Inhibition of Oncogenes Affects the Expression of NKG2D Ligands in Cancer Cells

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NK cells are lymphoid immune cells that participate in innate immunity to protect against foreign pathogens and transforming cells. It is known that the activity of NK cells is regulated by a balance between activating and inhibitory signals rather than specific antigens. One important activating signal is mediated by the NKG2D receptor, which recognizes NKG2D ligands on cancer cells. Therefore, tumor cells that express sufficient amounts of NKG2D ligands could be eliminated by NK cells, including NK cells. Oncogenes drive tumor cells to apoptosis resistant and uncontrolled proliferation by altered expression of many critical genes. Therefore, the expression of NKG2D ligands may be affected by oncogenes. This study focused on increasing the susceptibility of cancer cells to NK cells by regulating the expression of NKG2D ligands influenced by three oncogenes: k-ras, wnt, and c-myc. We demonstrated that inhibition of k-ras and c-myc increased the expression of NKG2D ligands and enhanced the susceptibility of cancer cells to NK cells. On the contrary, inhibition of the wnt pathway decreased MICA and ULBP1 transcripts. Although the decreased transcription of NKG2D ligands by inhibition of the wnt pathway, surface proteins of NKG2D ligands were not changed, and the susceptibility of HCT-116 cells was unaffected. The results demonstrate that the transcription of NKG2D ligands are regulated differentially by the k-ras, c-myc, and wnt pathways and that the cytotoxicity of NK cells solely depends on the amount of surface NKG2D ligands.

Key words : NK cells, NKG2D ligands, oncogenes

Introduction

Cancer cells were driven to uncontrolled proliferation and resistance to apoptosis by oncogenes. The most important event in transforming cells is mutation of normal proto-oncogenes which regulate cell growth and cell differentiation or apoptosis. This transforming cells has the increased expression of natural killer group 2, member D (NKG2D) ligands and nearly eliminated by NK cells through immune surveillance [15]. However it was known that this immune surveillance did not work in developed cancer patients. Because oncogenes maintain malignant phenotypes and alter the expression of many other genes in cancer cells and evade immune systems, we suspected that core genes

of transformation might affect the expression of NKG2D ligands through unknown regulation mechanisms in cancer cells. Although several oncogenes such as BCR/ ABL, PI3K and NF- κ B, were previously investigated to effect on the expression of NKG2D ligands, the three most frequent oncogenes, k-ras, wnt and c-myc, did not demonstrated [1,13]. Recently, many oncogene-targeted therapy has been developed and some of them evaluated as a success without critical adverse effects in cancer treatments such as imatinib, elrotinib, bevacizumab and bortezomib. Therefore, it will be needed to investigate to effect on immune systems by these inhibition of oncogenes. In this study, we investigated whether three oncogenes, k-ras, c-myc and wnt, affect the expression of NKG2D ligands and susceptibility of cancer cells to NK cells.

Materials and Methods

Cell lines and reagents

A549 human non-small cell lung cancer cell lines, K562 chronic myeloid leukemia and HCT116 human colon cancer cell lines were used in this study which were obtained from the Korean Cell Line Bank (Seoul, Korea). These cell lines

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were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. the NK-92 cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in alpha-Minimum Essential Modified medium supplemented with 12.5% (v/v) fetal bovine serum, 12.5% (v/v) horse serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 200 U/ml of recombinant human interleukin-2, 100 mg/ml streptomycin, and 100 U/ml penicillin. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Tipifarnib was purchased from SELLECK chemicals (Houston, TX, USA), and 10058-F4 and XAV-939 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Total RNA extraction and Multiplex Reverse Transcription (RT)-PCR

Total RNA extraction and RT-PCR were performed as previously described [11]. Briefly, total RNA was extracted using RNeasy® Mini Kit (Qiagen GmbH, Germany) from cell lines following the manufacturer's protocol. One microgram of extracted total RNA was used to synthesize cDNA using 100 pmol of random primers (Takara, Japan) and 100 U of M-MLV reverse transcriptase (Promega Co., Fitchburg, Wisconsin, USA). The resulting cDNA was used in the PCR reaction with QIAGEN® Multiplex PCR Kit (Qiagen GmbH). Seven pairs of primer sets were used to investigate the expression of genes including ribosomal protein L19 (RPL19), MICA, MICB, ULBP1-3 and β-actin (ACTB). ACTB and RPL19 were used as a loading control and degradation marker, respectively. The PCR products were analyzed using ethidium bromide-stained 2.0% agarose gel electrophoresis and quantitated by image analyzing software, Quantity One (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry

