Impact of IL-2 and IL-2R SNPs on Proliferation and Tumor-killing Activity of Lymphokine-Activated Killer Cells from Healthy Chinese Blood Donors

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Abstract

One of the goals of tumor immunotherapy is to generate immune cells with potent anti-tumor activity through in vitro techniques using peripheral blood collected from patients. However, cancer patients generally have poor immunological function. Thus using patient T cells, which have reduced in vitro proliferative capabilities and less tumor cell killing activity to generate lymphokine-activated killer (LAK) cells, fails to achieve optimal clinical efficacy. Interleukin-2 (IL-2) is a potent activating cytokine for both T cells and natural killer cells. Thus, this study aimed to identify optimal donors for allogeneic LAK cell immunotherapy based on single nucleotide polymorphisms (SNP) in the IL-2 and IL-2R genes. IL-2 and IL-2R SNPs were analyzed using HRM-PCR. LAK cells were derived from peripheral blood mononuclear cells by culturing with IL-2. The frequency and tumor-killing activity of LAK cells in each group were analyzed by flow cytometry and tumor cell killing assays, respectively. Regarding polymorphisms at IL-2-330 (rs2069762) T/G, LAK cells from GG donors had significantly greater proliferation, tumor-killing activity, and IFN-γ production than LAK cells from TT donors (P<0.05). Regarding polymorphisms at IL-2R rs2104286 A/G, LAK cell proliferation and tumor cell killing were significantly greater in LAK cells from AA donors than GG donors (P<0.05). These data suggest that either IL-2-330(rs2069762)T/G GG donors or IL-2R rs2104286 A/G AA donors are excellent candidates for allogeneic LAK cell immunotherapy.

Keywords: NK- LAK- IL-2-330 (rs2069762)T/G - IL-2R (rs2104286)A/G - blood donors - killer cells

Introduction

Biologically based immunotherapies are a new method for treating tumors using biological products in vivo to augment the tumor specific immune response of patients, maintain their physiological balance, or protect against tumors. Alternatively, in vitro methods can be used to expand or activate the cytotoxic effector cells that regulate the in vivo biological response. The goal of such in vitro methods is to enhance the anti-tumor activity of effector cells before infusing them back into patients where they kill the tumor. Erhan’s studies found that most cancers occur not to transferred malign cells out of the body, but by a malign transformation of self-cells (Erhan et al., 2013). But the effects of whole cell immunization for prevention of human cancers is not clear. New studies are needed to evaluate whole cell type immunization on cancer prevention. Lymphokine-activated killer (LAK) cells are widely used in immunotherapy. LAK cells, first described in the 1980s, are activated natural killer (NK) cells that are capable of recognizing cancer cells in a non-MHC-restricted manner (Archinbaud et al., 1991). LAK cells have been utilized in vivo in animals and in humans to treat cancer. These cells are a heterogeneous population consisting primarily of NK, NKT, and T cells, which are generated in vitro by culturing peripheral blood mononuclear cells (PBMCs) with interleukin-2 (IL-2) (Grimm et al., 1982). The predominant effector cells in the LAK cells are the NK cells. NK cells are derived from CD34+ cells and represent only 10-20% of total lymphocytes in the peripheral blood, but play an important role in the innate immune system. NK cells are a subset of large granular lymphocytes that have the phenotype CD3-CD16+ CD56+. NK cells are early innate immune effectors that can have nonspecific cytotoxic activity against tumor cells (Srivastava et al., 2008). LAK cells are traditionally prepared from isolated T cells cultured with IL-2. However, tumor patients generally have limited immune function and as a result, their T cells have reduced in vitro proliferation and tumor cell killing activity. This...
IL-2 is important for the proliferation of activated T-lymphocytes. It can also enhance activation-induced cell death in T-lymphocytes and eliminate self-reactive cells (Wang et al., 1997). IL-2 also contributes to terminating the lymphocyte response by inducing suppressive T-cells (Malek et al., 2003). A single nucleotide polymorphism (SNP) G-330T (rs206972) has been identified upstream of the IL-2 promoter-enhancer domain that reportedly influences protein production in healthy subjects, the G allele produced over three times the amount of IL-2 than their T/T and T/G counterparts (John et al., 1998; Hoffmann et al., 1999). The relationship between IL-2-330 (rs2069762) T/G and several infectious diseases, tumors, and autoimmune disorders has become an active area of research in China and abroad.

