunohistochemistry.

Murine model of JCP and SLIT

Mice received nasal instillation of 10 μ l each of Japanese cedar (*C. japonica*) pollen grains (5 mg/ml in PBS) onto both nostrils twice a week (12-13 times in total) as sensitization. The sensitized mice were divided into two groups as the mean IgE and pollen-IgG1 titers are comparable, and SLIT was started. Based on the therapeutic schedule of SLIT for JCP, gradually escalated doses of cedar pollen extract were applied onto the ventral surface of the tongue for continuous 4 weeks. Three days after the final SLIT application, challenge was performed by intranasal instillation for 7-8 days. Following awakening from the final challenge anesthesia, frequency of sneezing and duration of rubbing were recorded. Sixteen

hours after the final challenge, blood, spleens, RLNs (submandibular and cervical LNs), bronchoalveolar lavage fluid (BALF), and craniofacial tissues were collected for immunological assessment. Serum IgE and pollen-IgG1 in sera and pollen-specific cytokine production by splenocytes were measured by ELISA. Eosinophil infiltrations in BALF and coronal nasal tissue sections were evaluated by Diff-Quick and Luna staining. Expression of CD86 and MHC II in RLN-DCs and the percentages of Foxp3⁺ Tregs in blood and spleens were evaluated by flow cytometry.

<Results>

DCs in the BM, SLM, and dorsal surface of the tongue (DST) were assessed by MHC II and CD207 staining. Epithelial LCs co-expressed MHC II and CD207 and the distribution and numbers of MHC II⁺ versus CD207⁺ DCs within the epithelial layer were comparable in the three sites. Few CD207⁺ DCs were observed in the lamina propria (LP). These DCs are counterparts of dermal CD207⁺ DCs. Thus, we divided MHC II⁺ smDCs into CD207⁻ and CD207⁺ smDCs. The densities of SLM-DCs in all three subsets were lowest.

At 2 h after FITC painting, LCs and smDCs showed markedly increased MHC II signals in the BM and SLM. Despite the changes in signal strength and cell size, LC numbers were not affected until 10 h, and were markedly decreased at 24 h in both mucosae. FITC painting increased CD207⁻ smDC numbers in both mucosae. The density peaked at 6 h, and then decreased gradually. Notably, SLM CD207⁻ smDCs were almost exhausted at this time point. Our results show that despite the limited number of resident SLM-DCs, CD207⁻ smDCs were recruited quickly after topical painting, but disappeared at 24 h.

We next examined DCs after repeated FITC painting. MHC II⁺ smDC numbers were markedly decreased in the secondary painted SLM, but not in the BM. In the repeatedly painted SLM, some MHC II⁺ smDCs exhibited larger nuclei and a round morphology with less dendrites. These cells were preferentially located in the deeper LP region. Round-type cells were also observed in the FITC-painted BM, but their proportion was markedly higher in the SLM. To clarify phenotypic differences, we examined additional Ag expression. CD11b and CD206 were expressed in all smDCs, but the intensity of both Ags was higher and the expression of CD11c was lower in the round-type cells.

We established a new murine JCP model using whole cedar pollen grains without the use of adjuvant and performed SLIT using cedar pollen extract. Sensitized and challenged control mice exhibited severe sneezing and rubbing after the final challenge. These clinical manifestations were significantly inhibited by SLIT. Similarly, SLIT markedly reduced the elevated serum titers of total IgE and pollen-IgG1, the enlarged epithelial thickness and eosinophil infiltration in the nasal mucosa, and the eosinophil percentages in BALF. The increased secretion of the Th2 cytokines IL-4, IL-5 and IL-13 was significantly suppressed in the SLIT mice. Pollinosis and SLIT did not affect spleen and blood percentages of Foxp3⁺

Tregs.

To assess the local DC distribution after SLIT, SLM-DCs were evaluated at 10 h after the final treatment. In the control group, abundant MHC II⁺ smDCs were observed near the basal cell layers, which co-expressed CD11b, CD206, and CD11c. By contrast, markedly fewer smDCs were observed in the SLM after SLIT. Most cells expressed high levels of CD11b and CD206, but low levels of CD11c, and exhibited a round-type morphology. Despite markedly fewer MHC II⁺ smDCs after SLIT, the proportion of round-type cells was increased. The percentages of MHC II^{high}, CD86⁺, and MHC II^{high}CD86⁺ cells in CD11c⁺ DCs were significantly reduced by SLIT. FACS profiles revealed that the majority of MHC II^{high} DCs expressed CD86, suggesting a more activated status. These results indicate that RLN-DC activation was reduced by SLIT.

