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研究題目：The role of hypoxia responsive transcription factor DEC1 in periodontal inflammation

目　的：

The purpose of this study was to investigate mechanistic links between transcription factor DEC1 and pathways underlying inflammation, and to illustrate the link between hypoxia and inflammation in periodontal disease.

対象および方法：

Male *DEC1*KO mice and their wild-type littermates were used for the experimental periodontitis model. Measurement of alveolar bone resorption, micro-computed tomography, isolation of gingival mononuclear cells（GMCs）, flow cytometry and immunohistochemical analysis were used in this study. Human gingival fibroblast cells（HGF-1）were used for DEC1 over-expression and short interference RNA（siRNA）studies and and the cells were incubated for 48 h and then treated with LPS and/or hypoxia for various analyses, and quantitative real time polymerase chain reaction and western blot analysis were performed.

結果および考察：

*DEC1*KO mice were partially protected from periodontal inflammation compared with WT controls. Micro-CT analysis demonstrated that P. gingivalis caused a decrease in bone area compared with the vehicle-treated control group（Fig. 1）. Increased numbers of CD4＋RANKL＋T cells were seen 30 days after the last treatment when compared with the control group or the *DEC1*KO P. gingivalis-treated group（Figure 2）. Immunohistochemical staining showed that P. gingivalis treatment increased the expression of TNF-α, IL-1β, RANKL and cathepsin K in the inflammatory cell infiltrates（Figure 3）. Over-expression of DEC1 increased the levels of DEC1, TNF-α and IL-1β mRNAs and their expression levels were further increased by treatment with LPS（Figure 4A）. To elucidate the relevance of DEC1 in periodontal inflammation, HGF-1 cells were transfected with an siRNA targeting DEC1. DEC1 knockdown by siRNAs significantly reduced the expression of DEC1 protein and mRNA with or without LPS treatment, and siRNA knockdown of DEC1 decreased the levels of TNF-α, IL-1β, and TLR4 mRNAs with or without LPS treatment（Figure 4B）. siRNA knockdown of DEC1 significantly decreased the expression levels of LPS and hypoxia-induced DEC1 mRNA（Fig. 5A）, DEC1 and Notch1 protein（Fig. 5B）.

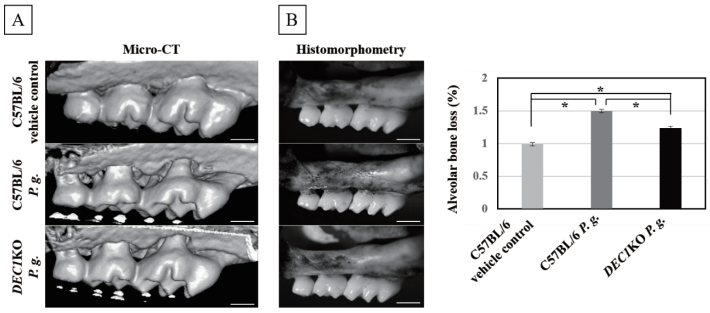

Figure 1 Inhibitory effect of *DEC1* on *P. gingivalis*-induced bone absorption. (A) After acclimating to the environment of the Animal Center and antibiotic treatment, 6-week-old WT or *DEC1*KO mice were treated orally five times with P. gingivalis ATCC 33277 or with vehicle as detailed in the Methods section. Images of the mandibular molar regions of each group were obtained after 30 days with a micro-CT apparatus under the following exposure conditions : tube voltage, 70 kV ; tube current, 100 μA ; voxel size, 17×17×17 μm. (B) The distance between the cemento-enamel junction and the alveolar bone crest was evaluated at seven buccal sites per mouse for horizontal alveolar bone loss on the left side of each maxilla. These results represent the means of data obtained from six mice in each group and are expressed as means ± SD. * P <0.05. *P. g.*, *P. gingivalis*. Scale bar : 200 μm.