Regarding the IL-2RA gene, different independent signals in this gene were identified by fine mapping of the region. The minor alleles identified in these regions were related to a heritable reduction in the circulating level of soluble IL-2RA (Sul et al., 2007; Smyth et al., 2008; Carr et al., 2009; Maier et al., 2009). IL-2RA is constitutively expressed on regulatory T cells, which potently suppress autoreactive T cells (Buckner et al., 2010). Some studies found that SNP has certain correlation with some disease (Bei et al., 2014). Understanding the normal distribution of IL-2 and IL-2R genotypes could provide an important baseline for additional work assessing the relationship between IL-2 SNPs and the occurrence and development of disease.

Immunotherapy using allogeneic cells is one of the focuses for tumor therapy research, but appropriately selecting donor cells remains a challenge. This study was designed to establish an optimized IL-2 SNP detection method by analyzing IL-2 SNP with HRM-PCR (High Resolution Melt PCR); to acquire the distribution profiles of IL-2-330 (rs2069762)T/G and IL-2R (2104286)AA, AG, GG. The SNP position 2069762 T/G and several infectious diseases. This study was approved by the Institutional Ethics Committee of China Medical University and followed institutional guidelines.

Reagents
K562 (human erythroblast leukemia cell line) cells were purchased from the Shanghai Cell Bank. The Type-it HRM PCR Kit (USA) and DNA extraction kit (Germany) were purchased from QIAGEN. The following reagents and flow cytometry antibodies were purchased from BD (USA): FITC-anti-CD3 monoclonal antibody, PerCP-anti-CD16 monoclonal antibody, PE-anti-CD56 monoclonal antibody, and red blood cell lysis buffer. In addition, we used the following: lymphocyte isolation buffer (Tianjin Haoyang Biological Manufacture Co., Ltd.), IL-2 (Amoytop, China), 1640 culture medium (Sigma, UK), fetal bovine serum (Sigma, UK), viable cell counting kit (CCK-8) (KeyGen, China), and an IFN-γ ELISA kit (BD Biosciences, UK).

DNA isolation
Genomic DNA was extracted from 200μL of whole blood with the QIAamp DNA Micro Kit according to the manufacturer’s instructions. The extracted DNA was resuspended at 30ng/μL and stored at -80°C (Thermo Fisher Scientific, USA).

IL-2 and IL-2R SNP detection by HRM-PCR
A 50μL reaction system was used to detect SNPs by HRM-PCR containing: 10×PCR buffer (5μL), 2.5 mmol/L dNTP solution (4μL), DNA extraction solution (5μL), primers (1μL) (Table 1), Taq DNA polymerase (1.5 U), and sterile deionized water to bring the final volume to 50μL. The amplification conditions were: pre-denaturation 5 min at 94°C, denaturation 40 s at 94°C, annealing 40 s at 50°C, and extension 60 s at 72°C, for 40 cycles. Then HRM analysis was performed, with dissolution curves obtained at 0.3°C/s from 45°C to 98°C. Amplification products were subject to gene sequencing. Three genotypes were identified: IL-2 (2069762) TT, TG, GG and IL-2R (2104286) AA, AG, GG. The SNP position of each sample was also identified.

Detection of NK cells in peripheral blood
Flow cytometry was used to detect NK cells in the peripheral blood. Briefly, 100μL of peripheral blood was stained in an eppendorf tube using FITC-anti-CD3, PerCP-anti-CD16, and PE-anti-CD56 antibodies. The cells were incubated with antibody for 15 min at room temperature in the dark. Then, 2mL of erythrocyte hemolysin was added, incubated for 10 min at room temperature in the dark, and centrifuged for 5 min at 1, 200 rpm. The supernatant was removed, and the cells were washed with 2mL of PBS and

Materials and Methods

Subjects
Healthy blood donors (n=400) were recruited from Liaoning Province Blood Stations and the First Affiliated Hospital of China Medical University healthy blood donors. The donors included 203 males and 197 females with a mean age of 39.65±12.90 years. Samples were obtained after receiving informed consent. The donors did not have hepatitis, HIV, syphilis, or other infectious diseases. This study was approved by the Institutional Ethics Committee of China Medical University and followed institutional guidelines.

Table 1. Primer Sequences and Positions

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP (rs no.)</th>
<th>Position in Gene</th>
<th>Sequence (5'-3')</th>
<th>Temperature (°C)</th>
</tr>
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| IL-2 | rs2069762   | Promoter        | Forward: TCCCAAGTGATTTAGAGGATAA  
Reverse: TGGGTGGAAGCAGACAGTAA | 60 |
| IL-2R| rs2104286   | Exon            | Forward: GTGAGGGAGGAAAGGCATAGA  
Reverse: CTGGGATAGAGGAGAACAG | 60 |
centrifuged for 5 min at 1, 200rpm. The cells were then resuspended in 500μL of PBS. The frequency of CD3-CD16+CD56+ NK cells was measured by flow cytometry.

