

# 学位論文の要旨

氏名 石川 悟

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学位論文名 Age-Associated Impairment of Antitumor Immunity in  
Carcinoma-Bearing Mice and Restoration by Oral Administration  
of *Lentinula Edodes* Mycelia Extract

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著者名 Satoru Ishikawa, Yasunori Matsui, Satoshi Wachi, Hiroshi  
Yamaguchi, Nanae Harashima, Mamoru Harada

## 論文内容の要旨

### INTRODUCTION

Age-associated impairment of T cell immunity should be considered at the setting of immunotherapy. Although several reasons have been proposed to explain immune dysfunction in the aged, the precise mechanisms have not been fully elucidated. Alternatively, the cancer-bearing state is accompanied by inflammation. Particularly, interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  are the main mediators of cancer-associated inflammation. These cytokines are also increased in the aged. IL-6 inhibits the type 1 helper (Th1) response and induction of cytotoxic T lymphocytes (CTLs). Additionally, inflammation and IL-6 increase myeloid-derived suppressor cells (MDSCs), and MDSCs suppress the Th1 response.

We recently reported that oral ingestion of *Lentinus edodes* mycelia (L.E.M.) extract mitigates immunosuppression and restores melanoma-reactive CD8<sup>+</sup> T cells in melanoma-bearing mice. Additionally, oral ingestion of L.E.M. extract can suppress an increase in the serum level of IL-6 in mice that were inoculated with colon carcinoma into the cecum, suggesting that this reagent has the potential to attenuate inflammation with either cancer and/or aging. In this study, we tried to elucidate the mechanisms of impaired antitumor immunity in aged hosts by comparing CT26 colon carcinoma-bearing young and aged mice and determined whether oral administration of L.E.M. extract could mitigate impaired antitumor immunity in aged hosts.

## **MATERIALS AND METHODS**

BALB/c and BALB/c nu/nu female mice (H-2<sup>d</sup>: 6–7 weeks old (W) ) were used as young mice. Retired BALB/c mice (45 or 60 W) were used as aged mice. Experiments were performed according to the ethical guidelines for animal experiments of the Shimane University Faculty of Medicine (IZ26-5) and for animal experiments of the Kobayashi Pharmaceutical Co., Ltd. CT26 is a colon carcinoma cell line derived from BALB/c mice. Dried powdered extract of L.E.M. was prepared with hot water before germination and after culturing mycelia in a medium composed of bagasse and rice bran. L.E.M. extract was diluted in PBS and was orally administered using gavage. For immunization, doxorubicin (DTX)-treated CT26 cells ( $1 \times 10^6$ ) were injected to the flank of mice. After 2 weeks, spleen cells were harvested and cultured with an H-2L<sup>d</sup>-binding tumor peptide in the presence of IL-2 (20 U/mL) for 4 days. Thereafter, its cytotoxicity was measured using a 5-h <sup>51</sup>Cr-release assay. To assess the subsets of the spleen, the cell suspension was stained with mAbs and analyzed using flow cytometry. Serum levels of IL-6 and TNF- $\alpha$  were determined using the ELISA Development Kit. To neutralize either or both IL-6 and TNF- $\alpha$  *in vivo*, anti-mouse IL-6 antibody and/or anti-mouse TNF- $\alpha$  antibody were injected i.p. twice, one day before and simultaneously with vaccination of cancer cells. To compare protective antitumor immunity, mice were vaccinated s.c. in the left flank with  $1 \times 10^6$  DTX-treated CT26 cells. Two weeks later, these mice were inoculated s.c. in the right flank with  $5 \times 10^5$  viable CT26 cells. The L.E.M. extract was administered orally 1 week prior to and 1 week after vaccination for a total of 2 weeks. Data were evaluated using unpaired two-tailed Student's *t*-test and ANOVA with Bartlett's test. A *P* value of  $< 0.05$  was considered to indicate statistical significance.

## **RESULTS AND DISCUSSION**

First, we compared the growth of CT26 in young (7 W) and aged (45 or 60 W) BALB/c mice. The tumor size was larger in aged mice compared with that in young mice. We next measured the serum levels of IL-6 and TNF- $\alpha$  in young (7 W) and aged (60 W) mice that were naïve or inoculated s.c. with  $5 \times 10^5$  CT26 cells 10 days prior. The results showed that the serum levels of IL-6 and TNF- $\alpha$  were significantly elevated in aged mice compared with those of young mice and that the tumor-bearing state showed the tendency to increase IL-6. Such difference in tumor growth was not observed between young (7 W) and aged (45 W) nude mice,

whereas the level of IL-6, but not TNF- $\alpha$ , was elevated in the serum of aged nude mice. We compared the vaccine efficacy of inactivated cancer cells in the *in vivo* priming of tumor-specific CTLs between young and aged mice. Although tumor-specific CTLs were induced successfully from the spleen cells of vaccinated young mice, the vaccine efficacy was weak in aged mice. In addition, neutralization of TNF- $\alpha$ , but not IL-6, at the timing of cancer vaccine suppressed the induction of CT26-specific CTLs from aged mice. The percentages of CD45<sup>+</sup> immune cells were higher in aged mice than in young mice. However, there was no difference between young and aged mice in the percentage of MDSCs among CD45<sup>+</sup> cells in tumor sites.

