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Restoration of P-glycoprotein Function is Involved in the Increase of Natural Killer Activity with Exogenous Interleukin-15 in Human Immunodeficiency Virus-infected Individuals

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Abstract

A depressed level of natural killer (NK) activity is one of the various immunologic abnormalities in human immunodeficiency virus (HIV) infection. Interleukin-15 (IL-15), an immunotherapeutic candidate in HIV infection, increases NK activity and induces the excretion of CC-chemokines from divergent immune cells, but the mechanisms of NK activity enhancement by IL-15 stimulation is not clearly established in HIV infection. This study examined whether CC-chemokines, which are known to increase NK activity, are secreted adequately in HIV-infected individuals, and also investigated whether P-glycoprotein is involved in NK activity enhancement after IL-15 administration. NK activity increased with IL-15 stimulation in NK cells of HIV-infected individuals, as it does in normal NK cells. IL-15 stimulates NK cells to secrete CC-chemokines, such as, macrophage inflammatory protein-1 α (MIP-1 α), macrophage chemotactic protein-1 (MCP-1) and regulated upon activation, normal T cells expressed and secreted (RANTES) in both HIV-infected individuals and controls with no significant difference. P-glycoprotein expression and function is decreased in HIV-infected individuals and restored only in NK cells of HIV-infected individuals after IL-15 stimulation. P-glycoprotein may play a role in the mechanism of increased NK cell activity in HIV-infected individuals after IL-15 stimulation.

Key Words: Interleukin-15, NK cells, NK activity, HIV, P-glycoprotein

INTRODUCTION

Human immunodeficiency virus (HIV) infection involves a complex interplay of viral replication and immune degradation that gradually progresses to acquired immune deficiency syndrome (AIDS), which thus allows various opportunistic infections and malignancies to occur.[1] Various ways to overcome this disastrous disease are being investigated, these include, immunotherapy, antiretrovirals and gene therapy.[2] Depressed natural killer (NK) activity level is one of the various immunologic abnormalities of HIV infection,[3] and the restoration of NK activity may functionally contribute to the host's innate defense against viral infections and tumor cells. Defective NK cell functions are known to be partially restored *in vitro* by a cytokine, interleukin-2 (IL-2).[4] Interleukin-15 (IL-15), discovered after IL-2, shares IL-2R β and IL-2R γ and has similar functions.[5,6] IL-15 is a 15 kD cytokine, and is known to express IL-15mRNA in many different cells such as monocytes, lymphocytes and fibroblasts and various normal tissues such as the muscle, heart, lung, liver and kidney.[7] IL-15 shares several biological properties with IL-2, such as, the induction of the stimulatory activities of T, B, and NK cells.[8] Although the functional activities of IL-2 and IL-15 overlap, their sequence homologies are different, as do their sources.[9] IL-15 has been found to be less toxic and to have a better therapeutic index than IL-2 in animal models.[10] These properties of IL-15 encourage its study as a therapeutic candidate for HIV infection. IL-15 enhances NK activity *in vitro*,[11] and CC-chemokines are secreted from NK cells during such enhancement.[12] Several chemokines

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are known to induce chemotaxis of NK cells,[13] and among the CC-chemokines, macrophage inflammatory protein-1 α (MIP-1 α), macrophage chemotactic protein-1 (MCP-1) and regulated upon activation, normal T cell expressed and secreted (RANTES) are not only involved in the chemotaxis of NK cells, but also enhance NK cytotoxicity.[14] IL-15 enhancement of NK activity and CC-chemokines secretion from NK cells and the association between these CC-chemokines and a further enhancement of NK activity is well known. In the case of HIV infection, the immune system significantly perturbed, the relationship between IL-15, CC-chemokines and NK activity is only partially understood, and moreover, the mechanism of IL-15 enhancement of NK cell activity is obscure.

The intracellular mechanism of cytokine or chemokine NK cell cytolytic enhancement involves the initial binding of the cytokine or chemokine to GTP-binding protein and a subsequent series of intracellular signals,[15] cytolytic enzymes such as granzyme B, perforin and serine esterase degranulates in the cytoplasm[16] and are transported out of the cell via transmembrane proteins such as P-gp.[17,18] P-gp is a 170 kD gene product of *mdr1*, which exists in the cellular membrane as a glycoprotein and acts as an ATP-dependent efflux pump. This glycoprotein pours out hydrophobic drugs, and is well known as a causal substance of multidrug resistance in malignancies.[19,20] The role of P-gp in NK cell-mediated cytotoxicity has been confirmed to be critical.[21] There are reports that P-gp expression and function is insufficient in HIV infection.[22,23] To examine whether NK activity enhancement, by exogenous IL-15, is related to this protein's expressional and functional recovery, we observed changes of P-gp expression after IL-15 stimulation by flow cytometric analysis using anti-MRK16 antibody and P-gp function by rhodamine 123 efflux assay.

