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論　文　名	「Basic study of canine dendritic cells：*In vitro* generation, characterization and regulatory factors（イヌ樹状細胞の基礎研究： *In vitro* における分化誘導、特性の解析および制御因子）」
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論文要旨

Introduction

Dendritic cells (DCs) are specialized to take up, process and present antigens, and have the capacity to stimulate naïve T cells and initiate the primary immune response. Since patients with growing tumor are impaired to recognize the abnormal cells as non-self, these properties of DC can be utilized to recall immune responses against tumor cells. Furthermore, since the highly specific immune responses against tumor cells are persistently memorized and take place wherever the immune cells can arrive at, the DC based immunotherapy is expected to cope with tumor relapses and metastasis.

For approaching to the immune therapy, much effort has been done to establish the

optimal method to induce DCs in vitro from monocytes and bone marrow precursors using purified cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4, to overcome the scarcity of DCs in blood. In human, using the *in vitro* developed DCs, many clinical trials have been carried out against variety of tumors. However, in most of the cases the DC based therapy has not been enough succeeded, and many of patients has showed progress of the disease. Recently it has been found that the *in vitro* developed DCs were inactivated along with reverse maturation in the *in vivo* environment where the stimulation for DC activation and maturation was not available. Furthermore, it has also found that some tumors produce factors to suppress the DC activation. Therefore, it is necessary for the success of the DC based therapy to constitute the *in vivo* environment to support the development and activation of DCs.

Suffering from common types of cancer with humans, success in DC therapy against canine cancers promises not only advancement in cancer treatment in veterinary medicine, but also provides effective information that advances DC therapy in humans. However, the development and characteristics of DCs in the dog has not been subjected to a thorough study. Therefore, a series of experiments were conducted in order to understand *in vitro* differentiation, characterization of canine DCs and regulatory

factors for optimizing their activity in the tumor therapy. First, the method to effectively induce fully matured DC from canine peripheral blood monocytes (PBMOs) was established. Second, using biophysical phenotypes, hematopoietic progenitor cells from canine bone marrow (BM) was isolated to be used ultimately as sources of DC. Third, genes of canine cytokine were cloned and expression vector including them were constructed to constitute the *in vivo* environment.

Chapter 1: Generation of canine DCs from peripheral blood monocytes without using purified cytokines

Since the DC population in blood and tissues is very small, recent work has aimed to generate DCs *in vitro* from PBMOs or BM precursors, using granulocyte-macrophage (GM)-CSF, IL-4 and maturing factors in the human and mouse. But this generation method is not applicable in animal species for which recombinant cytokines are not available. The differentiation and maturation of canine DCs from PBMOs also have not yet been well characterized, since appropriate conditions for the *in vitro* differentiation were not known. However, it has been reported that monocytes differentiate into DCs *in vivo* in the T cell area of lymph nodes, and that maturation of DCs takes place in the lymphoid tissues through their interaction with T cells. Therefore, it is suggested that

the conditioned media produced by activated T cells provides suitable conditions for differentiation and maturation of DCs. To verify this hypothesis and to cope with limitations in purified cytokines of the dog, canine T cell conditioned medium (TCCM) was developed by culturing peripheral T cells with immobilized anti-canine CD3 mAb, and the differentiation of DCs from PBMOs (isolated $CD14^+$ cells) was examined by morphology, phenotype and functional aspects.

There has been tremendous progress in characterizing DCs in both mice and humans. In all these studies, mature DCs have been identified based on their morphological and functional characteristics: 1) Large cells with long dendritic processes; 2) significant expression of molecules for antigen presentation, such as MHC (particularly MHC II) and CD1a; 3) potent ability to stimulate allogeneic T cells in MLR; and 4) poor phagocytotic activity. In addition, DCs produce cytokines such as IFN-γ, TNF-α and IL-18, to augment immune responses. In the present study morphological and functional characteristics of cells derived from the TCCM-culture of PBMOs was investigated, and found that the day 12 cells from the TCCM culture provided feature completely according with the DC-characters described above. Furthermore, the effects of TCCM are reproducible over different batches. It follows from these results that TCCM provide optimal condition for the *in vitro* differentiation of DCs from PBMOs. In

this study, a model for DC generation without using pure cytokines is exhibited.

Chapter 2: Isolation of hematopoietic progenitor cells in canine bone marrow

The *in vitro* differentiation of DC was first found from BM progenitor cells. From the results of the study described in Chapter 1, it is suggested that efficiency of DC generation is affected by the purity of precursor. Therefore, as the first step of investigating the canine DC-differentiation in the BM cell route, hematopoietic progenitor cells (HPCs) in canine bone marrow were isolated using phenotypes concerned with physiological properties: Cell density, surface glycoproteins which bind to wheat germ protein (WGA) and capability for efflux of vital dye, Rhodamine-123 (Rh) which is mediated by multidrug resistance genes such as P-glycoprotein.

