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学位授与の要件	医歯薬学総合研究科機能再生・再建科学専攻 （学位規則第４条第１項該当）
学位論文の題目	Inflammatory macrophages modulate mesenchymal stem cell function via TNF-α in regeneration process of the tooth extraction socket （抜歯窩骨再生における間葉系幹細胞の機能は、炎症巣に存在する TNF-αによって調節される）
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学位論文内容の要旨

Introduction

Bone healing comprises inflammatory, repair, and remodeling phases. Inflammation is essential for the initial inflammatory phase, where systemic and local reactions influence the immune cells. Macrophages are immune cells that reach the healing site and contribute to inflammation. Macrophages recruit other immune cells and regulate bone regeneration. Studies have reported that depletion of macrophages by clodronate liposome administration impaired bone formation and healing in the tibial metaphyseal drill holes and screws. Macrophages secrete cytokines and exhibit functional plasticity, which can switch between inflammatory (M1) and anti-inflammatory (M2) macrophages. Studies have reported that M1 macrophages secrete IL-6 and TNF-α, proinflammatory mediators that recruit inflammatory and immune-related cells. Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts and have immune-modulatory functions essential for tissue regeneration after tissue damage. As M1 macrophages were reported to have the highest positive impact on MSCs' osteogenic differentiation during co-culturing, macrophages seem a key player to modulate MSC functions in osteo-induction and immune-modulation. Interestingly, TNF-α (one of the famous M1 macrophage-producing proinflammatory cytokines) receptor-deficient and TNF-α knockout mice have been reported to show delayed bone formation *in vivo*. Therefore, in this study, the detailed macrophage/MSC interaction mechanisms were investigated, especially to clarify how macrophages or TNF-α protein existing in the early stage of healing at the extraction socket emphasize or regulate the MSC functions at the injured site.

Materials and methods

To investigate macrophage/MSC interactions during bone healing, we created a maxillary first molar tooth extraction model with C57BL/6J (5 weeks old, female) mice and compared following

findings between clodronate and control groups after temporal clodronate liposome (12.5 mg/kg per mouse) and saline injections. Mice were sacrificed on day1, 3, 5, 7, and 10 after tooth extraction (n=4). Micro-CT was used to evaluate regenerated bone volume, and accumulated cells in the tooth extraction socket (TES) were histomorphometrically evaluated. Anti-CD80 (M1 marker), anti-TNF-α, and anti-PDGFRα antibodies were used for frozen immunofluorescence staining.

The region of interest (ROI) was defined by drawing lines from mesial and distal alveolar crests to the interradicular septum. The area under these lines to the root tip was used for area measurement. This ROI was used for all histological and immunofluorescence images. ImageJ software automatically counted the number of positive cells and new bone formation areas. As an *in vitro* experiment, femoral bone marrow-derived MSCs were isolated and cultured. TNF-α-stimulated (10 ng/ml, 24 h) and unstimulated MSCs underwent bulk RNA-sequencing (RNA-seq) to identify TNF-α stimulation-specific MSC transcriptomes. Real-time PCR (RT-PCR) was performed to evaluate gene expression levels after MSCs osteogenic differentiation for 1 week. Statistical significance was tested using one-way ANOVA, Tukey's multiple comparison tests, and unpaired t-test (two-tailed).

Results

Micro-CT and H&E staining analyses revealed that the regenerated bone volume and bone area were significantly decreased in the clodronate group when compared to the control group at day7 and day10 post-extraction (micro-CT day7 control vs clodronate: 0.02 vs 0.01 mm^3, day10 control vs clodronate: 0.06 vs 0.04 mm^3, H&E day7 control vs clodronate: 54 vs 42% area in ROI, day10 control vs clodronate: 58 vs 51% area in ROI). From the immunohistochemical analysis of CD80, TNF-α and PDGFRα, the clodronate group showed significantly lower positive cell number at day5 post-extraction (clodronate vs control: 307 vs 559 positive cells, $p<0.0001$; 281 vs 544 positive cells, $p<0.0001$; 365 vs 633 positive cells, $p<0.0001$, respectively), while positive cell numbers of CD80, TNF-α and PDGFRα were increased at day7 post-extraction (clodronate vs control in total TES: 493 vs 396 positive cells, $p=0.0131$; 479 vs 385 positive cells, $p<0.0001$; 593 vs 473 positive cells, $p=0.0058$, respectively). The RNA-seq analysis, investigating TNF-α stimulation-specific MSC transcriptomes, showed that 94 deferentially expressed genes were identified after sorting using Log_2 fold change (Log_2FC), which is greater than 3 or less than -3. Among the 94 genes, 81 genes were up-regulated and 13 genes were down-regulated, and 10 hub genes that were connected to many other genes in the gene network of MSCs (*Il1a*, *Il1b*, *Il6*, *Ccl2*, *Ccl5*, *Cxcl10*, *Cxcl9*, *Cxcl5*, *Cxcl1*, and *Ccl20*) were identified. In the 10 hub genes, *Ccl20*, *Ccl5*, and *Cxcl9* showed the top 3 highest fold-change differences between TNF-α-stimulated and control groups. To determine the expression changes of these three genes during osteogenic differentiation, RT-PCR analysis was performed and only *Ccl20* was significantly reduced in the TNF-α-stimulated MSC group (control vs TNF-α: 1.08 vs 0.58).