Since immunologic status in HIV infection is very different from that of a normal person, the effect of IL-15 needs to be re-assessed in the HIV setting before any clinical applications can be identified. The mechanism of NK activity enhancement by IL-15 must be understood fully before it is applied in medicine, because of the possible adverse consequences that it may exhibit in the immune system. Our study involved the investigation of the relationships between IL-15, NK activity and CC-chemokines to determine the extracellular effects of IL-15 on NK cells in HIV infection. To observe the intracellular mechanism of IL-15 induced NK activity enhancement in HIV infection, we observed P-gp expression and function by flow cytometric analysis and rhodamine efflux assays.

MATERIALS AND METHODS

Subjects

Twelve HIV-seropositive adults (group I) who were confirmed by Western blot analysis and 10 healthy blood donors (group II) were enrolled in this study. Informed consent was obtained from all participants. All HIV-seropositive patients were on anti-retroviral therapy. The average CD4+ T cell count was $538 \pm 23/\mu l$, and the average HIV-RNA level was $2,813 \pm 6,705$ copies/ml.

NK cell preparation

Venous blood from HIV-seropositive individuals and healthy seronegative donors was collected in heparinized Vacutainer tubes (Becton Dickinson, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ, USA) density gradient centrifugation. PBMCs were incubated for 2 hours in 5% CO_2 RPMI-1640 medium at 37°C, containing 10% FCS (fetal calf serum; Sigma Chemical Co., St. Louis, MO, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine for monocyte adherence. Nonadherent cells negatively selected using a NK Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Purified NK cells were contaminated with $<5\%$ of $CD3^+$, $CD14^+$, $CD19^+$ cells as measured by FACS (FACS, USA) analysis.

Cytokines, antibodies and reagents

Purified recombinant IL-15 (Immunex Corporation, Seattle, WA, USA) was used throughout the study. The antibodies used were, Fluorescent isothiocyanate (FITC)-conjugated anti-MRK 16 antibody (mouse monoclonal IgG2a; Kamiya, Frankfurt, Germany), and phycoerythrin (PE)-conjugated anti-CD 56 antibody (mouse monoclonal IgG2a; Becton Dickinson).

Rhodamine 123 (Sigma Chemicals Co., USA) was used for rhodamine efflux assay.

Natural killer cell activity

PBMCs (10^6 cells/ml) were incubated (at 37°C, in 5% CO_2) in the presence or absence of IL-15 for 24 hours. They were then washed and used as effector cells for cytotoxicity assays. K562 cells (human chronic myelogenous leukemia, ATCC CCL243) were used for a 4 hour ^{51}Cr release assay to measure % cytotoxicity of NK cells.[24] Samples showing a spontaneous release of over 10% were discarded from the data. % cytotoxicity was calculated using the following formula, and all experiments were performed in triplicate.

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Measurement of NK cell chemokine production

NK cell production of chemokines, MIP1-α, MCP-1, RANTES were measured from cell-free culture supernatants using commercial ELISA kits, and following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Results represent the mean of duplicate wells ± SD.

P-glycoprotein expression and functional assay

P-gp expression was measured by flow cytometric analysis using FITC-conjugated anti-MRK 16 antibody (Kamiya) and PE-conjugated anti-CD56 antibody (Becton Dickinson, CA, USA). The control antibody was FITC-conjugated unrelated mouse IgG2a (Becton Dickinson). Antibodies (10 μl) were mixed at 4°C for 30 minutes with NK cells stimulated with or without IL-15 and washed twice for flow cytometric analysis. P-gp function was determined by rhodamine efflux assay. Rhodamine-123 0.5 μg/ml was mixed with 5×10^5 cells/ml at 37°C for 1 hour (influx phase), washed once and mixed with PE-conjugated anti-CD56 antibody at 4°C for flow cytometric analysis. After the influx phase, cells were incubated in the complete medium at 37°C for 2 hours (efflux phase), washed once and mixed with PE-conjugated anti-CD56 antibody at 4°C for flow cytometric analysis. % efflux was calculated using the following formula.

$$\% \text{ efflux} = \frac{A - B}{A} \times 100$$

A=% of positive rhodamine 123 cells among CD 56^+ cells during influx phase

B=% of positive rhodamine 123 cells among CD 56^+ cells during efflux phase

Statistical analysis

All values were expressed as mean SD. Statistical analysis was done by SPSS using Student's t test, Mann Whitney U test, and Pearson's correlation coefficient. $p < 0.05$ was considered significant.