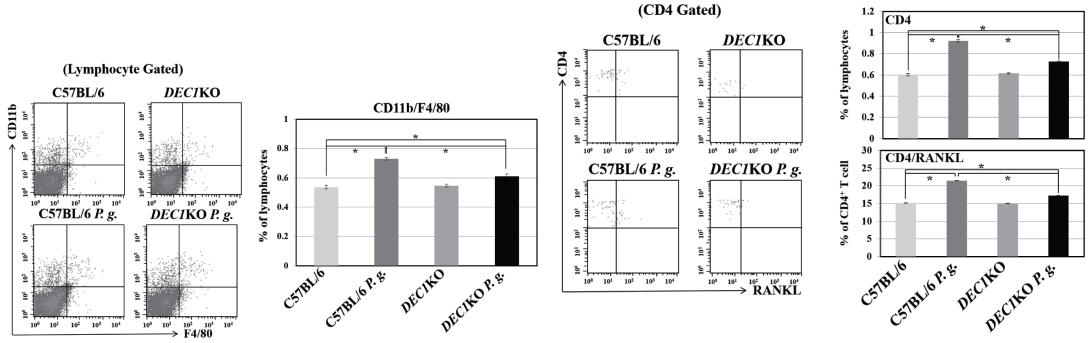

Figure 2 Expression of CD11b and F4/80 by GMCs. GMCs isolated from mice as noted 30 days after *P. gingivalistreatment* were carefully removed using microsurgical tweezers under a stereomicroscope, followed by an enzymatic dissociation with collagenase type IV. GMCs were purified on discontinuous Percoll gradients and stained with fluorescence-conjugated anti-CD11b and biotinylated anti-F4/80 mAbs, followed by PerCP-Cy5.5-streptavidin. Values are presented as the means ± SEM of six mice in each group ; * p<0.05 when compared with sham-infected WT and *DEC1*KO mice.

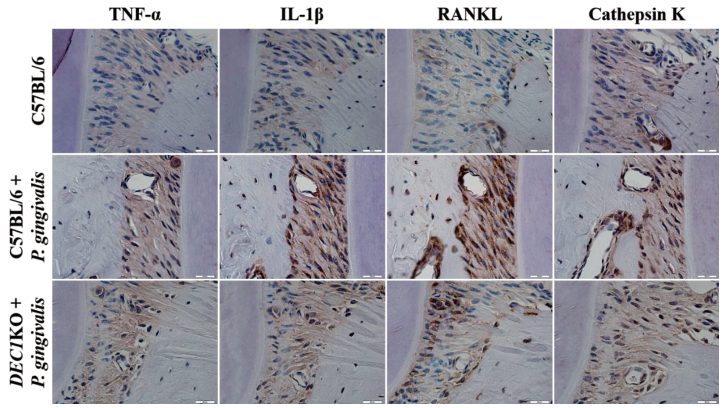

Figure 3 DEC1 decreases osteoclast formation in periodontal tissues. Sections of 4 μm thick formalin-fixed, paraffin-embedded specimens were deparaffinized and immunoreactivity was detected using a DAKO ENVISION Kit. TNF-α, IL-1β, RANKL and Cathepsin K were abundantly expressed in the *P. gingivalis* challenged WT mice tissues. Immunohistochemical analysis revealed higher expression of these genes in *P. gingivalis*-treated mice compared to the control and *DEC1*KO *P. gingivalis* mice. Scale bar : 20 μM.

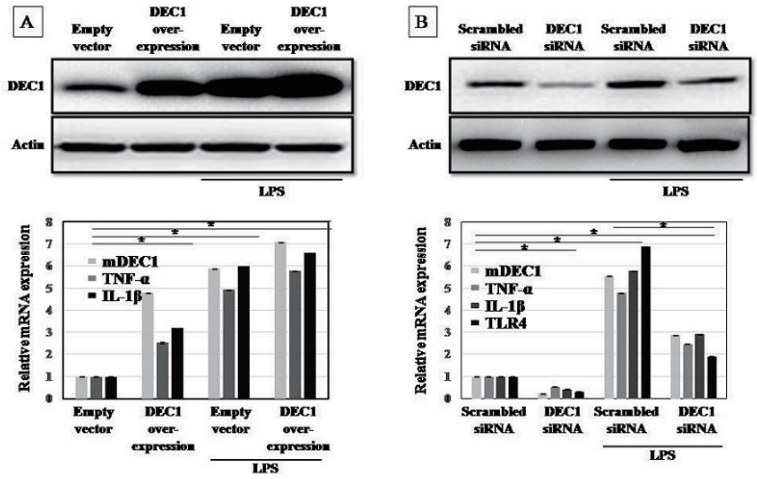

Figure 4 Effects of over-expression or knockdown of DEC1 on gingival inflammation. (A) HGF-1 cells were transfected with an empty vector or with the DEC1 expression plasmid, incubated for 4 h, treated with or without LPS (50 ng/mL) and then incubated for an additional 24 hours before being lysed. (Top) Lysates were subjected to Western blot analysis for DEC1 and actin ; the experiments were repeated three times. (Bottom) After transfection, total RNA was prepared and subjected to real-time PCR analyses of DEC1, TNF-α, IL-1β and β-actin ; each value represents the mean ± SD (bars) of mRNA levels relative to actin determined in three independent experiments. $^{*}P < 0.05$, compared with the control cells (empty vector and empty vector with LPS). (B) HGF-1 cells were transfected with a control siRNA or with an siRNA against DEC1, incubated for 48 hours, treated with or without LPS (500 ng/ml) and then incubated for 24 hours. (Top) Cell lysates were prepared and subjected to Western blot analysis for DEC1 and actin ; the experiments were repeated three times. (Bottom) After transfection, total RNA was prepared and subjected to real-time PCR analyses of DEC1, TNF-α, IL-1β and β-actin ; each value represents the mean ± SD (bars) of mRNA levels relative to actin determined in three independent experiments. $^{*}P < 0.05$, compared with the control cells (scrambled siRNA and scrambled siRNA with LPS).

