

Role of NKT Cells and α -Galactosyl Ceramide

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Abstract

Alfa-Galactosyl Ceramide was isolated from Ocean sponge which has antitumor effect against several tumors in *in vivo* animal model with no cytotoxicity. KRN7000(KRN) is the most potent α -Galactosyl Ceramide modified from the one isolated from Ocean sponge. KRN is also active against metastatic tumors through the activation of animal immune system. Research efforts in learning the mechanism of action, we found the important role of dendritic cells(DC) and NKT cells. NKT cells was first characterized in 1988 which is overlap some part with NK cells and T-Cells and majority is different from NK and T. KRN is active through the activation of DC and NKT in giving antigen specific immune stimulation in animal. This antigen specific stimulation is memorized by immune system and can reject second tumor challenge. KRN is not active in nude mice and NKT deficient animal. NKT cells level in blood is lower in patients with autoimmune disease, cancer, HIV positive or aplastic anemia. NKT rapidly releases IL-4 and IFN- γ at high level when activated. NKT is CD1d and TCR restricted. NKT plays important role in autoimmune disease such as Type 1 Diabetes, Scleroderma and Systemic Lupus Erythematosus, infections such as Mycobacteria, Listeria and Malaria, GVHD control and tumor rejection. NKT acts as double edge sword, aggressive and suppressive ways. KRN can prevent the onset of Type 1 Diabetes, inhibit replication of hepatitis virus B in liver and suppress malaria replication in activating NKT cells. KRN can activate NKT through DC and activated NKT activates NK, T and macrophage. KRN also expands NKT cells and expanded NKT has full function. Although the exact role of DC and NKT is not clear, KRN clinical study results in conjunction with DC and NKT cell activation are expected.

1. Introduction

We discovered a group of sphingo saccharides from marine sponge, *Agelas mauritanus*, through our screening program which used *in vivo* animal model against B16 melanoma [1]. These group of molecules, agelasphin, have no cytotoxicity and work through the stimulation of animal immune system. Those molecules did not work in nude mice. Experimental results indicated that the activity through immune stimulation was specifically memorized and the second challenge with the same tumor was rejected. The mechanism of action was not clear although it suggested that molecule stimulate dendritic cells (DC). We were able to synthesize those molecules and screened the most potent modified molecule using *in vivo* animal assay system. The molecule was named KRN7000 [2]. KRN7000 is effective against various tumors in animal as well as metastatic tumors.

NKT cells share characteristics with natural killer

cells (NK) and T-cells. NKT cell was first described in 1987 [3]. The role of NKT cells was not known during our early stage research work on mechanism of action. The role of NKT cells in immune system became revealed and the mechanism of action of KRN7000 became better understood. NKT has overlapped activity with T-Cells and NK cells in immune system. Collaborations with leading scientists in the world helped understanding the role of NKT and relationship with KRN7000. Taniguchi et al studied NKT knock in and knock out gene animal and showed the important role of NKT cells in KRN7000 treated mice [4]. Recently, the presence and the role of NKT cells have been studied intensively and the role of NKT cells became more clear a little by little. Number of NKT cells in peripheral blood was reported in patients with various diseases and in healthy volunteers. In some diseases patients, NKT cell level is lower than healthy volunteers. The role of NKT cells and the relationship with the treatment of disease with increasing NKT cell number is not

studied yet. KRN7000 was studied both in patients with cancer and normal healthy volunteers. Toxicity was mild and there is no dose limiting toxicity.

2. KRN7000

KRN7000 is a synthetic modified product of natural agelasphin group compounds, α -galactose-ceramide. This molecule was screened from various natural and synthesized agelasphin group of compounds using *in vivo* mice model.

2.1. Structure

Agelasphins and KRN7000 are a glycosphingolipid. It is a linked molecule of sugar and ceramide. There are various sugars and various ceramides which can be linked together. In active glycosphingolipid, sugar and ceramide has to be linked with α configuration. Majority of sugar ceramids found in various natural resources have β link configuration and have no such activities like Agelasphin. Galactose-ceramide has most potent activity among various sugars [4]. Agelasphin group compounds only could be screened using *in vivo* model. Because its activity is not due to direct action of molecule to tumor cells but indirectly through animal immune system. The longer chain ceramides have less water solubility.

2.2. Activity

2.2.1. Animal Model

KRN7000 has no cytotoxicity *in vitro* and no serious toxicity was found in animal pre-clinical and human clinical studies up to $4800\mu\text{g}/\text{m}^2$ [5]. It is active against B16 melanoma, colon 26, EL-4 lymphoma model in mice as well as systemic metastatic models. Production of Interferon γ (INF γ) and IL-2 is dose dependently increased following KRN7000 administration in mice and T-cells harvested from treated mice showed increased cell-lytic activity against Yak 1 cells dose dependently. KRN7000 is not active in nude mice or NKT deficient mice. It activates DC cells in animal and activated DC cells transfer the specific immune stimulation signal to other immune responsible cells which eradicate tumor cells. *Ex vivo* DC stimulation with KRN7000 and stimulated DC administration is also effective. KRN7000 can cure the mice with systemic metastatic tumor. But could not manage late stage tumors probably because of large tumor burden. Combination of KRN7000 and cytotoxic drug has synergistic effects even treatment started at later stage. Which suggest cytotoxic drug reduced tumor burden and remaining tumor was managed with immune system stimulated by KRN7000. Stimulation of immune system triggered by KRN7000 is specific and memorized by immune system. The animal challenged with tumor and cured with KRN7000 treatment, was challenged again with the same tumor but the tumor was rejected. If you challenge other tumor to the cured mice, it was accepted

and grew in the animal. This suggests that animal has memorized immune response to the tumor. KRN7000 activates DC to recognize tumor and to process its as target antigen. *Ex vivo* stimulation of DC with tumor lysate alone was not effective, but with KRN7000 DC could be activated to process tumor lysate as antigen and activated DC administered in to mice can eradicate the tumor.