<Discussion>

We demonstrated great differences in OMDC distribution between three mucosal sites. OMDC distribution seems to be closely correlated with exposure to commensal flora and dietary antigens and anatomical/ histological alternation. The higher flow of saliva may reduce the risk of such exposure. Our observation that the DC density in the SLM was markedly lower is reasonable. Most CD207⁻ smDCs in both mucosae disappeared at 24 h, suggesting migration to RLNs. The supply of new DCs to the SLM seemed to be very limited. Interestingly, we found morphologically different two types of smDCs. The proportion of round-type cells was higher in the SLM and repeated Ag painting and SLIT yielded a round-type cell-dominant status. Our round-type smDCs seemed to partly resemble the CD11b⁺CD11c⁻ cells identified by Mascarell et al.(2008). This subset functions as immunogenic APCs and has tolerogenic properties as assessed by IL-10 production and Treg generation. This may be due to the combined use of BM-DCs and SLM-DCs. CD11b⁺CD11c⁻ lingual cells co-expressed CD206 and high levels of mRNA for retinaldehyde dehydrogenase 2 (RALDH2), suggesting similarity to tolerogenic macrophages reported in the LP of the small intestine. Despite the similar levels of smDC recruitment after initial painting, secondary and repeated Ag painting failed to induce additional recruitment. The exhaustion of resident DCs and increased proportion of round-type cells seemed to closely correlate with this phenomenon.

SLIT in our JCP model efficiently inhibited Th1 and Th2 cytokines, and the regulatory cytokine IL-10. This suggests that the efficacy of SLIT cannot be explained by Th1/Th2 immune deviation. The exhaustion and phenotype conversion of local SLM-DCs and inactivation of RLN-DCs suggest a contribution of DCs to SLIT-induced tolerance. In humans, IL-10 production by monocytes and T cells was significantly higher in non-allergic individuals, and patients who received allergen-specific immunotherapy produced high levels of IL-10. In JCP patients who received SLIT, IL-10⁺Foxp⁺ Treg elevation is a good biomarker

in the improved-QOL group. In our mouse model, the proportion of Foxp3⁺ Tregs was not affected by pollinosis or SLIT. Few Foxp3⁺ Tregs as well as Foxp3⁺ T cells were detectable in the SLM (data not shown). Further studies are required to demonstrate the involvement of unique SLM-DC types in SLIT tolerance mechanisms.

<和文による要約>

舌下粘膜は、アレルゲンに対する免疫寛容を誘導する舌下免疫療法の抗原塗布部位として使用されている。しかしながら、舌下粘膜樹状細胞 (SLM-DC)の関与については明らかになっていない。本研究では、抗原の局所塗布あるいは舌下免疫療法後の SLM-DC の動態とフェノタイプを解析した。FITC 塗布後の SLM-DC を組織学的に検討すると共に、新規に樹立したスギ花粉症マウスモデルで、舌下免疫療法後の SLM-DC の変化を調べた。SLM-DC の密度は、頬粘膜および舌背粘膜と比較し明らかに少なかった。舌下粘膜への FITC 塗布は、6 時間をピークに粘膜下樹状細胞 (smDC)をリクルートさせたが、24 時間後にはほとんど消滅していた。舌下粘膜への反復抗原塗布は、SLM-DCs の枯渇と樹状突起の少ない円形の CD206^{high}CD11c^{low} 細胞優位状態へのフェノタイプ変化を誘導したが、この特徴は頬粘膜とは異なるものであることが明らかとなった。スギ花粉症モデルにおける舌下免疫療法は、臨床症状およびアレルゲンに対する免疫応答を効果的に抑制し、SLM-DCs 数を減少させ、円形タイプ優位のフェノタイプ変化をもたらし、所属リンパ節樹状細胞活性化を抑制した。舌下粘膜への抗原局所塗布は、smDCs の急速な枯渇とタイプ変化をもたらした。これらのユニークな SLM-DC の動態変化が舌下粘膜療法による免疫寛容誘導に関与していることが示唆された。