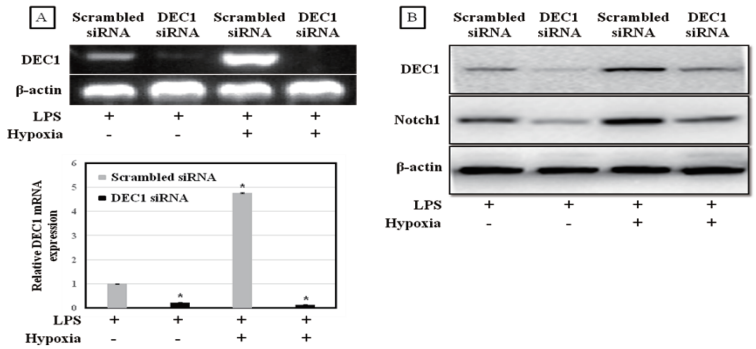

Figure 5 Effects of knockdown of DEC1 on periodontal inflammation. Human periodontal ligament cells were transfected with a control siRNA or with an siRNA against DEC1, incubated for 48 h, treated with or without LPS and/or hypoxia as noted, and then incubated for 24 h. (A) Total RNA was then prepared and subjected to RT-PCR and real-time PCR analyses of DEC1 and β-actin ; each value represents the mean ± SD (bars) of DEC1 mRNA levels relative to β-actin determined in three independent experiments. $^{*}P < 0.05$, compared with the control cells (scrambled siRNA). (B) Cell lysates were prepared and subjected to Western blot analysis for DEC1, Notch 1 and β-actin ; the experiments were repeated three times.

DEC1 is expressed in gingival and periodontal ligament cells to modulate one of the essential pathways regulating bone homeostasis, the osteoblast-dependent regulation of osteoclastogenesis. It does this by regulating P. gingivalis-derived LPS-induced expression of IL-1β, TNF-α and TLR4. Moreover, our findings may contribute to an improved understanding of the transcriptional regulation involved in alveolar bone homeostasis. We suggest that the mechanism to inhibit the LPS-induced production of inflammatory cytokines is related to DEC1. The explanation for these observations may be as follows：hypoxia augments LPS-stimulated DEC1 mRNA expression in human PDL as a direct effect of DEC1. Our results demonstrate that the LPS stimulated DEC1 expression in human PDL and hypoxia enhances the virulence of LPS to induce DEC1 expression using a DEC1-dependent pathway.

成果発表：

Presentations：

1. Role of stromal cell-derived factor 1 alpha and CXCR4 in *Porphyromonas gingivalis*-induced periodontal inflammation. Fengzhu Zhang, Ujjal K. Bhawal, Chieko Taguchi, Kazumune Arikawa, Ikuo Nasu, Hirohisa Arakawa, Koh Shibutani. 第 67 回日本口腔衛生学会・総会（May 18-20, 2018, Hokkaido）; poster presentation
2. 転写因子 DEC1 は実験的に誘導された歯周炎の制御に重要である。バワール　ウジャール、張　鳳洙、鈴木　正敏、藤田　裕、小林　良喜、平塚　浩一、渋谷　鑌。第 18 回口腔科学会（September 2, 2018, Matsudo）; oral presentation
3. Pathogenetic mechanisms in the initiation of Sjögren's syndrome in SATB1 conditional knockout mice. Yuriko Tanaka, Ujjal K. Bhawal, Fengzhu Zhang, Motonari Kondo. 第 60 回歯科基礎医学会学術大会（September 5-7, 2018, Kyushu）; oral presentation

Publication：

1. Transcription factor DEC1 is required for maximal experimentally induced periodontal inflammation. Fengzhu Zhang, Masatoshi Suzuki, Il-Shin Kim, Ryoki Kobayashi, Nobushiro Hamada, Fuyuki Sato, Ujjal K. Bhawal. *J Periodont Res* 2018；53：883–893.
2. The role of the hypoxia responsive gene DEC1 in periodontal inflammation. Il-Shin Kim, Fengzhu Zhang（co-first author）, Ujjal K. Bhawal. *J Hard Tissue Biol* 2018；27：227-232.
3. Circadian expression of differentiated embryonic chondrocytes expressed genes 1 and 2 in human oral squamous cell carcinoma HSC-3 cells. Kazumune Arikawa, Fengzhu Zhang, Chieko Taguchi, Ujjal K. Bhawal. *Int J Oral-Med Sci* 2018；17：33-37.