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| 論　文　名 | Generation of molecular-targeting peptides for selective inhibition of the interaction between CTLA-4 and B7 in the dog: a new immune checkpoint inhibitor for cancer therapy（イヌにおけるCTLA-4とB7間の相互作用を選択的に阻害する分子標的ペプチドの創出：がん治療に向けた新規免疫チェックポイント阻害剤の開発） | |
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論文要旨

**Introduction**

Two signal pathways activate T cells: the antigen-specific signal and the co-stimulatory signal. Most effective co-stimulatory signal is generated by binding of CD28 on T cell with B7 on dendritic cell (DC), a typical antigen-presenting cell. Following the activation, cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed on T cell, which takes B7 from CD28, and generates suppressive signals. This suppression of T cells by the CTLA-4 signal plays an important role to prevent an excess of immune responses as an immune checkpoint. However, in tumor immunity, the suppression of T cell response by CTLA-4 is a critical mechanism underlying tumor growth. Therefore, inhibition of the binding between CTLA-4 and B7 elicits enhancement of the T cell activity against tumors by restoring the binding between CD28 and B7, and progresses tumor immune therapy.

Treatment with monoclonal antibody specific for human CTLA-4 has emerged as an effective cancer therapy in humans. But therapeutic antibodies show some limitations such as high molecular weight with complex structure which make them difficult to synthesize, high immunogenicity, which requires a change of the framework from mouse immunoglobulin to that of recipient animal, low *in vivo* stability, and difficulty to improve affinity to targets. Those biophysical properties have prompted the extensive investigation of alternative binders. A

targeting peptide, which has low molecular weight, low immunogenicity, high *in vivo* stability, and the high possibility to improve affinity is a promising alternative tool for the therapy.

In this study, the author developed the molecular-targeting peptides against human (h)-CTLA-4 from the library of peptide with helix-loop-helix (HLH) structure, and increased the affinity to CTLA-4. Then the biological activity of the h-CTLA-4-targeting peptides was confirmed in assays using human immune cells. With the effective results in human, the biological activity of the h-CTLA-4-targeting peptides finally evaluated in canine immune system.

**Chapter 1: Generation of CTLA-4-targeting peptides**

To develop molecular-targeting peptides, as same in the case of antibody, the purified target protein is necessary. However, the cloning and the purification of recombinant canine CTLA-4 are difficult and take a long time. On the other hand, recombinant h-CTLA-4 is commercially available and clinically used for immune suppression. Therefore, the author at first examined the cross-reactivity between h-CTLA-4 and canine CTLA-4. The h-CTLA-4 specifically binds to canine DCs expressing canine B7 in flow cytometry (FCM), and inhibits the interaction between canine DCs and canine T cells expressing canine CD28 in allogeneic mixed lymphocyte reaction (MLR). These results suggested that the h-CTLA-4 has ability to bind canine B7 and biological activity to inhibit the binding between canine CD28 and canine CTLA-4. Therefore, the h-CTLA-4 was used as the target to develop the canine CTLA-4-targeting peptides.

For development of the CTLA-4-targeting peptides, yeast-displaying HLH peptides which bound to h-CTLA-4 were detected in FCM using fluorescence-labeled h-CTLA-4-Ig, and were isolated using fluorescence activated cell sorter (FACS). After sorting, three different h-CTLA-4-targeting peptides were obtained. Of the three, Y-2 showed the highest binding activity to h-CTLA-4, which was significantly decreased in the presence of B7-1. These results suggested that Y-2 and h-B7-1 share a same binding site on h-CTLA-4. Moreover, Y-2 showed no binding activities to other unrelated proteins such as h-IgG-Fc, h-TNF-α, h-EGF, suggesting that Y-2 will not show undesirable effects *in vivo*. However, the dissociation constant ($K_D$) of h-CTLA-4 binding to the Y-2-expressing yeasts was 1530 nM, and significantly higher than that to h-B7-1 (278 nM), indicating that the affinity of Y-2 to h-CTLA-4 was significantly low.

Therefore, as the second step, the author improved the binding affinity of Y-2 by randomly changing a few amino acids of Y-2. This random mutation was carried out by error-prone PCR based on the DNA sequence of Y-2. Consequently, three variants were generated after the PCR. The variants, named as ERY2-1, ERY2-4, and ERY2-6, showed significantly higher binding activities to h-CTLA-4 than that of Y-2. The $K_D$ of h-CTLA-4 binding to the ERY2-1-, ERY2-4- or ERY2-6-expressing yeasts was 20.75 nM, 18.5 nM or 20.7 nM respectively, about 1/75 of that to the Y-2-expressing yeasts. Also, the binding activities of the variants significantly decreased in the presence of human B7-1. Moreover, the variants showed no binding activities to other unrelated proteins, h-IgG-Fc, h-TNF-α, h-EGF. Thus, these results indicated that the variant peptides generated by random mutation of Y-2 significantly improved the binding affinities to CTLA-4 and that, as same as Y-2, variants had the same binding site of B7-1 on h-CTLA-4.

