Matrine regulates immune functions to inhibit the proliferation of leukemic cells

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Abstract: Aims: To investigate the roles of matrine in regulating immune functions and its effect on the proliferation of leukemic cells. Methods: Human leukemia K562, OUN-1, HL-60, U937, K562/AO2 cell lines and primary leukemic cells were used to detect the NKG2D ligands (NKG2DL) expression such as MICA/B, ULBP-1, ULBP-2, ULBP-3, and NK cells receptor NKG2D, CD158a, CD158b were detected by flow cytometry. Cell cytotoxic activity of human NK cells and CIK cells against K562 leukemia cells was detected using CFSE/PI double staining. Pro-inflammatory cytokines and adhesion molecules in K562 or NK cells supernatant after matrine treatment were detected. Results: Matrine could upregulate the expression of NKG2DL on leukemic cell lines, and primary leukemic cells and enhance the NK and CIK cytotoxicity targeted to K562 cells. After matrine treatment, pro-inflammatory cytokines and adhesion molecular such as IL-6, IL-1, IL-2, IL-4, IL-5, GRO and TNF-α in K562 leukemia cells was detected using CFSE/PI double staining. Pro-inflammatory cytokines and adhesion molecules in K562 or NK cells supernatant after matrine treatment were detected. Matrine could upregulate the expression of NKG2DL on leukemic cell lines, and primary leukemic cells and enhance the NK and CIK cytotoxicity targeted to K562 cells. After matrine treatment, pro-inflammatory cytokines and adhesion molecular such as IL-6, IL-1, IL-2, IL-4, IL-5, GRO and TNF-α in K562 cells supernatant were significantly decreased (P < 0.05). Flow cytometry analysis showed that the NKG2D expression was up-regulated significantly as well as the CD158a and CD158b expression decreased after treatment with different concentration of matrine in a dose-dependent manner in K562 cells. A significant decrease of supernatant concentrations of IL-1α, IL-5, IL-6, IL-10, IFN-γ, GRO and TNF-α in NK cells was also observed after exposure to the matrine. Conclusion: Matrine regulates immune functions to inhibit the proliferation of leukemic cells.

Keywords: Matrine, leukemia cells, immune functions, NK cells

Introduction

Acute myeloid leukemia (AML) is a group of clonal hematopoietic stem cell disorders in which both failure to differentiate, and over-proliferation in the stem cell compartment leads to accumulation of non-functional cells termed myeloblasts [1]. AML affects approximately 15,000 persons per year in the United States, more than half of young adult patients and about 90% of older patients still die of the disease and is the sixth leading cause of cancer-related deaths [2, 3]. But unlike other cancers, in the past 30 years, the improvement of patients with AML is contributable to refinement of supportive treatment rather than the introduction of new drugs [4]. The need to explore new agents other than chemotherapy has been highlighted in the last years in order to overcome drug related resistance and toxicity and a lot of investments have been done in the use of monoclonal antibodies (mAbs) and recently gene modified immune cells have been considered as an alternative approach whenever chemotherapy fails to eradicate the disease [5].

Nature killer (NK) cells play a key role in the immune response to infections and malignancies by direct cytolysis of infected or transformed cells and by secretion of potent immune mediators [6]. NK cell killing depends on the overall balance of inhibitory and activating signaling mediated by an array of surface receptors recognizing cognate ligands on putative targets. Cytokine-induced killer (CIK) cells are a
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Matrine is an alkaloid extracted from Sopgora flavescens Ait. with the molecular formula of C_{15}H_{24}N_{20}, has many biological activities, such as anti-inflammation, anti-virus, anti-fibrosis, anti-tumor and anti-immunosuppression, leading to wide clinic use in China [10-12]. In addition, matrine can induce apoptosis in leukemia K562 cells by the mechanisms of regulating the expression of proliferation- and apoptosis-related genes or proteins such as Fas, FasL, eIF4E, E2F-1, Bcl-2, Bax and caspases [13-15]. But the roles of matrine in regulating immune functions and the relationship with the proliferation inhibition in leukemic cells has not been investigated before.

