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論　文　名	Manipulation of tumor microenvironment by cytokine gene transfection enhances dendritic cell-based immunotherapy （腫瘍微小環境へのサイトカイン遺伝子導入操作による樹状細胞療法の増強）
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論文要旨

Introduction

In the tumor immunity, immature dendritic cells (DCs) take up antigens, maturate and migrate into lymph nodes. There, the mature DCs present antigens to naïve T cells in and induce differentiation into T helper 1 (Th1) and cytotoxic T lymphocytes (CTL) respectively. Also, DCs activate natural killer (NK) cells. These cells (Th1 system) exert cellular immunity against tumor. Recently, a number of DCs were able to be induced *in vitro* from bone marrow precursor or peripheral blood monocytes (PBMCs). Thus, DCs loaded with tumor antigens has been used in the therapy against tumors in human or animals. Although the DC therapy has almost no toxicity, the clinical outcome is not particularly successful. This is mainly due to degradation of DCs in the tumor microenvironment, which is comprised of tumor cells, stromal cells, and immune cells. Therefore, it is expected that the successful clinical outcome is elicited by constructing the microenvironment to enhance the DC maturation and the Th1 activation. Such requirement is thought to be satisfied by transfecting the gene of corresponding cytokines into tumor cells and expressing the cytokines from them.

In the present study, the author, at first, demonstrated in *in vitro* experiments that the immune responses against tumor cells was significantly enhanced by transfection with gene of interferon-gamma (IFNγ) which promotes both activation of cellular immunity and

maturation of DCs, then cloned genes of both canine and murine CD40 ligand (CD40L) and clarified that the cytokine promotes maturation and activation of DCs, and consequently, enhances immune responses, finally, using a novel gene-delivery system (GDS), examined the therapeutic effect by *in vivo* transfection of the IFNγ and CD40L genes into tumor cells growing in the mouse.

Chapter 1: Promotion of DC maturation and enhancement of immune responses against tumor by transfection of cytokine genes *in vitro*

The existence of Th1 responses in a tumor microenvironment elicits a better prognosis for the patients. Transfection of Th1 polarizing cytokines, such as IFNγ, into tumor cells is an effective way to set up an appropriate microenvironment. Using a novel type synthetic vector composed of polyamidoamine dendrons, we transfected canine IFNγ gene into canine tumor cell lines, and examined direct and indirect effects of DCs against tumor growth in vitro.

A cloned canine IFNγ gene expressed functional protein that induces maturation of DCs. When the canine IFNγ gene was transfected into canine tumor cell lines using the synthetic vector, most cells secreted canine IFNγ. Secretion of IFNγ reduced with time, but was maintained for 48 hours. DCs incubated with the IFNγ-transfected tumor cells exhibited greater suppressive activity and induced significantly higher cytotoxic activity against the tumor cells, relative to those incubated with untransfected tumor cells and comparable dose of IFNγ.

Successful transfection of IFNγ by the synthetic vector efficiently enhanced the anti-tumor immune function of DCs, and sets up a suitable microenvironment for improvement in tumor therapy.

Chapter 2: Production of CD40 ligand to induce maturation of dendritic cells

CD40L expressed by activating T cells is shown to induce maturation of immature DCs and this maturation is a vital part in DC based tumor immunotherapy. The author has constructed two expression vectors to produce mouse membrane bound CD40L and canine soluble form of CD40L (csCD40L) protein.

When canine PBMCs were incubated with appropriate cytokines, expression of CD86 was significantly elevated, but the majority of cells displayed the morphology of immature DCs. Following the addition of the expressed canine csCD40L to the DC-inducing culture, the cell morphology shifted to that of mature DCs, and expression of CD80, CD86, MHC class II and CD1a was significantly enhanced. This morphological change and enhancement of expression was observed even when the csCD40L was present only in the second half period of the culture. Furthermore, the csCD40L caused a significant increase in IL-12 production from DCs. Similarly, when mouse immature DCs when cultured with CHO cells expressing mCD40L, DCs were matured morphologically and by increase expression of costimulatory molecules such as CD80, CD86, CD 83 and CD11c.

These results indicate that the CD40L significantly promotes the maturation and activation of DCs.