To determine the surface expression of the NKG2D ligands on cancer cells, the cells were incubated with mouse anti-MICA, anti-MICB, anti-ULBP1-3 (R&D systems, Minneapolis, MN, USA), which were NKG2D ligand-specific monoclonal antibodies (mAbs), and the corresponding isotype controls at 10 µg/ml followed by incubation with the goat anti-mouse-PE conjugated (BD Pharmingen Inc., San Diego, CA., USA). The analysis was performed on the FACS Sort® (Becton Dickinson, Mountain View, CA., USA) using CellQuest software (Becton Dickinson) and the cell surface

expression was quantified by the value of the mean fluorescence intensities (MFI) obtained with the specific mAbs.

NK cell-mediated cytotoxicity assay

NK cell-mediated cytotoxicity was determined with the DELFIA® EuTDA Cytotoxicity Reagents (PerkinElmer Life Sciences, Waltham, MA, USA). The precise procedure was described previously [1]. Briefly, the target cells (1×10^6 cells/ml) were incubated with freshly prepared 10 µM of BATDA, an fluorescence enhancing ligand, in 2 ml culture medium for 30 min at 37°C, and washed. Next, 100 µl of BATDA-labeled target cells (5,000 cells) were transferred into a round-bottom sterile plate. These target cells were co-cultured with NK-92 cells for 2 hours at an effector/target ratio ranging from 2.5:1 to 10:1. After incubation, 20 µl of the supernatant from each well was transferred to the wells of flat-bottom 96 well plates. 180 µl of the europium (Eu) solution were added to form a highly fluorescent and stable chelates (EuTDA). The fluorescence of the EuTDA chelates was measured with time-resolved fluorometry (Victor3, PerkinElmer). The percent specific release was calculated from (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100(%). In the blocking experiments, the blocking anti-NKG2D mAb (R&D systems) was added to suspension of the NK-92 cells and incubated for 30 min before they co-cultured with the target cells. All experiments were done in triplicate.

Statistical analysis

To evaluate the altered level of gene expression, the mean fold of gene expression in EGFR inhibitor treated cells compared to untreated control. Standard deviation (SD) or standard error (SE) of the mean was calculated. For comparison of groups, the paired Student's *t*-test was performed. A *P* value below 0.05 was considered statistically significant in all experiments.

Results

Deferential regulation of the transcription of NKG2D ligands by inhibition of k-ras, c-myc and wnt pathways

The expression of NKG2D ligands at mRNA levels was analyzed using multiplex PCR and quantitated using Quantity-One software (Bio-Rad). In multiplex PCR reaction, we used 7 pairs of primer sets against five NKG2D ligands and two house keeping genes (Fig. 1A, 1B, 1C left and mid-

dle panels). RPL 19 was amplified with nearly fully length of the transcripts and it represented the integrity of isolated RNA. ACTB was used loading control and the expression level of five NKG2D ligands was normalized by the expression level of ACTB. The alteration of NKG2D ligands were represented by the ratio to the expression level in untreated cells (Fig. 1A, 1B, 1C right panel). We use the

Tipifarnib to inhibits ras activation cascade through farnesyl transferase inhibition, 10058-F4 to selectively inhibit c-myc through prevention of c-myc-max interaction and XAV-939 to selectively inhibit β -catenin through stimulation of β -catenine degradation by stabilizing AXIN. It was found out that Tipifarnib induced the expression of MICA, MICB, ULBP1 and ULBP2 at mRNA level in k-ras mutated A549

Fig. 1. Multiplex RT-PCR analysis of the transcripts of NKG2D ligands by treatment with k-ras, c-myc and wnt inhibitors at indicated doses for indicated durations. (A) A549 cells were treated with k-ras inhibitor, Tipifarnib, (B) K562 cells were treated with c-myc inhibitor, 10058-F4 and (C) HCT116 cells were treated with wnt inhibitor, XAV-939, at serial doses and duration. The alteration of NKG2D ligands transcription were quantitated and relative expression ratio to untreated control was demonstrated as graph (right panel).

cells (Fig. 1A). Also, 10058-F4 increased the expression of MICB, ULBP1 and ULBP2 (Fig. 1B). However, Inhibition of wnt pathway using XAV-939 in HCT116 cells decreased the transcripts of MICA, ULBP1 and ULBP3 (Fig. 1C).

