Intracellular Poly(I:C) Initiated Gastric Adenocarcinoma Cell Apoptosis and Subsequently Ameliorated NK Cell Functions

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Natural killer (NK) cells are granular lymphocytic cells that exert essential functions in viral infection defense and tumor immune surveillance. However, the functions of NK cells were impaired in cancer patients. Poly-cytidylic acid [poly(I:C)] has been used as an immune adjuvant to improve innate and adaptive immune responses. In this study, intracellular poly(I:C) could trigger gastric adenocarcinoma cells apoptosis quickly. Meanwhile, the sensitivity of poly(I:C)-treated gastric adenocarcinoma cells to NK cell cytotoxicity was increased, concomitant with the elevated expression of MICA/B and Fas. Furthermore, the cytotoxic activity of NK cells against tumor cells was augmented significantly by the supernatant from poly(I:C)-transfected tumor cells compared with NK cells treated by the supernatant from untreated tumor cells, as well as the proliferation and migration abilities of NK cells. In this process, the activating receptors and cytotoxicity-associated molecules of NK cells were up-regulated. Further investigation showed that type I interferon (IFN) produced by poly(I:C)-transfected gastric adenocarcinoma cells played an important role in this process. Our findings demonstrated that intracellular poly(I:C) not only triggered gastric adenocarcinoma cell apoptosis, but also enhanced NK responses via inducing type I IFN production by gastric adenocarcinoma cells. These functions make poly(I:C) a promising therapeutic medicine for gastric adenocarcinoma.

Introduction

EVIDENCE DEMONSTRATES THAT cancer formation could cause a variety of immunological disturbances, which will ultimately generate the immunosuppressive microenvironments and attenuate anti-tumor immunity (Tompkins 2007). Therefore, immunotherapy is considered a promising therapy against cancer. Through immunotherapy, the tumor microenvironment would be improved. Besides, the innate and adaptive anti-tumor immune responses would be enhanced, including augmenting the cytotoxic activity of CD8⁺ CTL and natural killer (NK) cells. Some strategies have been used for gastric carcinoma treatment, such as immunostimulants, tumor vaccines, adoptive immunotherapies, and cytokine therapies (Oldham and Dillman 2009; Meyer and Wilke 2011).

NK cells are important components of the innate immunity that belong to large granular lymphocytes and play essential roles in early defense against virus infection, tumor immune surveillance, and anti-inflammation (Vivier and others 2008; Lunemann and others 2009). After activation, NK cells kill target cells via Fas/TRAIL pathway, antibody-dependent cell-mediated cytotoxicity (ADCC) action, or re-

lease of granzyme and perforin. NK cells can also regulate the immune system by secreting several effective cytokines, such as TNF- α , interferon (IFN)- γ , and IL-12 (Farag and Caligiuri 2006; Vivier and others 2008). However, defects in NK cell activity can be found in many cancer patients. Evidence showed defects of NK cell activity in gastric carcinoma patients, with lower NKG2D expression in NK cells than that in healthy individuals (Oka and others 1993; Saito and others 2012). In cervical carcinoma, the expression of activating receptors NKp30, NKp46, and NKG2D was significantly decreased, leading to NK cell suppressed cytotoxic function (Garcia-Iglesias and others 2009). Therefore, the manner of enhancing the function of NK cells is critical for the development of novel and efficient anti-cancer immunotherapy.

Polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analog of double-stranded RNA, has been used as an immunostimulatory reagent and type I IFN stimulator for several years. Poly(I:C₁₂U) (Ampligen®), a GMP-grade synthetic analogue of poly(I:C), has been identified as promoting the maturation of dendritic cells (DC) and the secretion of IL-12 (Navabi and others 2009). Meanwhile, induction of endogenous type I IFN by poly(I:C) enhances the primary antibody response, thereby promoting the generation of long-term

antibody production and immune memory (Le Bon and others 2001). Moreover, there is evidence that poly(I:C) could elicit tumor cell apoptosis directly in TLR3 or an RIG-I/MDA5-dependent manner (Salaun and others 2006; Besch and others 2009; Peng and others 2009). However, whether NK cell functions would be improved as poly(I:C) was used to treat gastric carcinoma cells was still unclear.