We next determined whether oral administration of L.E.M. extract could ameliorate the impaired antitumor immunity in aged mice. CT26 growth in aged mice was significantly suppressed by oral administration of L.E.M.. In addition, oral administration of L.E.M. extract increased the CT26-specific cytotoxicity of DTX-vaccinated aged mice.

Since we observed that the growth of CT26 carcinoma was accelerated in aged mice, we compared the tumor growth between young and aged nude mice. However, no difference was observed. These results suggest that the difference in tumor growth between young and aged mice was ascribed mainly to the impaired antitumor T cell immunity in aged mice in this model.

What types of cells produce IL-6 and TNF- $\alpha$  in aged mice? The spleen cells from aged mice produced higher levels of IL-6 upon LPS stimulation than those from young mice. Regarding IL-6, LPS might trigger IL-6 production by macrophages and B cells in the spleen via TLR4-mediated signaling. We suppose that these cells in aged mice increase the potential to produce IL-6 as a result of an age-associated inflammatory environment. Of note is that serum IL-6, but not TNF- $\alpha$ , was elevated in aged nude mice. This finding indicates that IL-6 was increased in a T cell-independent manner in aged mice.

## **CONCLUSION**

We demonstrated that the age-associated increase in IL-6/TNF- $\alpha$  and early infiltration of MDSCs into tumor sites, at least partially, contribute to impaired T cell immunity in aged mice, and that oral administration of L.E.M. extract beforehand can retard tumor growth, probably via attenuating aging-associated inflammation.

論文審査及び最終試験又は学力の確認の結果の要旨

(甲)・乙	氏名	石川 悟
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学位論文審査委員	主査	中村 守彦
	副査	吉山 裕規
	副査	森田 栄伸



論文審査の結果の要旨

発がん率は加齢とともに増加するが、逆に免疫力は低下する。また、担がん状態は慢性炎症状態と見なせるが、加齢も炎症と関連する。申請者らは、シイタケ菌糸体 *Lentinula Edodes* Mycelia (L. E. M.) の経口摂取により、若齢担がんマウスの炎症が軽減することを報告している。今回申請者は、同系腫瘍 CT26 大腸がん細胞を皮下接種した若齢 (6-7W) マウスと加齢 (45Wまたは60-65W) マウスを比較して、加齢が抗がん免疫に及ぼす影響と、L. E. M. の経口投与による治療・改善の可能性を検討した。若齢マウスと比較して加齢マウスでは、皮下接種したCT26のサイズが大きく、血中の炎症性サイトカインIL-6とTNF- $\alpha$ も高値を示した。加齢nudeマウスでは、担がんとは無関係に血中IL-6だけが増加していた。また、加齢マウスでは、不活化したがん細胞を用いたワクチンによる、がん特異的T細胞の誘導が不十分であったが、ワクチン接種時の抗IL-6抗体による中和効果により回復傾向を示した。また、同マウスでは、がん接種5日後の局所へのCD45陽性免疫細胞の浸潤が促進しており、大部分(約85%)がmyeloid-derived suppressor cells (MDSC) であった。一方、CT26の皮下接種前からL. E. M. を経口投与した加齢マウスでは、血中IL-6が低下するとともに皮下のCT26の増殖も抑制された。さらに、がんワクチンによるがん特異的T細胞の誘導も回復した。

以上より、加齢担がんマウスでは、T細胞非依存的にIL-6が増加し、担がん早期におけるMDSCの局所浸潤が抗がんT細胞応答の誘導を抑制することを見出した。そして、L. E. M. の経口投与により局所の炎症が軽減し、加齢担がんマウスの抗がんT細胞応答が回復することを明らかにした。従って、L. E. M. の経口摂取が、がんワクチンとの併用療法になり得る可能性が強く示唆された。

最終試験又は学力の確認の結果の要旨

申請者は、加齢に伴う血中IL-6の高値に着目して、シイタケ菌糸体エキスL. E. M. の経口摂取により血中IL-6が低減して局所炎症が軽減し、抗がん免疫力を高めることを実証した。この知見は、がんワクチンとの併用療法の可能性を示唆する。審査において質疑応答も的確で、関連する知識も豊富であることから学位授与に値すると判断した。(主査: 中村守彦)

申請者は、がん細胞の増殖に対する抑制が、高齢者では働きにくい理由として血中IL-6が高値を示すことが挙げられることを、マウスモデルにおいて示した。それに対してL. E. M. の経口投与が血中IL-6レベルを低下させ、腫瘍特異的なT細胞を誘導し、腫瘍の増殖を抑制することを示した。関連知識も豊富で、質疑にも的確に答え、学位授与に値すると判断した。(副査: 吉山裕規)

申請者は、L. E. M. の抗腫瘍効果を明らかにするため、CT26大腸がん細胞をマウスに移植する系にてL. E. M. 経口摂取の影響を検討した。その結果、L. E. M. の経口摂取は加齢マウスにおいてIL-6の抑制作用を示し、それによるMDSCの機能抑制から抗腫瘍効果を示すことを明らかにした。綿密に遂行された研究であり、学位の授与に値すると判断した。(副査: 森田栄伸)

(備考) 要旨は、それぞれ400字程度とする。