Differential regulation of the expression of surface protein of NKG2D ligands by inhibition of oncogenes

The expression of NKG2D ligands at surface protein levels was analyzed using flow cytometry. The expression of NKG2D ligands were represented by histogram (Fig. 2A, 2B, 2C left panel) and the ratio of MFI to the surface expression in untreated cells (Fig. 2A, 2B, 2C right panel). The inhibition of k-ras increased the surface expression of MICA, MICB, ULBP1 and ULBP2 proteins in k-ras mutated A549 cells (Fig. 2A). The expression of MICB, ULBP1 and ULBP2 was increased by inhibition of c-myc according to the alteration of transcripts (Fig. 2B). However, inhibition of wnt in HCT116 does not affect the expression of surface protein significantly despite of reduction of the transcripts of MICA and ULBP1 (Fig. 2C).

The susceptibility of A549 to NK-92 cells by inhibition of k-ras, c-myc and wnt signaling

When cytotoxicity test was performed, the inhibition of k-ras and c-myc significantly increased the susceptibility of A549 and K562 cells to NK-92 cells, respectively (Fig. 3A, 3B), and inhibition of wnt did not affect the susceptibility of HCT116 cells to NK-92 cells (Fig. 3C). When NKG2D receptor was blocked by anti-NKG2D at prior assay, the cytotoxicity of NK cells were dramatically decreased. From these results, it was assumed that the cytotoxicity of NK cells solely depend on the expression level of surface NKG2D ligands.

Discussion

Cancer cells were different from normal cells on uncontrolled proliferation and resistance to apoptosis which were mainly derived by abnormal activation of oncogenes. Oncogenes are mutated form of normal genes, proto-oncogenes which typically regulate cell growth and cell differentiation or apoptosis. Human cancers usually evolve through a mul-

Fig. 2. Flow cytometric analysis of the surface expression of NKG2D ligands by treatment with k-ras, c-myc and wnt inhibitors. (A) A549 cells were treated with k-ras inhibitor, Tipifarnib, at 5 μ M for 24 hr, (B) K562 cells were treated with c-myc inhibitor, 10058-F4, at 15 μ M for 24 hr, and (C) HCT116 cells were treated with wnt inhibitor, XAV-939, at 10 nM for 24 hr. Thin line indicates untreated control and thick line indicated treated samples. The relative alteration of NKG2D ligands to untreated control were demonstrated as graph with standard deviation through triplicate tests (right panel). The significant alteration of NKG2D ligands was indicated as asterisk. **, p value < 0.01.

Fig. 3. Cytotoxicity assay using NK-92 cells. The susceptibility of (A) A549, (B) K562 and (C) HCT116 cells to NK-92 cells were measured after treatment with Tipifarnib at 5 μ M, 10058-F4 at 15 μ M XAV-939 at 10 nM for 24 hr, relatively. The percents of specific lysis of tumor cells were demonstrated with standard error through triplicate tests. The significant alterations of specific lysis were indicated as asterisk. . *, p value < 0.05 and **, p value < 0.01.

tistage process that can extend over a period of decades. This process includes the progressive accumulation of mutations and epigenetic abnormalities in expression of multiple genes including the proto-oncogenes [6]. These oncogenes drive the cells to malignant phenotypes and might alter the expression of many other genes including NKG2D ligands. This extensive damage of the genome of cancer cells is one of the important inductive stresses of NKG2D ligands and it was well accepted that the expression of NKG2D ligands was increased in transforming cells. Although NK cells play a role in cancer prevention and are remarkably cytotoxic to many different cancer cells *in vitro* and *in vivo*, cancer cells could evade the immune surveillance and proliferate through unclear mechanisms [3, 4]. Therefore it was thought that there might be unknown regulation mechanisms to prevent the sufficient expression of NKG2D ligands in cancer cells to evade immune systems. In this study we studied whether three most frequently founded oncogenes, k-ras, wnt and c-myc, affect the expression of NKG2D ligands. We showed that inhibition of k-ras and c-myc increased the expression of NKG2D ligands and enhanced susceptibility of cancer cells to NK cells. However inhibition of wnt pathway demonstrated the reduction of MICA and ULBP1 transcripts. Although, the decreased transcription of NKG2D ligands by inhibition of wnt pathway, surface proteins of NKG2D ligands were not changed and did not affect the susceptibility of HCT-116 cells. Although many discrepancies between mRNA and surface protein of NKG2D ligands like this study were reported, the reason is unclear [5, 9]. Certainly, the NKG2D ligands were strictly regulated in normal cells and there are many regulatory molecules involved in post-