This present study showed that poly(I:C)-liposome could disturb the immunosuppressive properties of gastric adenocarcinoma cells. Importantly, although poly(I:C)-induced type I IFN did not trigger gastric adenocarcinoma cell apoptosis directly, it could augment NK cell functions, which was favorable for anti-tumor therapy. As a result, poly(I:C) might be a potential immunotherapeutic drug against gastric adenocarcinoma.

Materials and Methods

Cell lines and cell culture

Human gastric adenocarcinoma cell lines (AGS cells) were cultured in F12 medium (GIBCO/BRL) containing 10% fetal bovine serum (FBS) (Fumeng). Human gastric adenocarcinoma cell lines BGC-823 cells were cultured in RPMI medium 1640 (GIBCO/BRL) containing 10% FBS. Human NK cells were grown in RPMI-1640 supplemented with 10% FBS (GIBCO) and 100 U/mL rhIL-2 (ChangSheng). Human NK-92 cells were cultured in α -MEM (GIBCO/BRL) containing 12.5% FBS (GIBCO), 12.5% horse serum (GIBCO), 100 U/mL rhIL-2, and 0.1 mM β -mercaptoethanol. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. All the cell lines were maintained in our lab.

Antibodies and reagents

Poly(I:C) was purchased from Sigma-Aldrich. Western blot primary antibodies used in this study were rabbit polyclonal antibodies against I κ B- α , and pNF- κ B p65 (Cell Signaling Technologies, New England BioLabs, Inc.). Mouse anti-human IFN- α / β receptor chain 2 (CD118) (clone MMHAR-2) was used for antibody blocking assay (PBL Biomedical Laboratories). rhIFN- β was obtained from Peprotech. Antibodies used in flow cytometry analysis, including PE-labeled anti-human HLA-ABC, NKp46, Perforin, IFN- γ , TNF, CD107a, PE-cy5-labeled anti-human CD95, and IgG₁, κ isotype controls, were obtained from BD Pharmingen; PE-conjugated anti-human NKG2A and IgG2a isotype control were purchased from R&D Systems; and PE-conjugated anti-human MICA/B, CD314 (NKG2D) were obtained from eBioscience. RNase was purchased from Sangon Biotech.

Transfection

AGS and BGC-823 cells were seeded in a 12-well plate (Costar) at a density of 1.6×10^5 /mL for 15 h and then transfected with poly(I:C) by Lipofectamine™ 2000 (Invitrogen, Life Technologies) according to the manufacturer's instructions.

Flow cytometry

For apoptosis assay, after transfection with 1 μ g/mL poly(I:C) for 12 h, the adherent and supernatant of AGS and BGC-823 cells were harvested and stained by using FITC-conjugated Annexin V and PI cell apoptosis detection Kit (Bestbio) following the manufacturer's instructions.

For cell surface protein analysis, cells were harvested and washed twice with phosphate-buffered saline (PBS), and incubated with antibodies for 1 h at room temperature. For intracellular protein assay, monensin was added to the culture medium for 4 h before being harvested at a final concentration of 6 μ g/mL. After fixation and permeabilization, cells were stained with antibodies at room temperature for 1 h. Results were measured by an FACS Calibur system (BD Biosciences) and analyzed with WinMDI 2.9.

RNA extraction and quantification

Total RNA of cells was extracted using Trizol (Invitrogen), and cDNA was synthesized using the M-MLV first-strand cDNA synthesis kit (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was detected with iCyclerIQ real-time PCR system (Bio-Rad). SYBR Green PCR Master Mix (QPK-201) was obtained from TOYOBO. The sequences of primer pairs specific for each gene were synthesized by BGI and shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/jir).

ELISA

Supernatants from BGC-823 cells were harvested after 0–30 h stimulation with 1 μ g/mL poly(I:C), and the levels of TNF- α were assessed by ELISA according to the manufacturer's protocol (Excellbio).

Protein preparation and western blot analysis

BGC-823 cells were lysed in lysing buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 50 mM NaF, 1% NP-40, and protease inhibitors. The SDS-PAGE, transferring protein to PVDF membranes, and blocking were performed according to standard methods (Han and others 2011). The membranes were incubated with the primary antibodies overnight at 4°C, and then washed with TBST (pH 7.4, PBS containing 0.05%, and Tween-20). After incubation with HRP-conjugated secondary antibodies and being washed again, blots were visualized by ChemiDoc™ XRS+ system (Bio-Rad).

