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論　文　名	Development of effective tumor immunotherapy using a novel dendritic cell-targeting Toll-like receptor ligand （樹状細胞を標的とした新規トル様レセプターリガンドを用いた効果的な腫瘍免疫治療法の開発）
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論文要旨

Introduction

Dendritic cells (DCs) capture tumor antigens (Ags), present in to naïve T cells and induce their differentiation into effector cells against the tumor, such as T helper (Th) 1 and cytotoxic T lymphocytes (CTLs), and activate them. The activated Th1 cells secrete interferon-gamma (IFNγ) to activate CTL and natural killer (NK) cells in the Th1 system, for enhancing the tumor immunity. In the tumor therapy, DCs loaded with tumor Ags *in vitro* and inoculated into patients to elicit the anti-tumor responses. However, the DC therapy has shown low therapeutic effect due to degradation of DC, suppression or inhibition of immune cells by immune-suppressive cells and cytokines present in the tumor environment. Therefore, in order to improve the DC based tumor therapy, enhancement and maintenance of the DC function is essentially required.

Toll-like receptors (TLRs) express mainly in immune cells and responsible for recognize pathogens which trigger critical stimuli to initiate immune response. TLR2 binds to bacterial lipopeptide and generate strong signals to activate various immune cells. TLR 2 expressed on DCs elicits the activation and maturation of DCs, which results migration of DCs into the draining lymph nodes (LNs) and initiate the immune responses. But TLR2 also express in neutrophils,

macrophages and some epithelial cells which do not migrate to LNs, thereby induce unnecessary inflammation at the injection site. A novel synthetic lipopeptide, h11c was made to avoid such undesirable effects, and has a TLR2 ligand and a human DC-targeting peptide which binds to CD11c highly expressed on DCs. Therefore, h11c is expected to give rise immune responses only to the Ags presented by DCs, and thus to be a promising tool for the DC-based tumor immunotherapy.

In this study, as the first step, the effect of h11c was examined on DC function. Then, the effects on the DC-based tumor immunotherapy were examined in mouse tumor models. With the optimal condition obtained in mouse experiments, the therapy with h11c was applied in to spontaneous tumors occurred in dogs and examined the effectiveness.

Chapter 1: Enhancement of dendritic cell function *in vitro* by h11c

Although the h11c contains a peptide targeting to human CD11c characteristically expressed by DCs, it has reported to have a significant affinity to murine CD11c. Therefore, the ability of the h11c to enhance the maturation and function of DCs was evaluated in murine. The h11c induced the maturation of murine DCs *in vitro* by enhanced expression of Ag presenting molecule (MHC II) and co-stimulatory molecules (CD80, CD86, CD40). In addition, the h11c significantly enhanced the expression of IL-12 and IL-6 from mouse DCs. However, the h11c-activated DCs also enhanced the expression of immune-suppressive IL-10. Similarly, DCs collected from LNs of h11c injected healthy mice exhibited significantly enhanced expression of the co-stimulatory molecules (CD80, CD86) and the cytokines (IL-12, IL-10). Moreover, DCs incubated with ovalbumin (OVA) and h11c showed significantly enhanced expression of OVA peptide on MHC class I molecules. Furthermore, LN cells collected from the mice immunized with DCs treated OVA and h11c showed significantly higher cytotoxic activity against the OVA-expressing tumor cells line, E.G7-OVA, compared with those from the group immunized with OVA alone.

Collectively, h11c was able to promote the activation and maturation of DCs, and thereby significantly enhanced the function of DCs to elicit immune responses against tumors. However, h11c also induced the production of immune-regulatory cytokine. Therefore, additional treatments were thought to be required to inhibit the undesirable function of h11c for using in the immunotherapy.

Chapter 2: Improvement of the DC therapy against mouse tumor model using h11c

Therapeutic effect of the h11c-treated DCs ([h11c-DC]) was evaluated at first in mouse tumor models. In order to inhibit the undesirable function of h11c, the treatment was combined with interferon-gamma (IFNγ), a typical activator of Th1 responses and promotor of DC-maturation, and COX-2 inhibitor (COX2-I) which has a critical role in preventing generation of suppressor cells such as myeloid derived suppressive cells (MDSCs) and regulatory T cells (Tregs).

