学 位 論 文 の 要 旨

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学 位 論 文 名	Generation of Antagonistic Monoclonal Antibodies Against the Neoepitope of Active Mouse Interleukin (IL)-18 Cleaved by Inflammatory Caspases.
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論 文 内 容 の 要 旨

INTRODUCTION

Interleukin 18 (IL-18) is a member of the IL-1 family and plays an important role in the innate and acquired immune systems. IL-18 is constitutively expressed as an inactive precursor protein (24 kDa) in various cell types, including monocytes, macrophages, intestinal epithelial cells, keratinocytes, and synovial fibroblasts. When cells receive a proinflammatory stimulus, a huge multiprotein complex called the inflammasome is formed, and followingly inflammatory caspase-1/4 is activated. As a result, the N-terminal of precursor IL-18 protein is cleaved, and mature IL-18 (18 kDa) is generated. Then it is finally released extracellularly through the process of pyroptotic cell death. Only mature IL-18 can bind specifically to IL-18 receptor 1 (IL-18R1), and this is followed by IL-18 receptor accessory protein (IL18Rap) recruitment and activation of the downstream NF-κB and MAPK signaling pathways.

Patients with a variety of inflammatory diseases, such as Crohn's disease, chronic obstructive pulmonary disease, multiple sclerosis, COVID-19, and also several kinds of cancers, have been reported the elevation of IL-18 levels in their blood. These findings suggest the involvement of IL-18 in the onset and progression of these diseases. Thus, inhibition of IL-18 function by anti-IL-18 antibody may contribute to the mitigation of inflammatory symptoms and treatment of diseases. To conduct the therapeutic experiments in mouse models of inflammatory diseases, we generated two novel antibodies against the N-terminal sequence (neoepitope) of mouse mature IL-18. We proved that these antibodies have an inhibitory activity from *in vitro* studies, and are both non-toxic for mice and stable in the blood from *in vivo* studies. Our results indicate that these antibodies will be a useful tool for mouse experiments.

MATERIALS AND METHODS

Monoclonal antibodies (mAbs) against the cleaved mouse IL-18 were produced by immunizing mice with the neoepitope peptide ^{36}NFGRLHCTT^{44}-C corresponding to the N-terminal end of cleaved mouse IL-18. Clonal populations of hybridoma cells producing the specific antibody against the neoepitope peptide were screened by ELISA. Finally, two types of hybridomas, 5-4.1 and 9-3.1 were established, and the antibodies produced by these hybridomas are referred to as mAbs 5-4.1 and 9-3.1, respectively. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University (ID: IZ29-53).

The binding ability of mAbs to mature mouse IL-18 protein were confirmed by immunoprecipitation assay. Epitope mapping was achieved by individually substituting each position of the neoepitope peptide with Ala. The antibody gene sequences of hybridomas were determined by SMARTer Human BCR IgG IgM H/K/L Profiling Kit. The complementarity determining regions (CDRs) were analyzed with the antibody gene sequence tool.

The expression of *IL-18 receptor 1* (*IL18R1*) and *IL-18 receptor accessory protein* (*IL18Rap*) genes in four mouse cell lines (YAC-1, P-815, RAW264.7, and Panc02) were examined by real-time quantitative PCR (RT-qPCR). After stimulation of P-815 cells with mature mouse IL-18, the expression level of *mIL-6*, *mCxcl1,* and *mCxcl2* genes was measured by RT-qPCR. The induction of mCxcl2 protein in P-815 cells after stimulation of cleaved mouse IL-18 and the inhibition by mAb 5-4.1 and 9-3.1 was measured using a mouse CXCL2/MIP-2 Quantikine ELISA kit. The 50% inhibitory concentration (IC50) values were determined using the nonlinear regression curve fit analysis of Prism 9.3.1.

Preclinical studies and data analysis about toxicity and stably of mAbs in mice (the single-dose toxicity test [Test No. 21546], the half-life of mAb 5-4.1 in a single intravenous infusion study [Test No. SR21138]) were performed by contract research organization based on the study protocols. The half-life of the mAb 5-4.1 was determined by using the nonlinear regression curve fit analysis of Prism 9.3.1.

RESULTS AND DISCUSSION

We needed to generate function-blocking mAbs against mature mouse IL-18 for therapeutic experiments in inflammatory model mice. Thus, we immunized mice with neoepitope peptide ^{36}NFGRLHCTT^{44}, corresponding to the N-terminal end of mouse IL-18 cleaved by inflammatory caspase-1/4. We finally established two hybridoma clones, 5-4.1 and 9-3.1. Both mAbs derived from these hybridomas adequately immunoprecipitated the bacterially expressed mouse IL-18 cleaved by caspase-4. Next, we performed alanine substitution scanning to determine which N-terminal amino acid residues of the neoepitope are important for mAb recognition. The single substitutions of N36A, F37A, and G38A remarkably reduced the detection levels, indicating that

these residues are required for recognition by these mAbs. We also revealed that mAbs 5-4.1 and 9-3.2 have unique amino acid sequences in their sequences of each CDR1, CDR2, and CDR3 of the heavy and light chains.

Since there was no simple and sensitive bioassay for mouse IL-18, we needed to develop a novel assay system in vitro. First, we analyzed the expression level of the mouse IL-18 receptor genes in mouse cell lines, and we found that the P-815 mouse mastocytoma cell line is highly expressing both the *IL18R1* and *IL18rap* genes. We also identified that the expression of *mouse Cxcl2* gene and the production of the mouse CXCL2 protein was up-regulated after mouse IL-18 treatment in P-815 cells. Finally, we determined using this bioassay system that the mouse IL-18-elicited CXCL2 release was dose-dependently inhibited by mAbs 5-4.1 and 9-3.1, with IC50 values of 255 nM and 199 nM, respectively. These results suggest that our newly developed system is useful for the accurate evaluation of the function-blocking activity of novel mouse IL-18 mAbs.

In order to use these mAbs to mouse experiments, it is necessary to assess their safety in mice bodies. To investigate the systemic toxicity of the mAb 5-4.1, it was administered intravenously to C57BL/6NJcl mice at a maximum dose of 200 μg/Body. On the day of the administration and throughout the subsequent rearing period, there were no changes in the general health conditions and no deaths of any of the animals. In addition, there was no statistical difference in body weights compared to control mice at either 3 or 14 days post-dose. At the time of necropsy two weeks after dosing, no abnormalities were found in the grossly observed organs and tissues of the bodies. These results indicate that a single intravenous administration of 200 μg/Body of the mAb 5-4.1 did not cause any toxic effects in mice.

Finally, we examined the half-life (t1/2) of the mAb 5-4.1 during a single intravenous infusion to provide a reference for the administration schedule of antibody treatment experiments in mouse models. The neoepitope mAb or control IgG antibody was administered intravenously at 200 μg/ Body dose. Blood samples were collected on days 1, 3, 7, and 14 after the administration of the test substance, and the half-life of the mAb 5-4.1 was consequently calculated as 1.29 days.

CONCLUSION

We generated two novel anti-mouse IL-18 neoepitope antibodies that specifically recognize the N-terminal of mature mouse IL-18. We also developed the functional evaluation system for mouse IL-18 using P-815 mouse mastocytoma cells and evaluated the inhibitory activity of these antibodies. Furthermore, we confirmed the safety and the half-life of the anti-mouse IL-18 neoepitope mAb 5-4.1 in mice bodies. These antibodies will be useful in mouse model experiments to examine the therapeutic strategies of inflammatory diseases.