RESULTS

Effect of rIL-15 on NK cell activity

Using K562 cells as targets, we investigated the

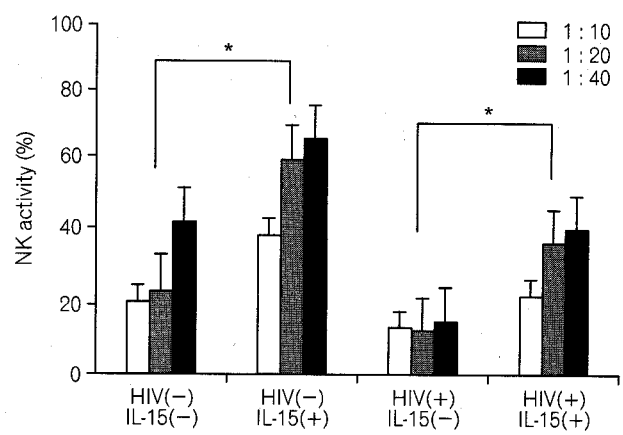

*Fig. 1. Effect of IL-15 on NK cell activity in HIV-infected individuals and controls according to effector to target cell ratios. NK cell activity was measured by chromium release assay before {IL-15 (−)} and 24 hours after IL-15 stimulation [IL-15(+); IL-15 100 ng/ml]. NK activity was measured by 4hr Cr release assay using K562 cells as target cells. Values are expressed as mean ± SD (*p <0.05). E, effector; T, target.*

NK cell activities of NK cells from HIV-infected individuals (group I) and healthy donors (group II) with or without the addition of rIL-15 to the assay wells at effector to target ratios of 10 : 1, 20 : 1, and 40 : 1. The results obtained from 12 HIV-infected individuals and 10 healthy donors are given in Fig. 1. The NK cell activity of group II increased as the E : T ratio was increased, and the addition of rIL-15 (100 ng/ml) further increased the NK activity as the E : T ratio increased. On comparing the NK cell activity of group II before and after rIL-15 addition, is was apparent that the NK activity is higher at each E : T ratio in the rIL-15 treated cells ($p<0.05$). NK cell activity of group I did not change with increasing E : T ratios, but the addition of rIL-15 (100 ng/ml) to NK cells of group I increased NK activity with increasing E : T ratios. By comparing the NK cell activity of group I before and after rIL-15 addition, it was found that the NK activity was higher at each E : T ratio in the rIL-15 treated group ($p<0.05$). No significant differences in NK activity were found between the rIL-15 treated NK cells of group I and rIL-15 untreated NK cells of group II.

NK cell activity of both groups I and II increased proportionally with increasing doses of rIL-15 (0, 10, 100, 1,000 ng/ml) at a constant E : T ratio of 20 : 1. NK cell activity of group I was lower than that of group II at all concentrations of rIL-15 (Fig. 2).

Effect of rIL-15 on the CC-chemokine secretion of NK cells

No significant differences in the levels of MIP1-α, MCP-1 and RANTES secretion from NK cells of group I and group II after rIL-15 (100 ng/ml) stimulation were apparent ($p>0.05$) (Fig. 3).

Effect of IL-15 on P-glycoprotein expression and function

P-gp expression was determined using anti-MRK16 antibody. Among the $CD56^+$ cells defined by flow cytometric analysis, anti-MRK16 antibody positive cells were $12.1\pm1.3\%$ of normal cells and $6.2\pm1.2\%$ of the cells of group I. After rIL-15 stimulation of NK cells, anti-MRK16 antibody positive cells were $13.1\pm2.7\%$ of normal cells and $11.5\pm2.8\%$ of the cells of group I. IL-15 did not increase P-gp expression in normal cells, but an increase in P-gp expression was shown in NK cells of group I upon IL-15 stimulation. P-gp function was determined using rhodamine efflux analysis. % efflux was calculated from the results of flow cytometric analysis involving the rhodamine 123 staining of NK cells. In normal cells, % efflux was $87.1\pm7.7\%$ and in cells of group I, % efflux was $48.4\pm5.3\%$, which was a lower than the % efflux of normal cells ($p<0.05$). After rIL-15

