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Short communication

Production of canine soluble CD40 ligand to induce maturation of monocyte derived dendritic cells for cancer immunotherapy

Viskam Wijewardana[a,1], Kikuya Sugiura[a,*,1], Mana Yahata[a], Takashi Akazawa[b], Daluthgamage Patsy H. Wijesekera[a], Shigeki Imamoto[a], Shingo Hatoya[a], Norimitsu Inoue[b], Toshio Inaba[a]

[a] Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano City, Osaka, Japan
[b] Department of Molecular Genetics, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka City, Osaka, Japan

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ABSTRACT

CD40 ligand (CD40L) expressed by activated T cells is shown to induce maturation of immature dendritic cells (DCs) and this maturation is a vital part in DC based tumor immunotherapy. We constructed an expression vector by cloning the extracellular domain of canine CD40L fused to the signal sequence of canine IL-12p40. When PBMCs were incubated with canine granulocyte-macrophage (GM) -CSF and IL-4, expression of CD86 was significantly elevated, but the majority of cells displayed the morphology of immature DCs. Following addition of the expressed canine soluble CD40L (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. These results show that the csCD40L significantly promotes the maturation and activation of canine monocyte derived DCs.

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1. Introduction

Because of their superior ability in inducing adaptive immunity, there is growing interest in utilizing dendritic cells (DCs) as cellular adjuvants in cancer immunotherapy (Palucka et al., 2011; Steinman and Dhodapkar, 2001). To overcome limitation in their availability, DCs have been generated in vitro from PBMCs, giving rise to monocyte-derived DCs (MoDCs) (Zhou and Tedder, 1996). PBMCs are cultured with granulocyte-macrophage (GM)-CSF and IL-4 in order to generate immature MoDCs, and various soluble factors are then used to induce maturation, including a commonly used cytokine cocktail comprising TNF-α, prostaglandin E_2 and IFNα. Unfortunately, this cytokine cocktail did not induce the production of IL-12, which is critical in generating protective antitumor immunity from matured DCs (Luft et al., 2002). CD40 ligand (CD40L, CD154) was found on activated T cells as a transmembrane protein (Armitage et al., 1992). CD40/CD40L interaction during antigen presentation plays a critical role in DC maturation and gave rise to DCs with high IL-12 production (Mackey et al., 1998). Moreover, maturation with CD40L generated a homogeneous mature DC

* Corresponding author at: Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku, Ourai-kita, Izumisano City, Osaka 598-8531, Japan. Tel.: +81 72 463 5379; fax: +81 72 463 5379.
E-mail address: sugiura@vet.osakafu-u.ac.jp (K. Sugiura).
[1] Both these authors contributed equally.

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population, which is essential in tumor immunotherapy (Würtzen et al., 2001). Recently, we succeeded in inducing mature DCs from dog PBMCs using dog T-cell-conditioned medium (Wijewardana et al., 2006). Also a soluble form of CD40L (sCD40L) in human T-cell-conditioned medium was found to be crucial in MoDC maturation (Kato et al., 2001). We therefore suggest that canine sCD40L is a promising tool for DC based tumor immunotherapy against dog tumors.

In this study we cloned the extracellular domain (ECD) of the canine CD40L gene and succeeded in constructing an expression vector for producing canine sCD40L by eukaryotic cells. We show that the expressed sCD40L induces maturation and activation of canine MoDCs.

2. Materials and methods

2.1. Construction of sCD40L expression vector

Total RNA was extracted from canine peripheral blood (PB) mononuclear cells activated by Con A (Sigma), and cDNA synthesis was carried out as described previously (Wijewardana et al., 2006). PCR amplification was done according to Sugiura et al. (Sugiura et al., 2008), and primers were designed to amplify the nucleotide sequence of the ECD in canine CD40L cDNA (NCBI accession number AY333790) (Supplementary Fig. 1A). Sequences recognized by *Nde*I were attached to the forward primer, and those recognized by *Hind*III to the reverse primers (Supplemental Fig. 1B). The PCR products were then inserted into a PCR-Blunt vector (Invitrogen) and amplified in the transformed *E. coli* DH5α. Sequences of the inserts were determined by thermal cycle sequencing, using BigDye® Terminator (Version 1.1; Applied Biosystems) and a sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). The clone having 100% homology with the desired sequence was selected. The canine sCD40L-expression vector was constructed as with the canine IL-18-expression vector, using pcDNA^{TM}3.1/myc-His(−) (Invitrogen) with canine IL-12p40 cDNA (IL-12p40-pcDNA) inserted (Sugiura et al., 2010). The expression vector sequence supplemented with the signal sequence was first prepared by cutting the IL-12-pcDNA with *Nde*I and *Hind*III. We then extracted the coding sequence of CD40L ECD from CD40L-pCR-Blunt by digestion with the same enzymes, and inserted it into the prepared vector to construct sCD40L-pcDNA (summarized in Supplementary Fig. 2).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.09.016.