Lymphocyte Isolation and LAK induction

Peripheral blood was obtained from healthy volunteers. PBMCs were isolated using standard density gradient centrifugation on Ficoll-Histopaque. LAK cells were induced from the isolated PBMCs by culturing the cells in complete RPMI 1640 medium supplemented with 10% FCS and IL-2 (1, 000 IU/mL) from 48h to 14d at 37°Cin 5% CO₂.

Proliferation analysis of LAK cells by flow cytometry

LAK cells were analyzed using FITC-anti-CD3 and PE-anti-CD56. The frequency of CD3+CD56-, CD3-CD56+, and CD3+CD56+ cells in culture were determined. Flow cytometry was performed using a BD FACSCalibur and analyzed using BD CellQuest Pro software.

In vitro LAK cell killing activity assay

K562 cells, a human erythroblast leukemia cell line, were maintained in RPMI-1640 supplemented with 10% FCS and 1% L-glutamine. For the killing assay, the K562 cells were treated with pancreatin and transferred onto a 96-well plate at 1x10⁶/cells/mL. After 24h, the mature LAK cells were added into the wells at an effector:target (E:T) ratio of 1:10, 1:20, or 1:40. After 48h, the number of viable cells was determined using the CCK-8 kit. The CCK-8 reagent was added to the culture for an additional 2h, and then the OD was determined using a microplate reader.

Cytokine detection in the supernatant of LAK cell cultures

The mature LAK cells were collected and cultured for an additional 2h. The supernatant was then collected and used to quantify the amount of IFN-γ by ELISA per the manufacturer’s instructions. Briefly, 96-well flat-bottom plates were coated with an anti-IFN-γ antibody, non-specific binding was blocked by incubating with a buffer containing 0.5% BSA. After blocking, 40μL of supernatant, 10μL of detection antibody, and 50μL of biotinylated-horseradish peroxidase (HRP) were added to each well and incubated at 37°C for 60 min. The plates were then washed five times with wash buffer, and 50μL of chromogen solutions A and B were added to each well and incubated at 37°C in the dark for 10 min. To terminate the reaction, 50μL of stop buffer was added into each well. The plates were immediately read using a Microplate Reader System (Rayto, China).

Statistical analyses

Data are presented as the mean±SD. One-way ANOVA or Student’s t-test were used to determine differences between groups where appropriate. The differences were considered statistically significant when the P value was <0.05. All statistical analyses were performed using SPSS version 17.0 software.

Results

Genotype distribution

Using HRM-PCR to analyse the SNP of IL-2 and IL-2R, showed that IL-2 rs2069762 TT genotype and IL-2R rs2104286 AA genotype were the highest of the proportion, secondly the IL-2 rs2069762 TG genotype and IL-2R rs2104286 AG genotype, IL-2 rs2069762 GG genotype and IL-2R rs2104286 GG genotype were in the proportion to a minimum (Table 2). These showed distribution of IL-2 and IL-2R SNP in the healthy population in Liaoning, China.

The frequency of peripheral blood NK cells based on IL-2 and IL-2R SNP genotypes

Donors were stratified into six groups based on their IL-2 and IL-2R genotypes. The percentage of CD3-CD16+CD56+ NK cells in the peripheral blood was determined by flow cytometry for each genotype. While there were slight variations between the groups, they were not statistically significant (Figure 1).

Frequency of LAK cell subtypes based on the IL-2 and IL-2R SNP genotype

The phenotype of the PBMCs before IL-2 treatment included a mixed population of CD3-CD16+ (LAK-NK), CD3+CD56+ (LAK-NKT), and CD3+CD56- (LAK-T) cells. After 14d in culture with IL-2, the frequency of LAK-NK, LAK-NKT, and LAK-T cells was compared between IL-2 and IL-2R genotype groups. LAK cells were composed of LAK-NK cells, LAK-NKT cells and LAK-T cells. So the frequency of LAK cells were the sum of the frequency of the three groups. Regarding the IL-2 rs2069762 T/G genotypes, the frequency of LAK cells in the GG group (89.2±8.1)% was higher than the TG (62.3±7.2)% and TT (69.1±7.1)% group. In the IL-2R rs2104286 A/G groups, the frequency of LAK cells was significantly greater in the AA (82.2±8.3)% group

