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学位論文の題目	Resident stroma-secreted chemokine CCL2 governs myeloid-derived suppressor cells in the tumor microenvironment (腫瘍間質由来 CCL2 は骨髄由来免疫抑制細胞の腫瘍微小環境への動員に関する)
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学位論文内容の要旨

Oral squamous cell carcinoma (OSCC) is one of the most common solid tumors of the head and neck. OSCC consists of two major components: malignant epithelium and stroma, which make up the tumor microenvironment (TME). The stromal components play an active role in supporting tumor cells for progression, invasion, and metastasis. The stromal components contain a heterogeneous population of cells that derive from resident stromal cells and recruiting cells, namely cancer-associated fibroblasts (CAFs), tumor-associated endothelial cells (TECs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs). MDSCs are bone marrow-derived immature myeloid cells possessing immune suppressive functions. CCL2 (C-C motif chemokine ligand 2), also known as monocytic chemotactic protein (MCP-1), and CCR2 (the CCL2 receptor) play a crucial role in MDSC migration. CCL2 is secreted by tumors such as prostate cancer, breast cancer, malignant glioma, colorectal cancer, and lung carcinoma. However, the role of stroma-secreted CCL2 in MDSC recruitment in OSCC remains unclear.

In the present study, we sought new insights into the interconnection between resident stroma and MDSCs in the TME. To this end, we established a novel patient-derived stroma xenograft (PDSX) model. We performed sequential transplantation in BALB/c nude mice, including (i) bone marrow transplantation (BMT) of GFP-expressing cells to trace the BMDCs and (ii) co-xenografting of patient-derived stroma (two cases, designated PDS1 and PDS2, isolated from two OSCC patients) with oral cancer cells (HSC-2) after 28 days of BMT. The PDSX model permits an *in vivo* recreation of the TME to reproduce the interaction between the resident stroma and MDSCs, generating a system resembling the human cancer-TME interaction under natural growth conditions. As a control, we established a co-xenograft model of a normal human dermal fibroblast cell line (HDF) with HSC-2 cells. As another control for this model, we xenografted recipient mice with HSC-2 alone. Using these animal models, we investigated (i) whether the resident stroma alters the infiltration of BMDCs into the TME; (ii) whether the resident stroma alters MDSCs recruitment; and (iii) the gene expression signature of stromal cells, a profile that is expected to be essential for the migration of MDSCs.

First, we evaluated whether the tumor-stroma interaction alters BMDCs migration *in vitro* and *in vivo*. We

performed a Transwell migration assay and wound closure assay for *in vitro* assessment and GFP IHC detection for *in vivo* assessment. The data provided that PDS1, PDS2, and HDF all promoted BMDCs migration *in vitro* and recruitment *in vivo*. Further, we evaluated whether the resident stroma alters MDSCs recruitment. Murine MDSCs are known to express CD11b and GR1 and to provide immunosuppression by producing arginase 1 (Arg1). Therefore, we identified MDSCs by performing multicolor fluorescent detection on Arg1-positive cells that exhibited co-staining with GFP, CD11b, and GR1. Notably, multicolor immunofluorescence revealed that the PDS co-xenografts recruited Arginase-1/CD11b/GR1/GFP quadruple-positive cells, which are myeloid-derived suppressor cells (MDSCs), to the TME, whereas the HDF co-xenograft did not. These findings suggested that patient-derived stroma promotes MDSCs infiltration into the TME. To clarify the underlying molecular mechanism by which co-xenografted stromal cells (PDS1 and PDS2) promoted MDSCs recruitment, we next performed microarray analysis to identify the differentially expressed genes (DEGs) in PDS1 and PDS2 compared to HDF. We observed that PDS1 and PDS2 expressed CCL2 mRNA (encoding C-C motif chemokine ligand 2) at higher levels than HDF.

