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学位論文（要約）

The Pathogenesis of Joint Destruction in Patients with Rheumatoid Arthritis and its Potential Therapeutic Agents

（関節リウマチにおける関節破壊の病態解明と治療ターゲットに関する研究）

2020 年 3 月

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Background and Purposes:

Chapter 1: Optineurin (OPTN) is an autophagy receptor which has been reported to act as a negative regulator of osteoclast differentiation *in vitro* where loss of OPTN increased osteoclast activity and bone turnover. OPTN regulates nuclear factor-kappa B (NF-κB) signaling, one of the most important inflammatory pathways in RA. Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) is one of the major effector cells in RA. Receptor activator of nuclear factor-κB ligand (RANKL)-RANK signaling regulates osteoclastogenesis and involves in bone erosions in RA. Further, RANKL expressed on RA-FLS is primarily responsible for bone erosions in RA. Given the possible involvement of OPTN in RA, we investigated the role of OPTN in RA-FLS, particularly its role in the expression of RANKL on FLS and subsequent osteoclast differentiation.

Chapter 2: The miR-9 has lower expression in RA compared to healthy controls. The miR-9 has been reported to target NF-κB1/p50 gene in bone marrow-derived mesenchymal stem cells (BMSCs) and hence inhibit the NF-κB signaling pathway. In RA synovium, RANKL contributes to articular destruction as well as to NF-κB signaling-induced inflammatory pathway. Therefore, we speculated that miR-9 might suppress NF-κB pathway in RA-FLS and preserve cartilage in inflammatory arthritis.

Materials and Methods:

Synovial tissues were obtained during synovectomy or total joint replacement surgery from patients with RA in Hokkaido University Hospital.

Chapter 1: RA-FLS with passages 4 to 8 were used in this study. Tumor necrosis factor-alpha (TNF-α) or interferon-gamma (IFN-γ) were treated in RA-FLS for their major involvement in the pathogenesis of RA. RA-FLS were incubated with/without TNF-α or IFN-γ at a concentration of 10 ng/ml or 100 ng/ml and the expression of OPTN was analyzed using real-time quantitative polymerase chain reaction (RT-qPCR) for mRNA levels and western blot for protein levels. RANKL and osteoprotegerin (OPG) mRNA expression were analyzed by RT-qPCR in RA-FLS transfected with OPTN siRNA with/without 4 hours/24 hours treatment of 10 ng/ml TNF-α, 100 ng/ml IFN-γ or both. Cell surface RANKL expression was analyzed by flow cytometry in OPTN-reduced RA-FLS after 3 days after the stimulation with TNF-α/IFN-γ. OPTN-reduced RA-FLS were cocultured with $CD14^+$ monocytes isolated from healthy individuals for 10 days and stained with Tartrate-Resistant Acid Phosphatase (TRAP). TRAP-positive cells with 3 or more nuclei were considered as osteoclasts. Matrix metallopeptidase-3 (MMP-3), interleukin-6 (IL-6), GATA-3, carbohydrate sulfotransferase 15 (CHST15), and hyaluronan synthase 1 (HAS1) mRNA levels were analyzed by RT-qPCR. Cytosolic IκBα and nuclear NF-κB1 were analyzed by western blotting to evaluate NF-κB signaling.

Chapter 2: RA-FLS with passages 3 to 8 were used in this study. 10 ng/ml TNF-α or/and 100 ng/ml IFN-γ were selected for treatment of RA-FLS. The binding of miR-9 to NF-κB1 3'-UTR was analyzed by luciferase reporter assay and immunoprecipitation. Chromatin immunoprecipitation (ChIP) assay was performed to identify the binding of NF-κB1 to RANKL promoter. Promoter activity of RANKL induced by NF-κB1 was evaluated using luciferase assay. FLS were treated with miR-9 or anti-miR-9 to evaluate cell proliferation using bromodeoxyuridine (BrdU) assay, the expression of RANKL mRNA by RT-qPCR and cell surface RANKL by flow cytometry. Therapeutic effect of intra-articular administration of miR-9 was evaluated in type-II collagen-induced arthritis (CIA) in rats.