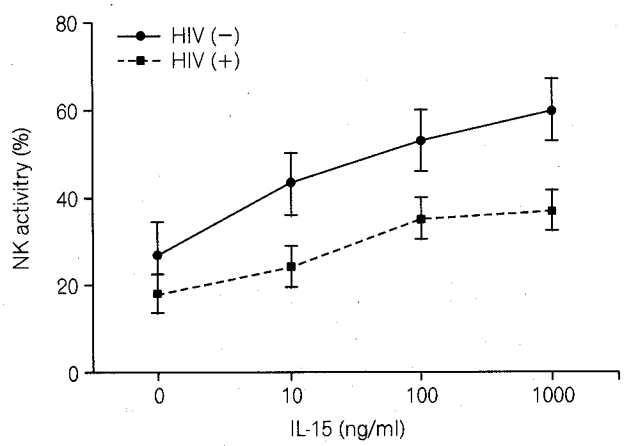

Fig. 2. Effect of IL-15 (0, 10, 100, 1,000 ng/ml) on NK cell activity in HIV-infected persons and controls with a constant effector to target cell ratio. NK cell activity was measured by chromium release assay after 24 hours of IL-15 stimulation. After IL-15 stimulation, NK cell activity of both HIV-infected persons and controls increase dose dependently. Values are expressed as mean ± SD.

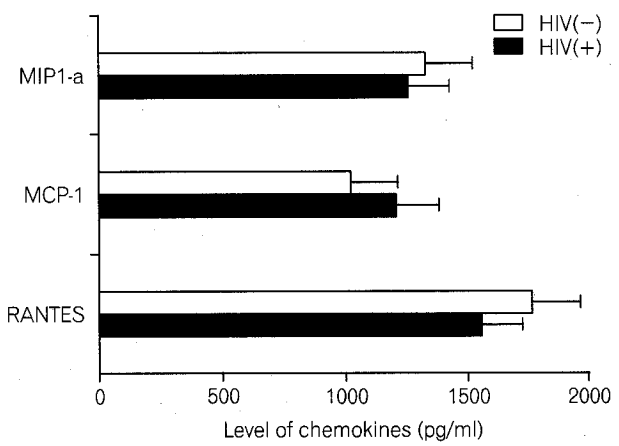

Fig. 3. Effect of IL-15 on CC-chemokine secretion from NK cells of HIV-infected individuals and controls. Levels of CC-chemokines (pg/ml) were measured by ELISA from supernatants of NK cell cultures 24 hours after IL-15 (100 ng/ml) stimulation. Values are expressed as mean ±SD. $p>0.05$ between HIV(-) and HIV(+) levels of chemokines.

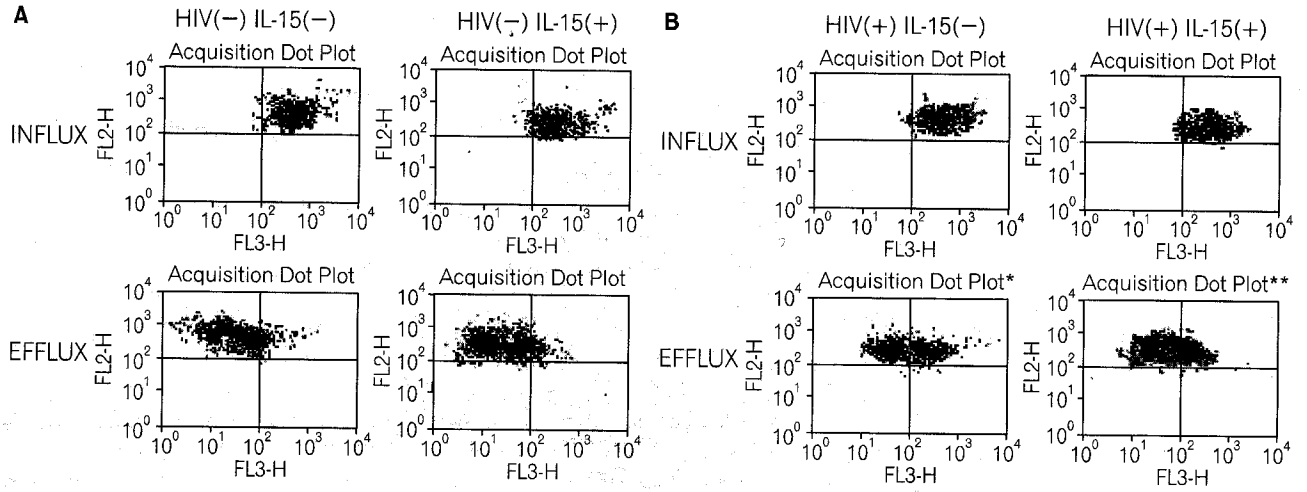

Fig. 4. Flow cytometric analysis of rhodamine 123 efflux assay. Differential Rh123 efflux ability is seen between CD56$^+$ NK cells from a control (A) and a HIV-infected individual (B). The X-axis shows the level of intracellular Rh123 and the y-axis represents the level of PE-conjugated CD56$^+$. Baseline incorporation of Rh123 (INFLUX, upper panels) and after an additional 90-min of incubation in a dye-free medium (EFFLUX, lower panels) are shown. Cells located to the left of the marker in the EFFLUX panels have effluxed the dye. Rh123 efflux is lower in the NK cells of the HIV-infected individual than the control ($p < 0.05$), and IL-15 (100 ng/ml) is shown to recover the efflux level in the NK cells of the HIV-infected individual (**$p < 0.05$).*