As the third step, the variant peptides were synthesized and investigated property of binding to

CTLA-4. The $K_D$ of the synthesized ERY2-1, ERY2-4, or ERY2-6 binding to h-CTLA-4 was 277.7 nM, 196.8 nM, and 571.7 nM, respectively. These values were comparable to that of h-B7-1 (278 nM). Since the variant peptides, especially ERY2-4 possessed a comparable affinity with B7-1 in binding to h-CTLA-4, and thus would be capable to inhibit h-CTLA-4 and h-B7-1 interaction. Due to the structural similarity between CD28 and CTLA-4, the CTLA-4-targeting peptides might bind to CD28. Because the binding between CD28 and B7 is essential for T cell activation, interruption of the binding thus induces immune suppression. However, it was confirmed by ELISA that ERY2-4 had significantly lower affinity to CD28 than h-B7-1. These results suggest that the ERY2-4 selectively binds to CTLA-4 for the inhibition of the CTLA-4/B7 binding without disrupting the CD28/B7 binding.

Taken results together, it is indicated that the CTLA-4-targeting peptide which is capable of inhibiting CTLA-4/B7-1 interaction was successfully generated.

**Chapter 2: Biological activity of CTLA-4-targeting peptides in human immune response**

To exert the therapeutic effect, CTLA-4-targeting peptides should functionaly block the interaction between CTLA-4 on DC and B7 on T cells. As the targeting peptide, ERY2-4 was generated against h-CTLA-4, the biological activity of ERY2-4 on human immune cells were at first evaluated. In the FCM assay, by addition of ERY2-4, the binding of h-CTLA-4 to DCs expressing B7 was significantly decreased. These results suggest that the CTLA-4-targeting peptides blocked the interaction between h-CTLA-4 and h-B7. Furthermore, to evaluate the functional blockade of human CTLA-4/B7-1 interaction, a mixed lymphocyte reaction (MLR) was conducted using human lymphocytes and human DCs. Allogeneic lymphocytes in the ERY2-4-treated cultures showed 2-fold enhanced proliferation compared to non-treated cultures. Taking these results together with results described in the Chapter 1, it is suggested that the CTLA-4-targeting peptides increased T cell responses by inhibiting the interaction between CTLA-4 expressed on T cells and B7 on DCs.

**Chapter 3: Biological activity of CTLA-4-targeting peptides in canine immune response**

As the final step of this study, the author investigated the effects of the CTLA-4-targeting peptides on the canine immune system, for estimating a possibility for clinical use as new immune checkpoint inhibitor in tumor therapy. In the FCM assay, the binding of h-CTLA-4 to canine DCs was diminished by the h-CTLA-4-targeting peptide, ERY2-4 as same as the results using human DCs. Furthermore, in MLR using canine lymphocytes and canine DC, allogeneic lymphocytes in the ERY2-4-treated cultures showed 1.6-fold enhanced proliferation compared to non-treated cultures. It was demonstrated in the study of Chapter 1 that the h-CTLA-4 has ability to bind canine B7 and biological activity to inhibit the binding between canine B7 and canine CTLA-4, and that ERY2-4 has a significantly high affinity and specificity to h-CTLA-4 in Chapter 2. Taking these results together, it is strongly suggested that the h-CTLA-4-targeting peptide enhanced canine T cell responses due to the blockade of the interaction between CTLA-4 expressed on canine T cells and B7 on canine DCs. These results also indicates the possibility of the h-CTLA-4-targeting peptides for the application of canine tumor therapy, in which the targeting peptides prevent the suppression of T cell activity by the CTLA-4/B7 interaction, providing the

affinity of the h-CTLA-4-targeting peptides to canine CTLA-4 will be further increased in future study.

**Conclusion**

The CTLA-4-targeting peptides was generated and the biological activity in human and in canine immune responses were evaluated. Based on the results, the following conclusions are drawn;

1. The CTLA-4-targeting peptide, Y-2 showed specific binding activity to h-CTLA-4Ig, but with a lower binding affinity compared to B7-1.
2. Production of Y-2 variants by random mutation significantly increased the binding affinities and specificities to h-CTLA-4. Among Y-2 variants, ERY2-4 showed the strongest binding affinity to h-CTLA-4, which is comparable to that of B7-1. Further, it showed no binding activity to CD28.
3. The h-CTLA-4-targeting peptide, ERY2-4 inhibited the interaction between h-CTLA-4 and B7 expressed on human DCs. Further, it enhanced human lymphocytes proliferation in the response to allogeneic human DCs.
4. ERY2-4 showed inhibitory activity against the interaction between h-CTLA-4 and B7 expressed on canine DCs, and enhanced canine lymphocytes proliferation in the response to allogeneic canine DCs.

Overall, the CTLA-4-targeting peptide was biologically active in both humans and dogs: it functionally blocked the immune checkpoint molecule, CTLA-4 to enhance the immune responses.