Indeed, when we confirmed the CCL2 protein expression in co-xenografts tissues, PDS co-xenografts contained significantly higher proportions of CCL2-positive stromal cells than the HDF co-xenograft. CCR2 has been reported to serve as the primary functional receptor for CCL2 in the TME, and the CCL2/CCR2 axis is known to play a crucial role in tumor progression. Therefore, we next examined whether the recruitment of CCR2-positive MDSCs to the TME in the co-xenograft models is different. CCR2/Arginase-1/CD11b/GR1 quadruple-positive MDSCs (receiver cells) were significantly recruited in PDSX models more than in the HDF co-xenograft. Consistent with these results, either treatment of tumor/stroma co-cultures with an inhibitor of CCL2 synthesis, bindarit, or treatment of BM cells with CCR2 antagonist, RS 102895, significantly inhibited the PDS-driven migration of BM cells *in vitro*. Further, to investigate whether blocking CCR2 in xenograft models inhibits the recruitment of MDSCs in OSCC TME, we injected CCR2 antagonist intraperitoneally (3mg/kg, the other day) into the tumor/stromal co-xenografts. Then, we evaluated the MDSCs by multicolor immunofluorescent staining. Interestingly, the CCR2 antagonist significantly reduced the infiltration of CCR2/Arginase-1/CD11b/GR1 quadruple-positive MDSC in PDSX co-xenografts than HDF co-xenograft.

Taken together, the CCL2/CCR2 axis mediates MDSCs recruitment to the TME in OSCC, and stroma-secreted CCL2 is important in the MDSCs recruitment. Therefore, targeting key stromal factors likely will serve as a crucial strategy for treating OSCC. The interaction of stroma-secreted CCL2 with CCR2-positive MDSCs is essential for three complex factorial crosstalk between cancer cells, stromal cells, and MDSCs, and thus promises to constitute an attractive target for OSCC treatment.

論文審査結果の要旨

Introduction: Accumulating evidence has shown that cancer stroma and bone marrow-derived cells (BMDCs) in the tumor microenvironment (TME) play vital roles in tumor progression. However, the mechanism by which oral cancer stroma recruits immunosuppressive subset of BMDCs especially myeloid-derived suppressor cells (MDSCs) remains largely unknown. Here we sought to identify MDSCs that is recruited by cancer stroma.

Materials and methods: We established a sequential transplantation model in BALB/c nude mice, including (i) GFP-expressing bone marrow transplantation and (ii) co-xenografting of patient-derived stroma (two cases, designated PDS1 and PDS2) with oral cancer cell line (HSC-2). As controls, xenografting was performed with HSC-2 alone or in combination with human dermal fibroblasts (HDF). First, we evaluated whether the resident stroma alters BMDCs migration in vitro and in vivo. We performed a Transwell migration assay and wound closure assay for in vitro assessment and GFP immunohistochemistry (IHC) for in vivo assessment. Further, we evaluated whether the resident stroma alters MDSCs recruitment. Murine MDSCs are known to express CD11b and GR1 and to provide immunosuppression by producing arginase 1 (Arg1). Therefore, we identified MDSCs by performing multicolor IHC on Arg1, CD11b, and GR1. We next performed microarray analysis to identify the underlying molecular mechanism for the relation of resident stromal cells and MDSCs.

Results: PDS1, PDS2, and HDF co-xenografts promoted BMDC migration in vitro and in vivo. Multicolor IHC revealed that the PDS co-xenografts recruited MDSCs to the TME more than HDF co-xenograft. Microarrays screening revealed that PDS1 and PDS2 expressed CCL2 mRNA at higher levels than HDF. Indeed, PDS xenografts (PDSX) contained significantly higher proportions of CCL2⁺ stromal cells and CCR2⁺ MDSCs than the HDF co-xenograft. Consistent with these results, either inhibition of CCL2 synthesis with bindarit, or antagonizing CCR2 with RS102895, significantly inhibited the migration of BM cells in vitro. Furthermore, intraperitoneal injection of RS102895 to PDSX models significantly reduced the CCR2⁺ MDSC infiltration.

Discussion: MDSC recruitment to TME differed according to the resident stroma types. CCL2 expression was also different among the stroma types: PDS1/2 (higher MDSC recruitment) showed the higher CCL2 expression than HDF (lower MDSC recruitment). In addition, CCR2⁺ MDSCs were recruited in PDSX. Taken together, CCL2/CCR2 axis mediated MDSC recruitment to the TME in oral cancer.

Conclusion: Oral cancer stroma-secreted CCL2 is a crucial signal for the recruitment of CCR2-positive MDSCs from bone marrow to the TME. Therefore, targeting key stromal factors likely will serve as a crucial strategy for treating OSCC.

This research provided insight into stroma-secreted CCL2 in MDSC recruitment to OSCC TME. The results are rich in novelty and serve as a foundation for the future OSCC treatment strategy targeting stroma-secreted CCL2. This paper has already been accepted by JCI insight and is internationally acclaimed. Therefore, the review committee recognizes the value of this dissertation as a dissertation for a doctoral degree (Dental Science).