2.2. Assay of protein expressed by transfected gene

The sCD40L-pcDNA was transfected into CHO cells using Lipofectamin 2000 (Invitrogen), and transfected cells were selected in a culture with neomycin (Nakarai tesque, Kyoto, Japan). The supernatant from the culture of sCD40L-pcDNA transfected CHO (sCD40L-CHO) or mock transfectant was concentrated using a centrifugal filter device (Amicon Ultra-15, Millipore). The concentrated solution was treated with Ni-agarose beads (Cosmogel® His-Accept: Nakarai Tesque) according to the manufacture's protocol so as to further concentrate the expressed protein. Protein expression by sCD40L-pcDNA was detected by loading the resulting solution in Western blotting using mAb to c-myc tag peptide (clone 9E10: Sigma–Aldrich) as described previously (Sugiura et al., 2008). To verify the structure of the expressed protein, the amino acid sequence predicted from the cloned cDNA was examined by Swiss model software (Arnold et al., 2006; Kiefer et al., 2009).

Bioactivity of the protein expressed as sCD40L was estimated from the ability to increase proliferation of B cells, as described by Mazzei et al. (1995). In summary, B cells were isolated from dog PB mononuclear cells by depletion of T cells and myeloid cells, using mAbs to canine CD3 (clone CA17.2A12, Serotec), canine CD4 (clone CA13.1E4, Serotec) and canine CD11b (clone CA16.3E10, Serotec), and magnetic beads coated with sheep-anti-mouse IgG (Dynabeads® M-450, Invitrogen). After the treatment about 70% of cells was CD21^{+} B cells. The B cell-enriched population (10^5) was incubated with 20 ng/ml canine rIL-4 (R&D Systems) and various concentrations of protein from the sCD40L-CHO culture in 0.2 ml RPMI1640 medium (Nakarai Tesque) supplemented with 10% FBS (Valley Biomedical Inc.) (complete medium). B cell proliferation was evaluated by measuring the radioactivity of [^{3}H]-thymidine incorporated into genomic DNA.

2.3. Culture of PBMCs and assay of DC maturation

CD14 positive PBMCs were isolated as described previously (Wijewardana et al., 2006). Using 24-well flat-bottom plates, the PBMCs (1×10^6/well) were cultured with various kinds of protein for 12 days in 2 ml complete medium. Four culture conditions were set up: (1) [Control] containing complete medium alone; (2) [GM-CSF+IL-4] containing rcGM-CSF (R&D Systems) at 10 ng/ml, canine rIL-4 at 10 ng/ml and protein from the mock transfected culture at 50 μg/ml; (3) [+CD40L-12d] containing rcGM-CSF (10 ng/ml), rcIL-4 (10 ng/ml) and protein from the sCD40L-CHO culture at 50 μg/ml; 4) [+CD40L-6d] containing initially rcGM-CSF (10 ng/ml), rcIL-4 (10 ng/ml) and, on day 6, with protein added from the sCD40L-CHO culture at 50 μg/ml.

In morphological analysis, the cultured cells were stained with May-Grünwald and Giemsa solution. In the assay for surface molecules expressed on DCs, the cultured cells were stained as previously described (Sugiura et al., 2010), with the following Abs: FITC-conjugated mouse anti-human CD1a mAb (clone NA1/34-HLK, Serotec), rat anti-canine MHC class II mAb (clone YKIX.334.2, Serotec), PE-Cy5-conjugated mouse anti-human CD14 mAb (clone TÜK4, Serotec), Biotin conjugated rat anti-mouse CD80 mAb (clone 1G10, Becton Dickinson), and mouse anti-human CD86 mAb (clone FUN-1, Becton Dickinson). Staining with biotin-anti-CD80 mAb was followed by incubation with Streptavidin-PE-Cy5 (Becton Dickinson). The stained cells were analyzed using a flow cytometer (FACS-Culibur, Becton Dickinson). The mean expression intensity (MEI) of the cell surface molecules was quantified as the mean fluorescent intensity, using appropriate software

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