Canine BM cells were separated by equilibrium-discontinued centrifugation, and HPCs, detected by *in vitro* colony formation, were significantly enriched in the relatively low density (LD) fraction. In flow cytometry, many CD34, a marker of human HPCs or MHC class II expressing cells were detected in the LD fraction, but these were not significantly enriched. When the LD cells were separated, using a cell-sorting method, into cells with high affinity of WGA (WGA^{high}) and cells with WGA^{low}, almost all multipotent HPCs (MHPCs) and HPCs committed to myeloid lineage were found in the

WGA^{high} population. However, forty percent of CD34 positive cells were found as WGA^{low}. Therefore, the WGA separation is superior to that by CD34 for isolation of DC progenitors. When the WGA^{high} population was further stained for Rh, almost all MHPCs were included in the dull population (Rh^{low}). Morphologically, most Rh^{low} cells were round blastic cells containing a large nucleus with nucleoli and narrow cytoplasm. Based on these results, it is suggested that all of the MHPCs in canine BM show the Rh^{low} WGA^{high} LD phenotype, and may contain hematopoietic stem cells, which are the primitive HPCs.

Chapter 3: Cloning and production of canine cytokine genes for generation and activation of dendritic cells *in vivo*

IL-12 shows potent ability to activate immune cells concerning in cellular immunity, such as cytotoxic T lymphocyte (CTL) against tumors. While IL-12 is produced by activated mature DCs, the cytokine in turn takes a role in maintenance of DC activity. On the other hand a combination of GM-CSF and IL-4 exert an effect to induce differentiation of PBMOs into DCs. Therefore, if an environment producing GM-CSF, IL-4 and IL-12 exists near or around tumor tissues, DCs will be continuously recruited by the differentiation from infiltrated PBMOs, and maintain the activity to prime

specific immunity against the tumor. The long term existence of such environment will be achieved by transfection of gene-expression vectors containing the cytokine genes. In this study, as the first step of the project, the molecular cloning of canine IL-4, GM-CSF and IL-12 gene was carried out, and the expression of functional cytokine by the gene was examined.

Full length of canine IL-4, GM-CSF, IL-12p35 and IL-12p40 cDNAs were amplified by PCR with specific primers, inserted into blunt-end vector and cloned in *E. coli* clone DH5α. After the certification of correct sequence, cDNA of IL-4, GM-CSF or IL-12 p35 (p35) was inserted into expression vector, pcDNA3.1, which contains neomycin-resistant gene and cDNA IL-12 p40 (p40) into pSVL, which does not contain neomycin-resistant gene. The vectors were transfected into CHO cells and the transfected cells were selected with neomycin.

For expression of IL-12, the p35-pcDNA3.1 and the p40-pSVL vectors were co-transfected into CHO cells. After the selection with neomycin, the expression of IL-12 was examined in immunofluorescence assay using anti-canine p40 antibody. About 50% of the CHO cells was successfully transfected with both the p35-pcDNA3.1 and the p40-pSVL vectors. Ninety to100% of CHO cells transfected with IL-4-gene or GM-CSF-gene was detected to express IL-4 or GM-CSF cytokine in flow cytometry.

Supernatants were collected from cultures of IL-4-expressing CHO (IL-4-CHO), GM-CSF-CHO and IL-12-CHO, and examined for functions of DC induction and maturation. When PBMOs were incubated with supernatants of GM-CSF-CHO and IL-4-CHO, cell morphology changed to that of DCs, and expression of MHC class II, CD1a, CD80 and CD86 on the cells significantly elevated. The expressions of CD80 and CD1a were significantly enhanced by addition of the IL-12-CHO supernatant. These results suggest that cloned canine IL-4 and GM-CSF genes express functional cytokines for DC induction, and IL-12 gene for DC maturation. In addition, the IL-12-CHO supernatant activated T cells and induced secretion of IFN-γ. Taken together, it is suggested that these cytokine gene vectors can be utilized to constitute the *in vivo* environment for recruiting DCs and maintaining DC activity against tumors.

Conclusions

1. Mature DCs can be successfully generated using TCCM from PBMO and this could be used as a model to develop DCs in any species especially where recombinant cytokines have not developed.
2. HPCs including DC progenitors were significantly enriched in the LD WGA^{high} Rh^{low} cells of the dog BM.