DCs were induced from bone marrow cells and incubated with the tumor lysate as tumor-Ags. One cycle of the treatment was composed with one time-injection of DCs (2×10^6) and three times injection of IFNγ (2 μg/kg) and a COX2-I, celecoxib (1.67 mg/kg) per week, and four cycles of the treatment was

performed. In the therapy, DCs, IFNγ and COX2-I were injected into subcutaneously (s.c.) grown E.G7-OVA tumors in C57BL/6 mice. The growth of tumors was significantly inhibited in the [h11c-DC]+IFN group, compared with the other groups. In order to examine the systemic effect of the [h11c-DC] experiments were performed using following tumor lines, LM8 that grow in the liver of C3H mice and CT26.WT that grow in the lung of BALB/c mice. In the treatments the [h11c-DC], IFNγ and COX2-I were s.c. injected. A significant improvement was elicited by the [h11c-DC]+IFN+COX2-I treatment in survival of mice inoculated with either of the tumor line. In addition, both monocytic and granulocytic myeloid derived suppressor cells (MDSCs) were significantly decreased by the [h11c-DC]+IFN+COX2-I. Moreover, the expression of co-stimulatory molecules and the production of IL-12 of DCs were significantly enhanced by the treatment, whereas no increase was observed in the immunosuppressive IL-10. Furthermore, a significant increase in the activated CTLs/NK cells and significant decrease of Tregs were observed in the tumors of the treatment group.

These results indicate that the [h11c-DC]+IFN+COX2-I treatment promoted tumor rejection by significantly enhancing the DC function and suppressing the immune-regulatory cells.

Chapter 3: Improvement of the DC therapy against tumors spontaneously generated in dogs using h11c

Since the [h11c-DC]+IFN+COX2-I treatment elicited successful results in murine tumor-models, the enhancing effects were examined in the clinical treatment for tumor patients of dog.

The h11c also showed high affinity to dog DCs as well as human and mouse DCs. Moreover, h11c significantly enhanced the activation of dog DCs, evaluated by production of IL-12.

In the dog treatment DCs were induced from peripheral blood (PB) monocytes. Injection of [h11c-DC] and dog IFNγ was repeated at weekly intervals and a COX2-I, firocoxib was orally administrated daily. Besides of the tumor size, the therapeutic effect was evaluated by the tumor-responding T cells (TRTCs) which express IFNγ in the response to the tumor lysate, Tregs and MDSCs in PB.

In the therapy in dogs, the [h11c-DC]+IFN+COX2-I treatment was performed against four different malignant tumors. In the case of a nonepithelial malignant tumor with soft-ball-size in the left shoulder and a multiple malignant fibrous histiocytoma (MFH), in which the [h11c-DC] and IFNγ was directly injected into tumors, the [h11c-DC]+IFN+COX2-I treatment elicited almost complete regression by the treatment for 198 and 56 days. In the treatment against the multiple MFH, treatment to two large tumors resulted in disappearance of all tumors. In the case of the bone metastasis of squamous cell carcinoma, the [h11c-DC] and IFNγ was injected around tumor, elicited a partial regression. In these three cases, TRTCs significantly increased in related to the decrease of the tumor size whereas Tregs and MDSCs reduced. However, in the case of lung metastasis of renal cell carcinoma, in which it was impossible to directly inject [h11c-DC] and IFNγ into tumor, the [h11c-DC]+IFN+COX2-I treatment was ineffective to inhibit the tumor growth. In this case, increased once at the beginning of the treatment, but decreased thereafter. In contrast, the Tregs and MDSCs once decreased at the beginning, but increased thereafter.

These results specify that the treatment of h11c-DC in combination with IFNγ and COX2-I

effectively induce strong response to overcome surface tumors and tumors which can be directly treated with DCs, but further improvement is required for overcoming and metastatic tumors.

Conclusion

The effect of the combined therapy was investigated on mouse tumor models and spontaneously occurred tumors in dogs. Based on the results, the following conclusions are drawn;

1. The DC specific TLR, h11c significantly enhanced the DC maturation with increased expression of immune-stimulatory molecules and cytokines. Thus, h11c increased DC function to induce tumor-specific immune responses.
2. The treatment with the h11c-DCs in the combination with IFNγ and COX2-I elicited a significant improved response against both surface and visceral tumors in mouse models. In agreement with the tumor suppression, significantly decrease in the immune-suppressive cells and significant enhanced DC function to activate T cells were found in the treated mice.
3. The treatment elicited significant clinical responses against spontaneously generated tumors in dogs when the DCs and IFNγ were injected directly into or near to tumors although the combined treatment was not so successful for visceral metastatic tumor.