## 審査結果の要旨

免疫反応の調節には、T 細胞と抗原提示細胞である樹状細胞との相互作用が必要不可欠である。T 細胞の活性化には、T 細胞の抗原受容体が樹状細胞から提示される抗原に結合することによって生ずる第 1 シグナルに加え、共刺激分子間の結合から生ずる第 2 シグナルが必要である。この第 2 シグナルの中でも T 細胞上の CD28 が樹状細胞上の B7 と結合して生ずるものが最も強力で、効果的に T 細胞を活性化する。一方、T 細胞が活性化すると、CTLA-4 の発現が始まる。CTLA-4 は、CD28 と同じく B7 と結合するが、抑制シグナルを発生する。また、CTLA-4 は B7 に対して CD28 の数十倍の親和性を持つため、CTLA-4 と B7 との結合が優勢となり、免疫反応が終息に向かう。よって、CTLA-4 のような分子は、過剰な免疫反応による組織の損傷を回避するため、免疫チェックポイントと呼ばれている。しかしながら、CTLA-4 による免疫チェックは、がん免疫においても同様に機能し、がん免疫治療の効果を著しく減弱する。

これに対し、ヒトでは、CTLA-4 の機能を阻害するモノクローナル抗体が作製され、がん治療に用いられている。しかし、これらの治療抗体は、分子量が大きいため、免疫原性を示すので、免疫グロブリンの分子骨格をマウスのものからヒトのものへ置き換える必要があり、また、合成することが困難である。さらに、作製後に標的に対する親和性を向上

させることができない。これらの問題を解決するため、免疫原性を示さない合成可能な低分子で、標的に対する親和性を向上できる分子標的ペプチドが次世代の治療薬として期待されている。このような背景を踏まえ、本研究では、イヌのがん治療に向けた新規免疫チェックポイント阻害剤の開発を目的として、helix-loop-helix（HLH）構造を持つペプチドライブラリーからヒト CTLA-4 を標的とするペプチドを選別し、標的分子に対する親和性を向上させ、最終的にイヌの T 細胞活性に対する増強効果を検討している。

第 1 章では、CTLA-4 標的ペプチドの作製を行っている。作製に先立ち、精製ヒト CTLA-4 がイヌ樹状細胞上の B7 に結合し、また、混合リンパ球反応においてイヌ T 細胞とイヌ樹状細胞との相互作用を阻害することを認め、ヒト CTLA-4 とイヌ CTLA-4 が機能的交差性を持つことを見出している。さらに、90%のアミノ酸相同性があることから、精製ヒト CTLA-4 をイヌ CTLA-4 標的ペプチドの選別、分離をするための標的として用い、以降の実験を実施している。

フローサイトメトリーにおいて、蛍光標識したヒト CTLA-4 を用いて酵母表層提示 HLH ペプチドライブラリーから標的ペプチド Y-2 を選別している。Y-2 は、ヒト CTLA-4 への特異的結合性を示すのみでなく、その結合が精製ヒト B7 によって競合的に阻害されることから、ヒト CTLA-4 において B7 と同じ結合部位をもつことを見出している。しかしながら、CTLA-4 の Y-2 提示酵母への親和性は、B7 に比べて非常に低いことを認めたため、error-prone PCR 法によって Y-2 中の数個のアミノ酸をランダムに置換し、CTLA-4 への親和性が Y-2 に比べて 75 倍高い標的ペプチド ERY-2-4 を得ている。ERY-2-4 は、Y-2 と同様、ヒト CTLA-4 に対して特異的に結合し、B7 と同じ結合部位を持つことが確認されている。さらに、合成された ERY-2-4 は、CTLA-4 への親和性が B7 と同じであるだけでなく、CD28 への親和性が B7 に比べて有意に低く、CD28 と B7 との結合を阻害しないため、T 細胞の活性化に影響を与えないことを明らかにしている。

第 2 章では、作製したヒト CTLA-4 標的ペプチド ERY-2-4 のヒトの免疫反応に対する影響を調べている。ERY-2-4 は、フローサイトメトリーにおいて、精製ヒト CTLA-4 のヒト樹状細胞への結合を阻害し、また、混合リンパ球反応において、ヒト樹状細胞のアロ抗原に対するヒト T 細胞の反応を有意に増強しており、これらの結果から、ヒト CTLA-4 標的ペプチ ERY-2-4 は、ヒト T 細胞上の CTLA-4 とヒト樹状細胞上の B7 の結合を阻害することによって、ヒト T 細胞の活性を増強することを明らかにしている。

第 3 章では、作製したヒト CTLA-4 標的ペプチド ERY-2-4 のイヌの免疫反応に対する影響を調べている。ヒトでの実験結果と同様、ERY-2-4 は、フローサイトメトリーにおいて、精製ヒト CTLA-4 のイヌ樹状細胞への結合を阻害し、混合リンパ球反応において、イヌ樹状細胞のアロ抗原に対するイヌ T 細胞の反応を有意に増強している。第 1 章においてヒト CTLA-4 はイヌ樹状細胞上の B7 に結合すること見出していることから、本章の結果と合わせ、ヒト CTLA-4 標的ペプチド ERY-2-4 は、イヌ T 細胞上の CTLA-4 とイヌ樹状細胞上の B7 の結合を阻害することによって、イヌ T 細胞の活性を増強することを明らかにしている。

以上、本研究の成果は、がん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の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。