3. IL-12 not only activates T cells but facilitates the DC maturation also.

4. All results in the series of experiments together will provide new strategy for the DC based tumor immunotherapy not only in dogs but also in human.

審査結果の要旨

樹状細胞は、抗原提示に特化した能力を持つ血液細胞で、抗原特異的免疫反応を惹起できる唯一の細胞である。他方、がん患者の体内では、がん細胞に対する免疫機能が著しく障害されて、本来排除されるべきがん細胞が排除されずに増殖している。そこで、生体外（*in vitro*）において骨髄中の前駆細胞や末梢血単球から樹状細胞を分化誘導させて育て、がん抗原を取り込ませて提示させた後に生体内（*in vivo*）に戻せば、がん細胞に対する免疫反応が賦活され、がんを退縮に導くことが期待される。現在、この考えに基づき、ヒトにおいて *in vitro* で育てた患者の樹状細胞を用いてがんの治療が試みられているが、*in vitro* で育てた樹状細胞を *in vivo* に戻すと樹状細胞の免疫活性化能が急激に低下するため、これまではかばかしい成果が得られていない。

これまでに、いくつかの研究グループによってイヌ樹状細胞の *in vitro* における分化誘導と特性の解析が試みられているが、樹状細胞の分化誘導に必要な精製イヌサイトカインが、入手困難なため、未だ分化誘導の至適条件が確立されていない。また、骨髄中の樹状細胞の前駆細胞を分離するための方法も確立されていない。

そこで本研究では、１）精製サイトカインを用いることなくイヌ樹状細胞を *in vitro* で分化誘導する方法の確立、２）樹状細胞の前駆細胞を含んだイヌ骨髄中の造血前駆細胞の分離方法の確立、および３）*In vivo* において樹状細胞の成熟、活性化を維持する環境を構

築するためのイヌサイトカイン遺伝子のクローニングを試み、以下の成果を得た。

１．*In vivo* では、免疫細胞が分泌する炎症性サイトカイン群によって樹状細胞の分化誘導が起こる。本研究では、培養中でイヌ T 細胞を活性化刺激することで、炎症性サイトカインの溶液（T cell conditioned medium：TCCM）を取得し、それを用いて単球から樹状細胞への分化誘導を試みたところ、12 日間の TCCM 培養によって、ほとんどの単球が①形態、②細胞表面マーカー、③免疫反応活性化能、④貪食能のすべてに樹状細胞の特徴を表し、成熟樹状細胞への分化誘導が証明された。

２．骨髄細胞を比重、細胞表面糖鎖への小麦胚芽凝集素（WGA）の親和性および生体染料であるRhodamine-123（Rh）の染色性（細胞外への排出性）によって分別したところ、樹状細胞の前駆細胞を含む多能性造血前駆細胞は、低比重で、WGAの親和性が高く（WGA^{high}）、Rhの染色性が低い（Rh^{low}）細胞集団中に有意に多く含まれることが判明した。

３．樹状細胞の分化誘導に必須とされる顆粒球・マクロファージ - コロニー刺激因子（GM-CSF）とインターロイキン - 4（IL-4）のイヌ遺伝子、および腫瘍免疫の活性化亢進に重要である IL-12 のイヌ遺伝子をクローニングした。これらを適切なプラスミドに組み込んで、哺乳類細胞発現ベクターを作製し、CHO 株細胞に導入したところ、それぞれのサイトカインタンパク質の細胞からの産生が認められた。イヌ末梢血単球を GM-CSF および IL-4 遺伝子導入細胞から産生されたサイトカインと培養すると、樹状細胞へ分化誘導され、その培養に IL-12 遺伝子導入細胞から産生されたサイトカインをさらに加えると樹状細胞の成熟、活性化が促進した。また、この IL-12 遺伝子導入細胞産生サイトカインは、腫瘍免疫を活性化するインターフェロンγのイヌ T 細胞からの産生を誘導した。これらの結果からこれらのサイトカイン遺伝子は、*in vivo* において樹状細胞の成熟、活性化を維持する環境を構築できることが明らかとなった。

以上のように本研究では、イヌにおける腫瘍免疫治療法の開発をめざして、*in vitro* にお

ける樹状細胞の分化誘導、特性の解析およびその制御因子を明らかにした。イヌは、ヒトと同じ生活空間を持ち、ヒトと同様な腫瘍の発生がみられる。したがって、本研究の成果に基づくイヌにおける新しいがん治療法の開発は、獣医学の発展のみならず、ヒトの臨床応用にもつながるものであり、本論文の審査ならびに最終試験の結果と併せて、博士（学術）の学位を授与することを適当と認める。