KRN7000 can delay the onset of Type I diabetes in NOD mice. NOD mice develop diabetes in 10 weeks after birth. Administration of KRN7000 from 4 weeks after birth can delay/prevent the onset of disease. When you start the treatment at $100\mu\text{g}/\text{kg}$ with KRN7000 from 10 weeks after birth, KRN7000 can not prevent the onset of the disease. But even start treatment with KRN7000 from 10 weeks after birth, higher dose at $250\mu\text{g}/\text{kg}$ can prevent the onset of the Type diabetes in NOD mice. IL-7 and KRN7000 co-administration has synergistic effect against the onset of diabetes. IL-10 antibody inhibits the activity of KRN7000 [6,7].

It is also effective against EAE model mice. The activity of KRN7000 is CD1d positive cell dependent. It is not active in IL-4 or IL-10 gene knockout mice [8].

Replication of Hepatitis B virus in liver was suppressed by NKT cell activation with KRN7000. Its activity against Hepatitis B virus was inhibited in IFN α gene knockout mice or by the antibody to IFN γ [9]. KRN7000 also inhibits the development of liver stage murine malaria [10].

NKT cells rapidly activate NK cells after KRN7000 administration [11]. DC pulsed with KRN7000 *in vitro*, expand NKT cells [12] and expanded NKT cells have normal function in producing Granzym B, Perforin, IFN γ or IL-4 [13]. Those data suggested that KRN7000 is responsible for both Th1 and Th2 T-cell response.

2.2.2. Clinical Data

Neutrophil increased in 6 to 24 hours and lymphocytes decreased 6 to 48 hours after KRN7000 administration in normal healthy volunteers. DC/ NKT cells were activated and migrated from peripheral blood. Level of IFN γ was elevated in some volunteers who had higher NKT cell numbers prior to KRN7000 administration. Phase I study in patients with advanced stage tumor showed the safety of KRN7000 up to $4800\mu\text{g}/\text{m}^2$ without any dose limiting toxicity. Stable Disease/No Change were observed in some patients with melanoma and kidney carcinoma. There is no human study in patients with auto immune disease yet. The benefit of KRN7000 treatment has to be explored.

3. NKT Cells

NKT cell was first described in 1987. Present knowledge of NKT cells were well reviewed by Godfrey et al [14]. NKT cells are family of T-cells which share various characters with T-cells and NK cells. Heterogeneity of NKT cells gave confusions to scientists at the early stage of NKT cell research. Function of NKT

cells show controversy, some times suppressive, some times aggressive in controlling T-cells and NK cells.

3.1. Characteristics of NKT Cells

NKT cells first studied are $\alpha\beta$ TCR⁺ T cells that are CD4 and CD8 double negative. Those expressed NK1.1 and have potential to produce IL-4, INF γ and TNF α . The development of TCR V α 14J α 281 cells are dependent on MHC class I like molecules. NK1.1+ $\alpha\beta$ CD4/CD8 double negative cells and NK1.1+CD4⁺ T-cells are part of the same lineage cells. NKT cells are usually identified by the expression of $\alpha\beta$ TCR and NK1.1 or V α 14 in mouse. Mouse and human NKT cell are sharing common features. Major Subsets are both CD4⁺, double negative (CD4 and CD8, $\alpha\beta$ DN). T-Cell receptor on α -chain is V α 14 in mouse vs. V α 24 in human. Restriction element is CD1d in mouse and human. Cognate antigen is both Glycolipid, α -Galactosyl Ceramide (KRN7000). Cytokine production is very specific to IL-4 and IFN γ . Thus you may predict human response in mouse system. KRN7000 binds to CD1d and stimulates CD4⁺/CD8⁺ Double negative NKT.

3.2. Presence of NKT

NKT cell is around 0.1-0.5% of peripheral blood leukocyte. Lower NKT cell number was reported in patients with cancer, HIV infection or autoimmune disease. Auto immune disease patients with tissue damages such as lupus, systemic sclerosis, rheumatoid arthritis, multiple sclerosis or aplastic anemia has lower NKT cell number in peripheral blood [15,16]. In animal model for lupus or Type 1 diabetes, KRN7000 can prevent or delay the onset of disease.

3.3. Role of NKT Cell

NKT dose work both Th1 and Th2 stimulation. Stimulation of Th1 or Th2 looks dependent on circumstances and stage of NKT cell maturation. Th2 stimulation leads to control of autoimmunity. Th1 stimulation leads to tumor rejection and infection control.

4. Summary

1. Role of NKT cell is still not clear but seems to have the important role in autoimmunity, infection, and malignancy. NKT cells may be able to separate GVL and GVHD.
2. KRN7000 expands NKT cells in vivo and in vitro through DC. Stimulated NKT seems to work for Th1 and Th2 both ways.
3. Clinical application of increasing NKT with KRN7000 has to be studied in patients with autoimmune disease, hepatitis infection and cancer.
4. Expanded NKT cells for therapeutic use also has to be studied.
5. NKT cell has double edge sword activity, TH1 and TH2 stimulation and KRN7000 activate NKT cells both ways [19].

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