transcriptional regulation of NKG2D ligands. Previously, we reported that inhibition of PI3K or NF- κ B, which are essential molecules to enhance the proliferation and survival of cancer cells, increased the expression of NKG2D ligands in K562 and SNU-C4 cells at transcription level and activation of PKC suppressed ULBP1 gene in lung and colon cancer cells [1, 2]. One of most extensively studied oncogenes, BCR/ ABL, could increased the expression of NKG2D ligands and BCR/ABL inhibitors, such as Imatinib, Nilotinib and Dasatinib, decreased the expression of NKG2D ligands and differentially affect NK cell reactivity [13]. However, the three most frequent oncogenes, k-ras, wnt and c-myc, have not been investigated to the effect on the expression of NKG2D ligands and susceptibility to NK cells in cancer cells. RAS (Retrovirus-Associated DNA Sequences) are oncogenes created by the somatic mutation of proto-oncogenes of H-RAS (originated from Harvey sarcoma virus), K-RAS (originated from Kirsten sarcoma virus) and N-RAS (identified in neuroblastoma). A single amino acid substitution of k-ras is responsible for most activating mutation in about one of five cancer cells. The transforming protein that results is implicated in various malignancies, including lung adenocarcinoma, ductal carcinoma of the pancreas and colorectal carcinoma [8]. Myc is a transcription factor that regulates many critical genes for cell proliferation, differentiation, and apoptosis such as cyclin-dependent kinases (CDKs) and repressing CDK inhibitors. Myc is one of the most prevalent oncogenes found to be altered in human cancer and its aberrant regulation was found in about 50% of tumors particularly in hematopoietic neoplasias [10, 18]. Abnormal activation of wnt canonical pathway caused by

β -catenin or APC mutations was found in human in numerous cancers, including lung and colon cancer [7, 14, 16, 17]. Approximately 85% of all sporadic and hereditary colorectal tumors show loss of APC function, resulting in stabilization of β -catenin [12]. These study showed that the transcription of NKG2D ligands were regulated deferentially by k-ras, c-myc and wnt pathways, and the cytotoxicity of NK cells solely depend on the amount of surface NKG2D ligands. Because NKG2D ligands was regulated complex and multi-level of gene expression and could be induced by variety of cell stresses, the oncogene-targeted therapy should be considered whether the susceptibility of cancer cells to NK cells were suppressed through the altered expression of NKG2D ligands.

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초록 : k-ras와 c-myc, wnt 억제에 의한 NKG2D 리간드의 발현변화

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자연살상세포(NK cells)은 림프구계의 세포로서 외부 침입 병원균을 막고 체내 형질변환세포를 제거하는데 참여하고 있다. 이러한 자연살상세포의 활성화는 특정한 항원이 필요 없고 활성화 신호와 억제성 신호의 균형에 의해 조절되고 있다. 자연살상세포의 중요한 활성화 신호 중의 하나는 NKG2D 수용체를 통한 것인데, 이 NKG2D 수용체를 통해 자연살상세포는 암세포에 있는 NKG2D 리간드를 인식할 수 있다. 지금까지 인간에서는 여덟개의 NKG2D 리간드가 밝혀져 있고 이러한 리간드의 발현은 다양한 기전을 엄격하게 조절되고 있다. 암세포는 암유전자(oncogenes)에 의해 세포내 다양한 유전자의 발현이 정상세포와 확연히 달라지는데, 이러한 암유전자에 의해서 NKG2D 리간드의 발현이 영향을 받을 것으로 생각되어 진다. 이 연구는 인간의 암세포에서 가장 자주 발현되는 세가지 암유전자 k-ras와 c-myc, wnt의 억제를 통해 NKG2D 리간드의 발현이 어떻게 변화되는 지를 알아보았다. k-ras와 c-myc의 억제는 NKG2D 리간드의 발현을 효과적을 증가시켰고 암세포가 자연살상세포에 더욱 잘 죽게 변화되었다. 그러나 wnt 억제는 MICA와 ULBP1의 전사를 감소시켰다. wnt 억제에 의한 NKG2D 리간드의 전사 억제에도 불구하고 세포막의 단백질 발현은 변하지 않아서 암세포의 자연살상세포에 대한 감수성은 별다른 변화를 보이지 않았다. 따라서 k-ras와 c-myc, wnt 억제는 각각 다른 반응을 보였으며 최종적인 자연살상세포에 대한 감수성은 NKG2D 리간드의 세포표면단백질 발현정도에 의해 결정됨을 알 수 있었다.