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A comparative study of the effects of inhibitory cytokines on human natural killer cells and the mechanistic features of transforming growth factor-beta

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ABSTRACT

The major factors and mechanisms by which natural killer (NK) cells are inhibited in cancer patients have not yet been well defined. In this study, we conducted a comparative analysis of the effects of TGF-β, IL-10, and IL-4 on primary NK cells, and it was demonstrated that (1) TGF-β most potently inhibited the overall function of NK cells. (2) It appears that TGF-β reduced the tyrosine phosphorylation of Syk and the expression of c-myc. (3) It was also found that the IL-2-induced promoter-binding activities of C-myb, AP-1, CREB, and AR were also completely suppressed upon TGF-β treatment. Interestingly, TGF-β also completely suppressed other transcription factors, which are constitutively activated. Among these factors, we further confirmed roles of AP-1 in NK-92 cell activation through c-jun and MEK1 inhibitor assay. Our study provides insight into the effects of TGF-β in modulating NK cell functions.

1. Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system that are immediately able to lyse tumor cells or virus-infected cells without prior sensitization [1]. NK cells directly recognize target cells through inhibitory or activating receptors. The balance between the signals transmitted by these receptors determines NK cell triggering. The major activating receptors recognize target cell ligands [2]. Inhibitory receptors interact with MHC class I molecules on target cells [2]. Once activated, NK cells kill tumor cells by releasing cytotoxic granules containing perforin and granzymes. Additionally, the apoptotic target cell death is induced by a direct interaction between death receptors of cancer cells and the death ligands of NK cells, such as tumor necrosis factor (TNF), Fas ligand, and tumor-necrosis factor-related apoptosis inducing ligand (TRAIL). Activated NK cells secrete several cytokines, such as interferon γ (IFN-γ) and tumor necrosis factor alpha (TNF-α), and indirectly contribute to immune response through crosstalk with dendritic cells [3].

NK cells hamper tumor growth and metastasis [3]. Several studies have indicated that tumor tissues are infiltrated by NK cells [4] and in many cases, the infiltration of NK cells is correlated with improved prognosis of cancer patients [5]. However, clinical investigations have revealed that NK cells of cancer patients display impaired functions [6,7] and impaired NK cell-function is related to poor prognosis [8]. Recently, several studies have reported that tumor cells make up a microenvironment that inhibits NK cell activity and promotes survival of the tumor cells. Tumor cells employ suppressive mechanisms, including secreting soluble factors such as TGF-β, shedding NKG2D ligands such as MICA and ULBP, and recruiting suppressive immune cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [9]. These factors are mainly known to cause NK cell dysfunction by causing defective expressions of activating receptors [10].

Thus, it would be highly beneficial to overcome the suppressive mechanisms of NK cells, which interrupt the anti-tumor immune responses of cancer patients. Currently, most of NK cell therapies aim only to improve NK activity, disregarding the suppressors derived from pathogenic circumstances of cancer. In addition, these types of therapies such as cytokine treatment and adoptive cell transplantation have limits to try [11]. Therefore, finding a suppressive mechanism and central biomarkers involved in NK cell inhibition would be a promising strategy for the maintenance of

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normal NK cell function. However, suppressive mechanisms of NK cells are not yet well known.

Among suppressive factors, we focused on three immune-regulatory cytokines, TGF-β, IL-10, and IL-4 (referred to as inhibitory cytokines hereafter). They have a higher serum level in cancer patients than in average individuals. TGF-β has been reported to be secreted at a level of 1 ~ 20 ng/ml in multiple cancer patients [12,13]. TGF-β reduces secretion of IFN-γ, the activating receptors, and the cytolytic effect of NK cells [14–18]. IL-10 is reported to be secreted 10 ~ 50 pg/ml in late stage cancer patients [19]. IL-10 is known to reduce secretion of IFN-γ and TNF-α, and to reduce NK activity in cancer patients [20]. IL-10 and TGF-β also induce Tregs [21]. IL-4 is detected at a level of 10 ~ 70 pg/ml in a malignant tumor [22]. Although the effects of IL-4 on NK activity are controversial, several studies have reported that IL-4 inhibits IFN-γ secretion, as well as proliferation of NK cells in a sex hormone-dependent cancer [23].

In this study, we conducted a systemic comparative study of the inhibitory effects of TGF-β, IL-10, and IL-4 on NK cell functions *in vitro*. Aims of this study were to investigate the most vulnerable condition for NK cell functions, and to identify signaling intermediates that target the condition. Results demonstrated that TGF-β was the key inhibitor of NK cell activity in both single and combination treatments. We also identified that TGF-β reduced tyrosine-phosphorylation of syk and total c-myc expression upregulated by IL-2. In addition, we found that promoter binding activity of transcription factors C-myb, AP-1, CREB, AR, and STAT5, which is enhanced by IL-2, were all suppressed upon TGF-β treatment.