論文審査の要旨および担当者

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<p>(論文審査の要旨)</p> <p>樹状細胞は、自然免疫および適応免疫応答をリンクさせ、T 細胞の抗原特異的な免疫応答と免疫寛容を調節する免疫応答制御に重要な細胞である。舌下粘膜は、アレルゲンに対して免疫寛容を誘導する目的で、舌下免疫療法(sublingual immunotherapy)における抗原反復投与部位として使用されている。同時に、舌下粘膜は、病原体に対する防御応答を増強させるための舌下ワクチン投与部位としても検討されており、マウスモデルでは免疫応答増強が報告されている。</p> <p>張 晨陽は、舌下粘膜が、相反する免疫応答と免疫寛容誘導の場として利用されることに視点を向け、その制御が舌下粘膜に存在する樹状細胞に起因するのではないかと考えた。そこで、舌下粘膜への抗原塗布前後の粘膜に存在する各樹状細胞サブセットの動態を、頬粘膜への抗原塗布と比較しながら、検討を行った。また、舌下免疫療法における舌下粘膜樹状細胞の変化を観察するために、スギ花粉粒を用いたマウススギ花粉症モデルを新規に樹立し、そのモデルで舌下免疫療法を実施し、その抑制効果を確認後、舌下粘膜および所属リンパ節における樹状細胞動態と表現型の変化を検討した。</p> <p>本研究結果は以下のとおりである。</p> <ol style="list-style-type: none"> 1.未刺激における舌下粘膜における3サブセットの樹状細胞（ランゲルハンス細胞、CD207 陽性および CD207 陰性粘膜下樹状細胞）密度は、頬粘膜や舌背粘膜と比較して有意に小さかった。 2.舌下粘膜への FITC ハプテン抗原塗布は、6 時間をピークに粘膜下樹状細胞の集積を促したが、24 時間後にはほとんど消失していた。 3.舌下粘膜への反復 FITC ハプテン塗布は、粘膜下樹状細胞を枯渇させると同時に、樹状突起の少ない円形タイプの CD206^{high}CD11c^{low} マクロファージ様細胞優位な状態へのフェノタイプ変化を誘導した。 4.スギ花粉粒を用いて、ヒト花粉症とよく似た症状を呈するマウス花粉症モデルを樹立した。 5.スギ花粉症モデルにおいて、花粉エキス濃度を漸増させながら舌下免疫療法を実施したところ、臨床症状およびアレルゲンに対する抗体産生および T 細胞免疫応答が効果的に抑制されていた。 6.舌下免疫療法での最終スギ花粉エキス塗布から16時間後の舌下粘膜を調べたところ、粘膜下樹状細胞数が減少し、円形でマクロファージ様細胞優位のフェノタイプ変化が起こっていた。 7.舌下免疫療法により所属リンパ節樹状細胞の MHC class II および CD86 発現が低下している 		

ことから、樹状細胞の活性化が抑制されていることが示唆された。

本研究から、張 晨陽は、頬粘膜、舌背粘膜、舌下粘膜で樹状細胞密度が異なること、また、初回の抗原経粘膜塗布では、頬粘膜と舌下粘膜共に新規樹状細胞が局所に供給されるが、舌下粘膜の樹状細胞は、早期に枯渇すると同時に、局所細胞のフェノタイプ変化を起こすことによって反復抗原塗布後の樹状細胞のリクルートを抑制するという舌下粘膜樹状細胞のユニークな興味深い特性を初めて明らかにした。このことは、舌下免疫療法による免疫抑制機構にこの特徴的なマクロファージ様細胞が関与している可能性を示すもので、舌下免疫療法のみならず、口腔粘膜経由に誘導される免疫応答の理解に新規の知見を与えるものである。本研究をさらに発展させることで、この特殊な細胞の機能が明らかになり、口腔粘膜を介した抗原投与方法を利用する舌下免疫療法や舌下ワクチンのより効果的な臨床応用開発につながる意義のある研究と考えられた。

以上より、本研究は、生物学および医歯学研究の発展に広く寄与すると考えられ、博士（学術）の学位を請求するに十分価値あるものと認められた。