Materials and methods

Leukemia cells

Human leukemia K562, OUN-1, HL-60, U937, K562/AO2 cell line was obtained from China center for type culture collection (CCTCC) and maintained in RPMI 1640 medium supplemented 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin/streptomycin (all from Gibco/BRL, USA). Primary leukemic cells were isolated using the density gradient method from either the peripheral blood or bone marrow aspirates containing > 90% leukemic cells of five patients with AML aged between 28 and 49 years. The leukemic cells were freshly prepared to use. All patients provided their informed consent for the use of their samples and this study protocol was approved by the institutional ethical committee.

Monoclonal antibodies and reagents

Matrine was obtained from Xi’an Botany Garden (Shanxi, China), and its purity was > 99% as assessed by high-performance liquid chromatography. Matrine stock solution was prepared in di-distilled water (ddH_{2}O) at 10 mg/mL. Cell Counting Kit-8 (CCK-8) was purchased from Shanghai Dojindo Molecular Technologies, Inc. (Shanghai, China). Monoclonal antibodies specific to MICA/B, ULBP-1, ULBP-2, ULBP-3 and anti-NK cell receptors (NKG2D, CD158a, CD158b) were purchased from R&D Systems (Minneapolis, MN, USA) or (BD Biosciences, San Jose, CA, USA). Human IL-1, IL-2, IL-4, IL-5, IL-6, GRO, TNF-α, MIP-1α/b, MMP-9, RANTES Quantikine ELISA Kit was obtained from R&D Systems, Inc.

Apoptosis assay by flow cytometry

Cells were stained with the appropriate antibodies or the respective isotype control antibodies, flowed by incubation with FITC-conjugated goat antimouse IgG. An Annexin V-PI apoptosis detection kit from BD Pharmingen was used according to the instructions of the manufacturer. Data acquisition and the flow cytometric analysis were carried out on a BD FACs Calibur using the CellQuest software (BD Bioscience, Franklin Lake, NJ, USA).

Purification of human NK cells

The Bioethics Committee of Changzhou No. 2 People’s Hospital, affiliated Hospital of Nanjing Medical University has approved the investigation protocols to draw blood from healthy volunteers after written informed consent for the purposes of preparation cells against tumor and deep sequencing. PBMCs and NK from healthy donors were isolated as described before. Briefly, peripheral blood lymphocytes were obtained after Ficoll-hypaque centrifugation and purified NK cells were negatively selected by using an NK cell isolation kit (Stem Cell Technologies, Vancouver, Canada). The purity of NK cell population was found to be greater than 90% based on flow cytometric analysis of anti-CD16 antibody stained cells. The levels of contaminating CD3+ T cells remained low, at 2.4% ± 1%, similar to that obtained by the non-specific staining using isotype control antibody throughout the experimental procedures. The adherent subpopulation of PBMCs was detached from the tissue culture plates and monocytes were purified using isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). Greater
than 95% purity was achieved based on flow cytometry analysis of CD14 antibody stained monocytes.

**Generation of CIK cells**

CIK cells were prepared from PBMCs which were isolated by standard Ficoll separation [16, 17]. PBMCs were cultured in RPMI 1640 growth medium at a density of 5 × 10^6 cells/mL. The RPMI 1640 growth medium for CIK contained 10% FBS, 2% L-glutamine and antibiotics. 1000 U/mL IFN-γ was added on the 1st day, after 24 h incubation, 50 mg/L monoclonal antibodies (mAb) against CD3 (BD Pharmingen, NJ, USA), 300 U/mL IL-2 (Gibco) and 100 U/mL rIL-1α (Gibco) were added. Fresh IL-2 (150 U/mL) and complete RPMI 1640 medium were added every 3 days to maintain a cell density of 1.5-2.0 × 10^6 cells/mL for 21 day.