Cytotoxicity assay

The cytotoxicity of NK cells was determined by using MTT assay. Target cells were placed in a 96-well plate at a density of 1.0×10^4 cells/well. NK cells were co-incubated at an effector-to-target ratio from 10:1 to 1:1 for 6 h. Then, 20 μ L MTT (5 mg/mL) per well was added and incubated for another 4 h. The absorbance at 570/630 nm was detected. The percentage of cytotoxicity was calculated using the following formula: cytotoxicity (%) = $1 - (\text{OD}_{\text{E+T}} - \text{OD}_{\text{E}}) / \text{OD}_{\text{T}} \times 100\%$ (OD_{E+T}: OD value of the effector cell and target cell group; OD_E: OD value of the effector cell group; OD_T: OD value of the target cell group).

Proliferation assay

After transfection with 1 μ g/mL poly(I:C) for 4 h in BGC-823 cells, the medium was removed and incubated with fresh medium. After 8 h, NK cells were incubated with the supernatants and fresh medium (1:1) for 48 h; then, cell proliferation ability was detected by MTT assay as cytotoxicity assay described earlier.

Migration assay

NK cells migration assays were determined in transwell culture inserts (BD Falcon) of 8- μ m pore filters. BGC-823 cells were transfected with 1 μ g/mL poly(I:C) for 4 h; then, the medium was removed and incubated with fresh medium. After 8 h, the supernatants were collected and added to the lower chamber; while serum-starved NKL cells were added to the upper chamber. After 8 h, the number of NK cells that migrated to the lower chamber was counted by Bio-Rad TC10 automated cell counter.

Statistical analysis

Data were presented as the mean $\pm$ SD from at least three independent experiments. Statistical significance was performed using a paired Student's *t*-test (**P* < 0.05; ***P* < 0.01 were considered statistically significant).

FIG. 1. Polycytidylic acid [poly(I:C)] triggered the apoptosis of human gastric adenocarcinoma cells. **(A)** BGC-823 cells and AGS cells were transfected with 1 μ g/mL poly(I:C) for 12 h, and then, the apoptosis rates of these cells were determined by Annexin V/PI method. One representative of at least three independent experiments; **(B)** statistic bar chart of the apoptosis percentage induced by poly(I:C). Data were represented as the mean $\pm$ SD from at least three independent experiments. ***P* < 0.01 versus Lipo group).

Results

Intracellular poly(I:C) directly induced gastric adenocarcinoma cell apoptosis

Gastric adenocarcinoma cell lines, BGC-823 and AGS cells, were transfected with 1 μ g/mL poly(I:C) for 12 h, and the apoptosis were observed significantly. In BGC-823 cells, the apoptosis rate (AN+) was increased from 6.09% to 29.74%, and for AGS cells, the apoptosis rate was elevated from 7.35% to 49.91% (Fig. 1A). A statistic bar chart of apoptosis rate was shown in Figure 1B.

The susceptibility of poly(I:C)-transfected gastric cancer cells to NK cell lysis was enhanced

Generally, NK cell functions were impaired in patients suffering from cancer. To elucidate whether NK cell cytotoxicity against gastric adenocarcinoma cells would be ameliorated, BGC-823 cells were treated with a low dose of poly(I:C) (200 ng/mL) for 36 h. Under this dose, poly(I:C) could not induce cell apoptosis (data not shown). Then, the cytotoxic activities of NK cells against these poly(I:C) pre-treated cells were evaluated. The results showed that the sensitivity of poly(I:C)-transfected BGC-823 cell to NKL cell or NK-92 cell cytotoxicity was stronger than that of Lipo-treated cells (Fig. 2A). Meanwhile, the expression of MICA/B (ligands of NK cell activating receptor-NKG2D) and Fas was up-regulated; while HLA-ABC, a ligand of NK cell inhibitory receptor NKG2A, was down-regulated by poly(I:C) treatment (Fig. 2B). These findings indicated that the sensitivity of human gastric cancer cells to NK cell lysis could be increased by treatment with poly(I:C).