2. Materials and methods

2.1. Antibodies and reagents

Anti-CD56, anti-CD107a, anti-CD25, anti-CD3, anti-CD14, anti-CD19, anti-CD16, anti-CD56, anti-NKp46, and anti-NKG2D antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Anti-NKp30 and anti-NKp44 were purchased from Beckman Coulter Company (Marseille, France). Anti-phospho-SMAD3, anti-phospho-p38, anti-phospho-STAT5, anti-ZAP70, anti-phospho-FAK, and anti-phospho-IKBα were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-ERK, anti-syk, anti-c-myc, and anti-α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine and anti-GRB2 were purchased from BD Transduction Laboratories (San Diego, CA, USA). Recombinant human (rh)-interleukin 2 (IL-2), rh-interleukin 4 (IL-4), and rh-interleukin 10 (IL-10) were purchased from ATgen (Seongnam-Si, South Korea). These cytokines were expressed in *Escherichia coli* as inclusion bodies, and purified through column chromatography after refolding *in vitro*. rh-human TGF-β1 was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell lines and cell culture

The human NK cell line NK-92 was purchased from ATCC (American Type Culture Collection) and maintained in minimum essential medium α (MEM α) (Gibco, New York, NY, USA) supplemented with 12.5% FBS, 12.5% horse serum (Gibco), 0.2 mM myo-inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, antibiotics, and 5 ng/ml of IL-2. For the inhibitor treatment, NK-92 cells were pretreated with 50 μM PD98059 (Enzo Lifesciences, Philadelphia, PA, USA) or 10 μM SP600125 (Enzo Lifesciences). As a negative control, cells were treated with a percentage of DMSO (v/v) equivalent to the inhibitor treatment. After 2 h, cytokine stimulation was conducted. The chronic myeloid leukemia cell line, K562, was purchased from ATCC and maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

2.3. Isolation of NK cells

NK cells were isolated from the whole blood of healthy donors by negative selection using a Rosettesep™ NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, B.C., Canada). In brief, 50 μl of the antibody cocktail per 1 ml of whole blood was mixed and incubated for 20 min. The sample was placed onto a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged. NK cells were collected, and washed twice with PBS containing 2% FBS.

2.4. Sandwich enzyme-linked immunosorbent assay (ELISA)

IFN-γ and TNF-α were measured using IFN-γ ELISA kits (ATgen) and TNF-α ELISA sets (BD Biosciences San Diego, CA, USA) by following the manufacturers' instructions. NK cells (5×10^5/well) or NK-92 cells (1×10^5/well) were dispensed in duplicate in a 24-well plate and incubated with appropriate cytokines. Then, culture supernatants were harvested as samples. Serial dilutions of standards, controls and samples were carried out in duplicate in 96-well microplates pre-coated with the appropriate antibodies. After incubation, each well was washed and a horseradish peroxidase-conjugated secondary antibody was added. Wells were washed and the substrate solution (TMB) was added. The absorbance was measured at 450 nm.

2.5. CCK-8 cell proliferation assay

Cell proliferation was measured using a CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). NK cells (1×10^5/well) or NK-92 cells (1×10^4/well) were dispensed in triplicate in a 96-well plate. 10 μl of CCK-8 solution was added to each well 4 h before measurement. The absorbance was measured at 450 nm.

2.6. Multicolor flow cytometry

Cells (5×10^5/sample) were washed twice with PBS containing 2% FBS and then stained with fluorochrome-conjugated antibodies for 30 min. The fluorescence was measured using a BD LSR II.

2.7. ^{51}Chromium release assay

50 μCi of ^{51}Cr (NEN, Boston, MA, USA) was added per 5×10^5 K562 cells and incubated for 2 h. ^{51}Cr-labeled K562 cells were washed and plated into a 96 well plate (5×10^3/100 μl). NK cells then were added to different Effector/Target ratios, in triplicate, in plate wells and co-cultured for 4 h. Supernatants were collected into gamma counter tubes (PerkinElmer, Boston, MA, USA), and radioactivity was measured by gamma counter (PerkinElmer). Total lysis was calculated by the formula; Percentage of specific cytotoxicity = (experimental cpm−spontaneous cpm) /(maximum cpm−spontaneous cpm) × 100.

2.8. CD107a degranulation assay

NK cells were incubated with an equal number of K562 target cells (5×10^4/100 μl) for 4 h and anti-CD107a was added. Cells were harvested and stained with anti-CD56. Flow cytometric analysis was then conducted using a BD LSR II.