Overall, the combined treatment gave rise to emphatic amelioration in DC-based cancer therapy.

審査結果の要旨

樹状細胞（DC）は、抗原提示に特化した免疫細胞で、抗原特異的免疫反応を惹起できる。この特性を利用し、患者から採取した末梢血単球あるいは骨髄細胞を生体外で DC に分化誘導し、腫瘍抗原を提示させて患者に戻すことにより、腫瘍に対する免疫反応を高める治療が行われてきた。しかしながら、多くの場合、腫瘍の成長を抑制できる強力な免疫反応を賦活できないため、高い治療効果が得られていない。他方、DC をはじめとするすべての免疫細胞は、数種類のトル様レセプター（TLR）を発現しており、リガンドとの結合により非常に強く機能を向上させる。近年開発された DC を標的とする TLR リガンドである h11c は、TLR-2 のリガンド部位と DC に強く発現する CD11c に結合する部位を合わせ持つリポペプチドで、DC が惹起する免疫反応のみを選択的に活性化するため、非特異的免疫反応による副作用を回避できる。本研究では、腫瘍免疫療法の向上を目的として、h11c を作用させた DC による腫瘍治療効果を検討している。

第 1 章では、h11c による DC の免疫活性化能に対する影響を調べている。*in vitro* におい

てマウス DC に h11c を作用させたところ、DC における T リンパ球刺激分子の発現および免疫反応を活性化するサイトカインの産生が有意に増加した。しかし、同時に免疫抑制性のサイトカインの産生も増加することが認められた。この傾向は、*in vivo* で h11c を投与したマウスから回収した DC においても認められ、h11c は、DC の免疫活性能のみでなく免疫抑制能をも向上させることが判明した。これらの結果から、h11c を DC 治療に用いるには、腫瘍免疫をさらに活性化する処置と免疫抑制を制御する処置を追加する必要があることが分かった。

第 2 章では、担癌マウスモデルを用いて、h11c を作用させた DC（h11c-DC）による治療効果を検討している。また、前章の結果を踏まえて、h11c-DC の処置に加え、腫瘍免疫を活性化するインターフェロン γ（IFNγ）、および免疫抑制細胞の誘導を制御するシクロオキシゲナーゼ 2 阻害剤（COX2-I）の投与を行った。これら h11c-DC、IFNγ および COX2-I の 3 処置によって、皮下に移植した腫瘍の成長を有意に抑制したのみでなく、肺および肝臓に腫瘍を形成する内臓腫瘍モデルにおいても生存を有意に延長させた。また、これらの処置をしたマウスの DC では、T リンパ球刺激分子の発現および免疫活性化サイトカインの産生が有意に増加したが、免疫抑制性サイトカインの産生は増加せず、さらに免疫抑制細胞も減少していた。これらの結果により、h11c-DC の処置に IFNγ および COX2-I 投与を組み合わせた免疫治療法により、腫瘍の成長を非常に効果的に抑制できることが示された。

第 3 章では、前章で効果が認められた免疫治療法によって、イヌに自然発生した悪性腫瘍に対する治療効果を検証している。治療を行った 4 症例のうち、h11c-DC を腫瘍内または腫瘍周囲に注入可能な 3 症例については、腫瘍の完全消失または縮小が認められたのみでなく、腫瘍反応性 T リンパ球の増加と免疫抑制細胞の減少が認められた。h11c-DC を腫瘍内または周囲に注入できない内臓転移腫瘍については､腫瘍の成長を抑制できなったが、治療初期において、腫瘍反応性 T リンパ球の増加と免疫抑制細胞の減少が認められた。

以上､本研究において DC を標的とする TLR リガンドを用いることにより非常に効果的に腫瘍を治療できることが明らかになった。これらの成果は、腫瘍免疫治療に改善をもたらし、臨床獣医学および臨床医学の研究の新たな展開に資するものであり、本論文の審査ならびに最終試験と併せて、博士（獣医学）の学位を授与することを適当と認める。