FIG. 2. The susceptibility of human gastric adenocarcinoma cell to natural killer (NK) cell lysis was increased by poly(I:C) treatment. **(A)** BGC-823 cells were transfected with 200 ng/mL poly(I:C). After 36 h, cells were co-incubated with NKL or NK-92 cells for 6 h, and the cytotoxicity of NK cells were measured by MTT assay. Data were represented as the mean $\pm$ SD from at least three independent experiments. **P* < 0.05, ***P* < 0.01 versus Lipo group; **(B)** BGC-823 cells were transfected with 200 ng/mL poly(I:C). After 24 h, MICA/B, HLA-ABC, and Fas were analyzed by flow cytometry. One representative of at least three independent experiments.

NK cells were activated by the products of poly(I:C)-treated gastric adenocarcinoma cells

When incubated with the supernatant from poly(I:C)-transfected BGC-823 cells, the cytotoxic activity of NKL cells was augmented by ~10% at the E:T ratio of 4:1 (Fig. 3A). At the same time, the expression level of CD107a, a marker for the cytotoxic activity of NK cells, was also significantly increased (Fig. 3B). In addition, the activating receptors NKG2D and NKp46, cytolytic granules perforin, as well as cytokines IFN- γ and TNF α were up-regulated; whereas the inhibitory receptor NKG2A was down-regulated (Fig. 3B). To determine whether NK cell activation was induced by possibly resided poly(I:C) in the media, before incubating NKL cells, the supernatant from poly(I:C)-transfected BGC-823 cells was treated with RNase (Poly(I:C)+RNase). By a comparison of cytotoxicity activity, there was a significant difference between Poly(I:C)+RNase-treated NKL cells and Lipofectamine+RNase-treated NKL cells (Supplementary Fig. S1), indicating that the activity of NK cells was induced

by the products of poly(I:C)-treated gastric adenocarcinoma cells. These data suggested that some cytokines secreted by poly(I:C)-treated tumor cells promoted NK cell function.

Poly(I:C) triggered the activation of NF- κ B pathway in human gastric adenocarcinoma cells

In order to analyze the changes of cytokine profile in gastric adenocarcinoma cells, mRNA levels of some inflammatory cytokines were tested after poly(I:C) treatment. As Figure 4A showed, the mRNA levels of proinflammatory cytokines TNF- α , IL-1 β , and IL-8 were upregulated; whereas immunosuppressive factor TGF- β was down-regulated in poly(I:C)-treated tumor cells. Meanwhile, after treatment with poly(I:C) for 30 min, NF- κ B was activated quickly, and the production of TNF- α was induced after 8 h as well (Fig. 4B). These data indicated that intracellular poly(I:C) treatment would activate NF- κ B pathway and resulted in the change of the cytokine profile in gastric adenocarcinoma cells.

FIG. 3. The supernatant of poly(I:C)-treated gastric cancer cells increased the activation of NK cells. **(A)** BGC-823 cells were transfected with 1 μ g/mL poly(I:C) for 4 h; then, poly(I:C) were removed and cells were incubated with fresh medium. After 8 h, the supernatants were collected and used to treat NKL cells for 12 h. These NKL cells were used as effectors to cytotoxicity-untreated BGC-823 cells at a ratio of 4:1, 2:1, or 1:1 for 6 h, and the cytotoxicity was assayed by using MTT assay. Data were represented as the mean $\pm$ SD from at least three independent experiments. * P < 0.05, versus Lipo-group; **(B)** flow cytometric analysis of molecules associated with NK cell lysis in these NKL cells. One representative of at least three independent experiments.

Type I IFN did not contribute to poly(I:C)-induced apoptosis of gastric adenocarcinoma cells

Since type I IFN pathway activated by poly(I:C) could mediate apoptosis directly in many cell lines (Chen and others 2001; Morrison and others 2001), we want to understand whether type I IFN was involved in poly(I:C)-induced gastric adenocarcinoma cell apoptosis. Type I IFN pathway-associated genes IRF3/7/8, type I IFN, and representative anti-viral genes ISG15/56 were elevated (Fig. 5A), among which IFN- β was extrusive ($>1,000$ -fold). Similar results were observed in AGS cells (Supplementary Fig. S2). After treatment with a blocking antibody against the IFNAR receptor for 2 h, these BGC-823 cells were transfected with poly(I:C). The results showed that the apoptosis rate of BGC-823 cells treated with anti-IFNAR was similar to that of cells treated with isotype control (Fig. 5B). A statistic bar chart of apoptosis rate was shown in Supplementary Figure S3A. Similarly, exogenous IFN- β was unable to induce BGC-823 cell apoptosis under the doses from 500 to 1,000 U/mL (Fig. 5C and Supplementary Fig. S3B). These results suggested that IFN- β did not play a direct role in poly(I:C)-mediated the apoptosis of gastric adenocarcinoma cells.