**Flow cytometric assay of cytotoxicity**

NK and CIK cells were used as the effector, and K562 cells served as target, effector and target cells were mixed in complete RPMI 1640 medium at NK: K562 = 5:1 and CIK: K562 = 10:1 in triplicates, with 6000 target cells added per well. In briefly, K562 cells were collected and rinse twice with PBS, then incubated with 10 µM/L CFSE at 37°C and 5% CO₂ incubator. After that, add ice cold complete RPMI 1640 medium to the cells and stay at 4°C for 5 min followed by centrifugate at 1000 rpm for 5 min. The cells were incubated at 37°C for 4 h in 96-well U-bottomed plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) in a final volume of 200 µL well in a humidified 5% CO₂ with different concentration of matrine (0.2 mg/ml, 0.5 mg/ml, 0.8 mg/ml) and presence or absence of NK and CIK cells. At the end of the incubation time, the total contents of the U-bottom plates were transferred to 12 × 75 mm Falcon tubes (Becton Dickinson Labware, Lincoln Park, NJ). The tubes were then put on ice and incubated with 15 µL of 4 µM PI for 15 min, followed by flow cytometric analysis within 1 h. CFSE/PI double stained target cells were excited by an argon ion laser emitting at 488 nm. CFSE were detected in the FL1 channel (530/30 nm band pass filter), while PI were detected in the FL3 channel (670 nm Long Pass). All samples were analyzed on a FACS Calibur (BD Bioscience, USA), using software CellQuest ver.5.2.1 (BD Bioscience) for the acquisition and data analysis. The total number of events (cells) was determined by analyzing the data using a dot plot and rectangular regions to define the cell populations.

**Enzyme linked immunosorbent assay (ELISA)**

The secretion of proinflammatory cytokines (IL-1, IL-2, IL-4, IL-5, IL-6, GRO, TNF-α) and adhesion molecules (MIP-1α/b, MMP-9, RANTES) were measured by an indirect ELISA. Briefly, cells were plated in 6-well plates. After treated with matrine for indicated time, supernatant was harvested and stored at -80°C until analyzed. Supernatant was measured using ELISA protein assay kit (R&D Systems, USA) according to the manufacturer’s instructions. Color development was measured using a microplate reader (Thermo, MK3) at 450 nm.

**Measurement of leukemia cell NKG2D ligands and NK surface receptors**

The cultured leukemia cells were washed twice using PBS and incubated with purified anti-human antibodies specific for MICA/B, ULBP-1, ULBP-2, ULBP-3 and the NK cells with CD158a, CD158b and NKG2D (BD Biosciences, San Jose, CA, USA) for 30 min at 4°C followed by incubation with phycoerythrin labeled goat anti-mouse IgG antibody for 20 min. PBS containing 1% BSA was used throughout the experiment for blocking Fc receptors. After that, the cells were washed and finally resuspended in 0.5% paraformaldehyde and acquired in BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), the data was collected from BD FACSCalibur was analyzed using CellQuest software.

**Statistical analysis**

Data are expressed as means ± standard deviation of the mean of separate experiments (n ≥ 3). Student’s t-test was applied for comparison of the means of 2 groups, and ANOVA was used for the means of multiple groups. Values of P < 0.05 were considered statistically significant.

**Results**

**Upregulation of NKG2DL on leukemic cell lines and primary leukemic cells by matrine**

Several myeloid and lymphoid leukemic cells were examined for the expression of MICA/B,
Figure 1. Upregulation of NKG2D ligands (MKG2DL) expression on leukemic cell lines and primary leukemia cells by treated with matrine. A, B. Change in the mean fluorescence intensity (MFI) levels of MICA/B, ULBP-1, ULBP-2 and ULBP-3 in various leukemic cell lines and primary leukemia cells after matrine treatment. Each column represents the difference in the MFI level calculated by subtracting the MFI level of untreated cells from that of matrine treated cells.
ULBP-1, ULBP-2 and ULBP-3 before and after incubation in the presence and absence of matrine in order to identify the upregulation of NKG2DL expression. Two myeloid cell lines, K562 and OUN-1 showed apparent upregulation of both ULBP2 after incubation with matrine, OUN-1 showed also upregulation of MICA/B, HL-60 showed increase of ULBP-1 expression. However, U937 and K562/AO2 showed no change or decrease in the NKG2DL expression (Figure 1).

**Enhancement of NK and CIK cytotoxicity to K562 cells after matrine treatment**

To investigate the effect of matrine in direct capacity of NK cells to lyse target cells we have performed cytotoxic assays using K562 cells as targets. Significantly higher target cell necrotoxic rates were detected when NK cells exposed to a combination of IL-2 and matrine in comparison to IL-2 alone. These data confirm that matrine increased the capacity of NK cells to cytotoxic their targets.