Figure 1. Frequency of NK Cells Based on IL-2 and IL-2R SNP Genotype The frequency of NK cells in the peripheral blood was assessed by flow cytometry. Donors were divided into the following groups based on their IL-2 or IL-2R genotype: IL-2 rs2069762 GG, IL-2 rs2069762 TG, IL-2 rs2069762 TT, IL-2R rs2104286 AA, IL-2R rs2104286 AG, and IL-2R rs2104286 GG. The bar shows the mean and the error bars indicate the SD.
The frequency of NK cells (CD3- CD56+) and NKT cells (CD3+ CD56+) in the peripheral blood was inversely correlated with the risk of developing cancer (Imai et al., 2000). The importance of NK cells in tumor immunity has made NK cells an attractive target for immunotherapy (Ruggeri et al., 2014). During tumor development, NK cells can directly kill tumor cells by secreting perforin and granzyme, and can regulate the function of other immune cells by secreting cytokines. NK cells can selectively recognize and kill cells that express NK-cell-activating receptors and “non-self” tumor cells that have reduced MHC expression. Resting NK cells circulate through the peripheral blood, while cytokine-activated NK cells can penetrate blood vessels, and migrate into infection sites or tumors (Chan et al., 2010; Lomonaco et al., 2011).

In this study, we determined the frequency of NK cells in the peripheral blood of healthy volunteers based on their IL-2 and IL-2R SNP genotype. We found that the NK cell frequency was similar regardless of the donor genotype. NK cell activation and cytolytic function is regulated by many mechanisms, including expression of activating or inhibitory receptors on the surface of NK cells, cytokine signals, such as IL-2 or IL-15, and transcription factors associated with NK cell maturation and effector function (Waldmann et al., 2006). NK cell-mediated tumor cell killing has been shown to induce a secondary tumor-specific T-cell response that enhances production of IL-2, IL-12, IL-18, and IL-21 (Koh et al., 2001) to regulate the function of other immune cells. Thus, NK cells can bridge the innate and adaptive immune responses (Kelly et al., 2002; Lanier et al., 2009). An 11-year epidemiological survey showed that in adults, the degree of NK cell activation in the peripheral blood was inversely correlated with the risk of developing cancer (Imai et al., 2000). The importance of NK cells in tumor immunity has made NK cells an attractive target for immunotherapy (Ruggeri et al., 2007).
Activated NK cells are the primary effector cells in the LAK cells used in cancer immunotherapy. They are used as an effective method of malignant effusion treatment (Shubina et al., 2008). LAK cell therapy may become more widely used in combination with surgery and/or chemotherapy, as it is a highly effective prophylactic treatment for tumors that are otherwise insensitive to immunotherapy, such as lung cancer (Kimura et al., 2008).

IL-2 is a critical cytokine for stimulating T-cell proliferation and is produced by activated T cells. IL-2 binds to the IL-2R on T-lymphocytes in an autocrine and paracrine manner. As one of the most important regulating factors of immune system, IL-2 can promote the proliferation and differentiation of T cells and B cells, activate the tumor killer cells (LAK, TIL) and improve production of TNF (Brady et al., 2010; Grimm et al., 1982; Archimbaud et al., 1991). Inducibility of lymphokine activated killer (LAK) cells in patients with acute myelogenous leukaemia in complete remission and IFN-γ production. IL-2 can trigger NK cells to release IFN-γ and promotes their cytotoxic activity against tumor cells. Thus, it is plausible for the IL-2 SNP genotype to affect the killing activity of LAK cells.

This study demonstrated that the frequency and killing activity of LAK cells derived from healthy volunteers were related to the IL-2-330T/G and IL-2R rs2104286AA groups. This suggests that there is an association between the frequency and cytolytic activity of LAK cells and IFN-γ production. IL-2 can trigger NK cells to release IFN-γ and promotes their cytotoxic activity against tumor cells. This is consistent with our previous results, IFN-γ production was greatest in the IL-2-330T/G GG and IL-2R rs2104286 AA groups. This suggests that there is an association between the frequency and cytolytic activity of LAK cells and IFN-γ production. IL-2 can trigger NK cells to release IFN-γ and promotes their cytotoxic activity against tumor cells. Thus, it is plausible for the IL-2 SNP genotype to affect the killing activity of LAK cells.

We then investigated IFN-γ production in LAK cell cultures based on the IL-2 and IL-2R SNP genotype. Consistent with our previous results, IFN-γ production was greatest in the IL-2-330T/G GG and IL-2R rs2104286 AA groups. This suggests that there is an association between the frequency and cytolytic activity of LAK cells and IFN-γ production. IL-2 can trigger NK cells to release IFN-γ and promotes their cytotoxic activity against tumor cells. Thus, it is plausible for the IL-2 SNP genotype to affect the killing activity of LAK cells.

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