stimulation, in normal cells, % efflux showed no significant change to 89.1±7.0%, but in cells of group I, % efflux increased significantly to 77.2± 6.8% ($p < 0.05$). These results indicate that P-gp is under-expressed in NK cells of group I compared to the group II normal controls, and that P-gp function is lower in the NK cells of group I than those of group II. Moreover, they demonstrate that IL-15 recovers the expression and function of P-gp in NK cells of group I (Fig. 4).

DISCUSSION

The underlying cause of NK cell dysfunction in HIV infection is unclear. Studies have presented evidence in favor of a direct interaction between HIV and NK cells, and an indirect effect via diminished cytokine production.[25] In this respect, a means of restoring NK cell dysfunction may be beneficial. Whether this is achieved by enhancing the cells' antiviral activity, thus allowing them to overcome the effects of direct interaction with HIV, or by enhancing their cytokine activity enabling them to fulfill the necessary cellular stimulations to overcome the deficient immune function. The former concept is associated to the progress of anti-retrovirals, and the later concept is a part of the rationale of immunotherapy in HIV infection.

IL-15 stimulates NK cells and increases NK activity in HIV infection.[11] It can also stimulate T cells. In this process, CC-chemokines are secreted from NK cells as well as T cells as a result of IL-15 stimulation.[12,26] These CC-chemokines not only induces chemotaxis of immune cells, but also increases NK activity by stimulating the NK cells.[14] This is the extent of our knowledge of how IL-15 enhances NK activity in the non-pathogenic immune system, that is by direct NK cell stimulation and the secretion of CC-chemokines which ultimately stimulate NK cells. In HIV infection, we know that IL-15 enhances NK activity directly,[11] and that CC-chemokines are secreted from NK cells by IL-15 stimulation,[27] but only a limited number of studies have examined whether these CC-chemokines are secreted adequately in HIV infection. Our results show that CC-chemokines were secreted upon IL-15 stimulation in NK cells of both HIV-infected individuals and normal controls with no significant difference, which implies that the secretion abnormality of CC-chemokines may not be the defective step that contributes to the low NK activity of HIV-infected individuals.

P-gp could be one of the membrane proteins which function as transporters of cytotoxic materials in lymphocytes and NK cells.[17] In an experiment with two MDR reversing agents, nicardipine and AHC-93, the

role of P-gp in NK cell-mediated cytotoxicity was confirmed to be critical.[21] Other reports claim that P-gp expression and function are insufficient in HIV infection.[22,23] Morphologically, P-gp is found to be rearranged during the process of cell-mediated cytotoxicity and appears to be clustered in the cell-to-cell contact regions in normal immune systems. By contrast, in HIV-infected individuals, this rearrangement is reported to be hindered. These results seem to be associated with cytoskeletal network alterations of the cell-mediated killing process occurring in AIDS patients.[23] To examine whether NK activity enhancement is related to this protein's recovery in expression and function by exogenous IL-15, we observed the change of P-gp expression after IL-15 stimulation by flow cytometric analysis using anti-MRK16 antibody and P-gp function by rhodamine 123 efflux assay. A recovery in P-gp expression was observed and a statistically significant increase in P-gp function was observed after IL-15 stimulation only in the NK cells of HIV-infected individuals. This shows that P-gp expression and function is lower in the NK cells of only HIV-infected individuals, and only in these cells does IL-15 recover their function. We have demonstrated that IL-15 enhances NK cell activity in HIV-infected individuals. Moreover, it was found that during this process CC-chemokines are secreted from NK cells by IL-15 stimulation to similar extents by both normal NK cells and those of HIV-infected individuals. P-gp expression and function was lower in the NK cells of HIV-infected individuals, and as a result of exogenous IL-15, P-gp expression showed recovery and P-gp function was restored significantly only in the NK cells of HIV-infected individuals. Therefore, P-gp may play a role in the mechanism which increases the NK cell activity of HIV-infected individuals after IL-15 stimulation.

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