Chapter 3: Enhancement of DC-based immunotherapy by *in vivo* transfection of cytokine genes

The tumor microenvironment strongly influences the clinical outcome of immunotherapy. Intratumoral (i.t) injection of IFNγ, a cytokine which induces Th1 polarization and DC maturation, showed satisfactory clinical results in dog tumor therapy. This manipulation is expected to be more effective and persistent by transfecting genes of the relevant cytokine into tumor cells *in vivo*. To verify this hypothesis, we transfected genes of cytokine into tumor cells grown in mice, and examined the effect on DC therapy. For the transfection, the author used a GDS composed of a cationic lipid bound with pH-sensitive liposomes, which effuses enclosed DNAs from endosome into the cytoplasm. Therapeutic experiments were performed by transfection of IFNγ and CD40 ligand genes into transplanted LM8 osteosarcoma tumors via intravenous (i.v) or i.t routes. It was followed by i.t injection of DCs presenting the LM8 antigens. With this four week cycle of treatment, tumor growth was significantly suppressed by i.v treatment of IFNγ gene and by both i.v and i.t treatment of CD40 ligand gene with DCs compared to untreated mice. The CD40L gene treatment led to significantly improved survival. It is noteworthy that a hundred percent survival by the designed end point in the group transfected with both IFNγ and CD40L genes followed by DC treatment and that more than half of the mice in the group showed complete remission. Moreover, the group showed much significant increase systemically in innate NK activity, as well as adaptive immunity against the bearing tumor. Furthermore, a significant higher number of CTLs and the significantly lower number of Tregs were found in tumors of the group

Therefore, it is indicated that the tumor microenvironment to promote tumor rejection is constructed by transfection of the genes of the cytokine to augment DC function, followed by inoculation of DC.

Conclusion

The experiments performed in this thesis resulted following interesting findings

1. *In vitro* IFNγ gene transfection significantly enhanced both the activation of cellular immunity and the maturation of DCs against tumor cells.
2. Cloned canine and murine CD40L gene transfection promotes DC maturation and immune responses.
3. *In vivo* transfection of IFNγ or/and CD40L gene into tumor cells effectively enhances the therapeutic effect of DC based immunotherapy.

The results in the entire series of experiments together show a promising novel strategy to improve DC based immunotherapy in dogs as well as in humans in the future.

審査結果の要旨

樹状細胞（DC）は、初回の抗原特異的免疫反応を惹起できる唯一の血液細胞である。従来、がん免疫療法の一つとして、患者から採取した末梢血単球あるいは骨髄細胞から DC へ分化誘導し、成熟・活性化した DC に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、その治療効果は低く、大部分の患者でがんの進行を抑制できないのが現状である。この主な原因として、成熟・活性化した DC を患者体内において維持できないことが報告されている。一方、腫瘍細胞を取り巻く微小環境が最近注目され、DC や細胞障害性 T 細胞（CTL）が多く、制御性 T 細胞（Treg）が少ない状態では、腫瘍の排除が良好に行われることが分かってきた。

本研究では、がん免疫療法の改善を目的とし、サイトカインの遺伝子を腫瘍細胞に導入して発現させることで、腫瘍微小環境を腫瘍排除に有利な状態に作り変えることによる DC 治療効果の増強を検討している。

第 1 章では、*in vitro* の実験において腫瘍細胞にサイトカイン遺伝子を導入し、DC の直接的および間接的腫瘍増殖抑制能を向上させることを試みている。がん免疫の主体である細胞性免疫の活性化と DC の成熟・活性化を亢進するインターフェロンガンマ（IFNγ）をイヌ腫瘍細胞株に導入し、DC による直接的および間接的な腫瘍細胞の増殖抑制効果に対する影響を調べ、IFNγ を添加した場合に比べて、より効果的に腫瘍細胞の増殖を抑制することを明らかにした。

第 2 章では、活性化 T 細胞が産生するサイトカインであり、DC を効率的に成熟させる DC40 リガンド（DC40L）の遺伝子をクローニングし、形態的および機能的解析から、その発現産物が DC を成熟させ、免疫活性化能を増強させることを明らかにした。

第 3 章では、担癌モデルマウスにおいて、人工ベクターを用いて緑色蛍光蛋白

（GFP）遺伝子を腫瘍内（IT）あるいは静脈内（IV）投与した場合、いずれも腫瘍組織にのみ特異的かつ高効率に導入されることを確認した。その後、上記 IFNγ および CD40L の遺伝子を腫瘍細胞に導入し、DC 治療の効果に対する影響について検討している。特に、IFNγ と CD40L の両遺伝子を用いた治療と DC 治療の併用では、生存期間の有意な延長がみられ、半数以上のマウスで腫瘍が完全に寛解した。本治療初期に腫瘍組織内の浸潤細胞を調べたところ、CTL が有意に増加し、Treg が有意に減少していた。これらの結果から、IFNγ および CD40L の遺伝子の腫瘍への導入によって腫瘍微小環境内における DC の免疫活性化能を高め、CTL の活性化を亢進させることにより、治療の効果を有意に向上させることを明らかにした。

以上、本研究において腫瘍微小環境へのサイトカイン遺伝子導入により DC 療法が増強されることが明らかになった。これらの成果は、免疫治療の改善だけでなく、本治療における腫瘍微小環境の新たな重要性を提唱し、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。