**Inhibition of pro-inflammatory cytokines and adhesion molecules in K562 cells supernatant after matrine treatment**

Figure 2A shows K562 cells supernatant concentration of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, GRO and TNF-α between the control group and the 0.5 mg/ml matrine treatment group. After 0.5 mg/ml matrine treatment, supernatant concentrations of IL-1α (P < 0.01), IL-1β (P < 0.01), IL-2 (P < 0.01), IL-4 (P < 0.05), IL-5 (P < 0.01), IL-6 (P < 0.01), GRO (P < 0.01) and TNF-α (P < 0.01) were significantly decreased.
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A

NKG2D

control  0.2mg/ml MA  0.5 mg/ml MA  0.8mg/ml MA

2013-11-15 NK-NKG2D-NK C01

NK02D APC-A

128  157  265  201

B

CD158a

2013-1-29 NK-KU S

Count

158a PE-A

576  463  447  354

C

CD158b

2013-1-29 NK-KU S

Count

158b PE-A

38037  35063  33847  26928
Figure 3. Effects of matrine on NK cells NKG2D, CD158a, CD158b and proinflammatory cytokines expression levels. A. Histograms of NKG2D expression on NK cells after treated with different concentrations of matrine. B. Histograms of CD158a expression on NK cells after treated with different concentrations of matrine. C. Histograms of CD158b expression on NK cells after treated with different concentrations of matrine. D. Effects of matrine on NK cells proinflammatory cytokines expression levels. Results showed a significant decrease of these cytokines in matrine-treated NK cells compared to untreated cells. Results are reported as mean ± SD of experiments conducted in triplicate. Statistical significance was reported as *P < 0.05, **P < 0.01.
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Figure 2B shows K562 cells supernatant concentration of MIP-1a, MIP-1b, MMP-9, RANTES between the control group and the 0.5 mg/ml matrine treatment group. After 0.5 mg/ml matrine treatment, supernatant concentrations of MIP-1a ($P < 0.01$), MIP-1b ($P < 0.05$), MMP-9 ($P < 0.01$) and RANTES ($P < 0.05$) were significantly decreased.

Expression of activating and inhibitory NK receptors after matrine treatment and inhibition of pro-inflammatory cytokines and adhesion molecules in NK cells supernatant after matrine treatment

Nature killer cells express the NKG2D receptor and leukemic cells express the MIC A/B ligand. It has been shown that matrine can regulate the MICA/B which expresses on leukemic cells, and can enhance the NK cells cytotoxicity on K562 cells. We predicted that matrine might have increase the expression of NKG2D receptor in NK cells. Flow cytometry analysis showed that NK cells expressed NKG2D, and after treatment with different concentration of matrine, the NKG2D expression was up-regulated significantly (Figure 3A).

Representative flow cytometric histograms showing the expression of CD158a and CD158b from NK cells were presented (Figure 3B and 3C). The CD158a and CD158b expression were decreased after treated by matrine in a dose-dependent manner.

Figure 3D shows NK cells supernatant concentration of IL-1α, IL-5, IL-6, IL-10, IFN-γ, GRO and TNF-α between the control group and the 0.5 mg/ml matrine treatment group. After 0.5 mg/ml matrine treatment, supernatant concentrations of IL-1α ($P < 0.01$), IL-5 ($P < 0.01$), IL-6 ($P < 0.01$), IL-10 ($P < 0.05$), IFN-γ ($P < 0.01$), GRO ($P < 0.01$) and TNF-α ($P < 0.01$) significantly decreased.

Discussion

Matrine is a major alkaloid component found in the roots of Sophora species. In vitro studies have demonstrated that matrine can inhibit the growth of various human tumor cell lines. In vivo studies have shown that matrine inhibits the growth of various types of cancer cells in mice. The anti-tumor response of matrine has been further demonstrated through several clinical studies. Matrine has been shown to attenuate the side effects of chemotherapy and radiotherapy, therefore improving quality of life. By regulating immune function, matrine can produce synergistic effects when combined with chemotherapy or radiotherapy treatments, further improving patient outcome. Although the anti-tumor effects of matrine have been demonstrated in vitro and in vivo on various cancers, there are very few studies exploring the mechanisms underlying its actions.