Results:

Chapter 1: OPTN levels were upregulated after TNF-α or IFN-γ stimulation at mRNA and protein levels. Cell surface RANKL was significantly increased following treatment with TNF-α, IFN-γ or both and the effect was further pronounced in OPTN-reduced RA-FLS compared to control RA-FLS. The mRNA levels of RANKL were also increased in OPTN-reduced RA-FLS while OPG levels remained unchanged. $CD14^+$ monocytes cocultured with OPTN-reduced RAFLS had higher levels of differentiation into $TRAP^+$ multinucleated cells (MNCs) compared to those cocultured with control RA-FLS. MMP3 and IL-6 were upregulated while GATA-3, CHST15 and HAS1 were downregulated in OPTN-reduced RA-FLS. IκBα degradation and the translocation of NF-κB1 into nuclei following TNF-α treatment was prolonged in OPTN-reduced RA-FLS.

Chapter 2: The miR-9 bound to the 3'-UTR of NF-κB1 and downregulated NF-κB1. NF-κB1 directly bound to RANKL promoter and increased the promoter activity of RANKL. The expression of RANKL in mRNA/protein levels in RA-FLS were negatively regulated by miR-9. Proliferation rate of FLS increased by inhibiting miR-9 in RA-FLS. Intra-articular injection of miR-9 mimics dampened experimental arthritis by lowering the inflammatory state and by reducing RANKL and osteoclasts formation.

Discussion:

Chapter 1: We newly identified the role of OPTN in one of the RANKL-expressing cells, RA-FLS. The proliferation rate had no difference in OPTN-downregulated and control RA-FLS which suggest that the increased osteoclast formation was mediated by the increased RANKL expression at single cell level. Our further investigation by downregulating OPTN in RA-FLS confirmed the protective role of OPTN against joint destruction as the pathogenic molecules MMP-3 and IL-6 were increased while GATA3, CHST15 and HAS1 were decreased. Among them, the expression of MMP-3 was most obviously changed in OPTN-downregulated RA-FLS with approximately 5-fold higher mRNA levels compared to control RA-FLS. These findings suggest the direct role of OPTN to protect the joint by inhibiting expression and activity of MMPs. Previous studies have already shown the

regulation of NF-κB signaling by OPTN in nerve cells and indicated its involvement in neurodegenerative diseases. Our current study has confirmed this regulation in RA-FLS.
Chapter 2: We confirmed the binding of miR-9 to the 3'-UTR sites of NF-κB1 and hence the reduction of NF-κB1 expression in RA-FLS. Thus, miR-9 suppresses inflammatory nature of RA-FLS via degradation of NF-κB1 transcripts. Our ChIP analysis revealed that NF-κB1 binds onto the promoter region of RANKL and acts as a transcription factor that enhances RANKL promoter activity. In luciferase assay, wildtype RANKL promoter had the highest level of activity when it was co-transfected with NF-κB1. We further analyzed the effects of miR-9 on the expressions of RANKL in RA-FLS. We identified that miR-9 has the ability to reduce RANKL expression which we considered as an indirect effect via the reduction of NF-κB1. Finally, we aimed to confirm the protective effect of miR-9 against destructive arthritis using CIA rat model. $TRAP^+$ osteoclasts and RANKL expression were significantly dampened in miR-9 treated joints compared to those treated with control miRNA. The intact cartilage in miR-9 treated CIA rats suggests the proliferative effect of miR-9 on chondrocytes, in consistency to the previous report. The inflammatory parameters in clinical, micro-CT images and histology specimens further emphasized the importance of miR-9 regulation in RA. RANKL expression in RA-FLS is suppressed, resulting in impaired osteoclastogenesis by miR-9. We also demonstrated the protective effects of miR-9 in preventing joint destruction and reducing inflammation *in vivo* arthritis model.

Conclusion:
Chapter 1: OPTN plays a protective role against joint destructions in RA with its upregulation when immersing with pro-inflammatory cytokines in RA-FLS. Absence of OPTN might worsen RA by generating joint destructive state including increased RANKL expression on RA-FLS, subsequent osteoclast differentiation, and the dysregulation of molecules involved in joint homeostasis. These roles of OPTN may contribute to our understanding of the mechanisms of joint destructions in RA.
Chapter 2: Our findings reveal a previously unrecognized regulatory network mediated by NF-κB1-RANKL axis in RA-FLS and miR-9 deactivates the inflammatory arthritis by downregulation of NF-κB signaling. These findings propose therapeutic implications of miR- 9 in RA.