Discussion and summary

Clodronate administration in mice caused temporal macrophage depletion and delayed bone healing

in the tooth extraction socket, followed by concomitant recovery of TNF-α expressing cells and MSCs in the tooth extraction socket. Since TNF-α was known one of the essential factors to make MSCs contribute to bone regeneration, an *in vitro* study stimulating MSCs with TNF-α was performed to identify TNF-α stimulation-specific MSC transcriptomes. This result identified the top ten candidate hub genes differentially expressed in the TNF-α stimulated MSCs. RT-PCR-based validation analysis of TNF-α-stimulated MSCs *in vitro* revealed *Ccl20* can be one of the TNF-α stimulation-specific MSC genes.

論文審査結果の要旨

Introduction: Previous studies have shown that mesenchymal stem cells (MSCs) and macrophages play important roles in bone regeneration. Inflammatory M1 macrophages seem to be key players in modulating MSCs' function. Tumor Necrosis Factor (TNF)-α knock-out mice also showed delayed bone formation *in vivo*. However, the detailed mechanisms of interaction between macrophages and MSCs have not been still elucidated. In this study, we determined how macrophages and TNF-α proteins present during the early stages of tooth extraction socket (TES) healing accentuate and regulate MSC function at the site of injury.

Materials and methods: The tooth extraction model was prepared by extracting the maxillary first molar in C57BL/6J mice. Saline-injected controls and experimental groups were used with clodronate liposome and injected intraperitoneally once the day before tooth extraction. Samples were chronologically harvested and performed Micro-CT and H&E staining. For frozen immunofluorescence staining, anti-CD80, -TNF-α, and -PDGFRα antibodies were used. For *in vitro* experiments, cultured bone marrow-derived MSCs were stimulated with TNF-α and underwent bulk RNA-sequencing (RNA-seq). Real-time PCR (RT-PCR) was performed to evaluate gene expression levels after MSCs osteogenic differentiation for 1 week.

Results: Micro-CT and H&E revealed significantly decreased bone volume and area in the clodronate group at day 7 and day 10. From the immunohistochemical analysis of anti-CD80, -TNF-α, and -PDGFRα antibodies, the clodronate group showed significantly lower positive cell numbers on day 5, while higher on day 7. RNA-seq analysis showed 94 differentially expressed genes were identified after using|Log2FC| >3, $p<0.05$, and 10 hub genes were identified. From 10 hub genes, *Ccl20*, *Ccl5*, and *Cxcl9* were the top 3 highest fold-change differences. For expression changes of these three genes during osteogenic differentiation, RT-PCR analysis was performed, and only *Ccl20* was significantly reduced in the MSCs with osteogenic group.

Discussion and conclusion: Clodronate administration in mice caused temporal macrophage depletion and delayed bone healing in TES, followed by concomitant recovery of TNF-α expressing cells and MSCs in TES. Since TNF-α was one of the essential factors that made MSCs contribute to bone regeneration, an *in vitro* study stimulating MSCs with TNF-α was performed to identify TNF-α stimulation-specific MSCs transcriptome. This result identified the top ten candidate hub genes in the TNF-α stimulated MSCs. RT-PCR-based validation analysis of TNF-α stimulated MSCs *in vitro* showed *Ccl20* can be one of the TNF-α stimulation-specific MSCs genes.

This research provided insight into inflammatory macrophages secreted TNF-α stimulate MSCs specific gene *Ccl20*, which may regulate MSCs' immunomodulatory properties by maintaining MSCs stemness during bone regeneration. The results are rich in novelty and serve as a foundation for future treatments in bone regeneration and stem cell therapies targeting TNF-α and Ccl20.

The defense committee hereby accept this article as a doctoral dissertation in philosophy.