Cell surface proteins major histocompatibility complex (MHC) class I-related chain A (MICA) and UL16-binding proteins (ULBP) 1, 2, and 3 are up-regulated upon tumor transformation and can active human natural killer (NK) cells [18]. Nature killer cells play an essential role in the eradication of myeloid leukemia cells after allogeneic stem cell transplantation [19, 20]. Unlike cytotoxic T lymphocytes (CTL), NK cells do not require antigen-specific recognition to lyse their target. Upon recognition of activating ligands (MICA/B, ULBPs) on AML cells, NK cells contribute to lyse leukemic blasts through the activity receptor NKG2D and inhibitory receptor CD158a, CD158b and the secretion of proinflammatory cytokines such as IFN-γ and TNF-α. NKG2DL expression on leukemic cells plays a key role in the anti-leukemia effect by NK cells because NKG2D can mediates cytotoxicity depending on the NKG2DL expression levels on leukemia cells [21]. Tumor cells have a low expression levels of NKG2D ligands, to evade immune attack, preventing NK cells recognize and kill them [22]. Some reports shows that the expression level of NKG2DL on leukemia cells affects the sensitivity of the leukemia cells to killing by NK cells [23, 24]. Various agents have shown their inducibility of NKG2DLs on leukemia cells to augment the NK cell-mediated anti-leukemia effect [25]. This is the first time we report that matrine increase the expression level of ULBP1, ULBP2, ULBP3 and but not by MICA/B in AML cell lines and primary leukemia cells in vitro. After treated by different concentrations of matrine, NK cells and CIK cells also showed an increase in the NKG2D and decrease in the CD158a and CD158b expression.

Annexin V/PI double staining was measured to assess the degree of apoptosis of K562 cells induced by NK cells [26]. The results revealed...
significant NK cells cytotoxicity on K562 cells after treated by matrine by a dose-dependent manner. This phenomenon reminds us that matrine enhanced cytotoxicity of NK cells against K562 cells by upregulating the expression of NKG2DLs on the leukemia cells surface and downregulating the CD158a/b expression on NK cells.

NK cells induce the apoptosis of AML cells and contribute to amplify the anti-tumor immune response through the release of pro-inflammatory cytokines such as IFN-γ and TNF-α [27, 28]. But in this study, we found that treated with 0.5 mg/mL matrine, the expression of pro-inflammatory cytokines of NK cells was decreased significantly. Matrine inhibited the IFN-γ and TNF-α release in NK cells, while it increased the NKG2DLs expression and active the NK cells. Our finding, provide evidences that matrine regulate the NK cells immune response activity by regulate the pro-inflammatory cytokine release and receptor expression through different mechanisms.

In the present study, we observed that decreased expression of pro-inflammatory cytokines and adhesion molecules in a matrine treated K562 cells. More interestingly, matrine can regulate the JAK/STAT3 pathway [29], while the JAK/STAT pathway plays an important role in the immune/inflammatory response [30]. So, one possible mechanism involving in the anti-tumor effects of matrine is that matrine down-regulates the levels of pro-inflammatory cytokines, which subsequently inhibit the JAK-STAT3 signaling pathway.

Presently, no reports exist describing the effect of matrine on the immune function and its inhibition on the proliferation in leukemia cells. In the present study, we found that matrine could regulate immune function to inhibit the proliferation of leukemia cells. These findings were associated with the up-regulation of NKG2DLs expression of leukemia cells, enhancement of cytotoxicity of NK/CIK cells on leukemia cells, decrease the expression of pro-inflammation cytokines and adhesion molecules, and finally regulate the immune activity of NK cells. Our results have demonstrated for the first time that the underlying mechanism of matrine anti-leukemia activity by the activation of immune response on NK cells. Although further investigation is required for elucidating the direct molecular mechanisms of matrine on NK cells, it is conceivable that matrine may be useful as an immune therapeutic treatment for leukemia and other malignancies.

Disclosure of conflict of interest

None.

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