Poly(I:C)-treatment for gastric cancer enhanced the activation of NK cells

Furthermore, we speculated whether poly(I:C)-induced type I IFN could mediate the activation of NK cells. Indeed, the enhanced cytotoxicity activity of NKL cells by the supernatant from poly(I:C)-treated BGC-823 cells was reduced to the original level by blocking type I IFN receptor (Fig. 6A). These results indicated that poly(I:C) treatment could break the immune tolerance of gastric adenocarcinoma cells via the induction of type I IFN, which would be favorable to control tumor progression.

Finally, we determined whether the supernatant from poly(I:C)-treated gastric cancer cells could enhance other features of NK cells. The proliferation and infiltration of NK cells are important for anti-tumor immune response. So, by MTT method, proliferation capacity of NKL cells was evaluated, and the results showed that it was enhanced about 22% and 36% by the supernatant from 1 to 5 μ g/mL poly(I:C)-treated BGC-823 cells, respectively (Fig. 6B). Moreover, the migration ability of NKL cells was also increased by 11% (Fig. 6C). These results indicated that the proliferation and migration abilities of NK cells could be improved indirectly when poly(I:C) was used for gastric cancer therapy.

Discussion

Evidence indicated that poly(I:C) could enhance specific anti-tumor immune responses. For instance, poly(I:C) increased the cytotoxicity of splenic lymphocytes from both bladder tumor-bearing and control animals (Droller and Gomolka 1982) in the mammary adenocarcinoma-induced lung metastasis rat model; a low dose of poly(I:C) increased the numbers of NK cells and profoundly protected marginating-pulmonary NK cells from suppression resulting by surgery (Rosenne and others 2007). In these publications, poly(I:C) was administrated systemically. In order to avoid the potential adverse effects (Levine and Levy 1978), an intratumoral injection of poly(I:C) might be safe and efficient,

FIG. 4. Poly(I:C)-activated NF- κ B pathway in human gastric adenocarcinoma cells. BGC-823 cells were transfected with 1 μ g/mL poly(I:C). (A) After 4 h, mRNA levels of inflammatory cytokines TNF- α , IL-8, and IL-1 β , and immunosuppressive cytokines IL-10 and TGF- β were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR); (B) activation of NF- κ B in BGC-823 cells stimulated by poly(I:C) at different time points was examined by western blotting (upper), and the protein levels of TNF- α induced by poly(I:C) in BGC-823 cells were tested by ELISA (lower). Data were represented as the mean $\pm$ SD from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus Lipo-group.

which would be transferred by targeting delivery systems. In this study, we focused on whether NK cell functions could be improved as gastric adenocarcinoma cells treated by poly(I:C) intracellularly.

The anti-tumor function of poly(I:C) has been well demonstrated for several years (Cheng and Xu 2011). As an agonist of TLR-3, RIG-I, and MDA5, poly(I:C) influences cell

FIG. 5. Type I interferon (IFN) did not contribute to poly(I:C)-induced apoptosis of gastric adenocarcinoma cells. **(A)** BGC-823 cells were transfected with 1 μg/mL poly(I:C) for 4 h. qRT-PCR was performed to analyze type I IFN pathway-associated genes. The relative expression was calculated to mock-treated BGC-823 cells (Lipo). Data were represented as the mean ± SD from at least three independent experiments. * $P < 0.05$ versus Lipo group. **(B)** BGC-823 cells were treated with 2.5 μg/mL IFNAR blocking mAb or isotype control for 2 h before transfection with 2 μg/mL poly(I:C). After 4, 8, and 12 h, cells were harvested and stained with Annexin V and PI. **(C)** BGC-823 cells were treated with 500 U and 1,000 U IFN-β, respectively, while 2 μg/mL poly(I:C) was used as a positive control. After 10 h, these cells were harvested and labeled with Annexin V and PI. One representative of at least three independent experiments.

survival via inducing multiple pathways, including ERK, NF-κB, and IRF3/7 pathways, and generates effectors, especially proinflammatory factors and type I IFN in specific-type cancer cells (Salaun and others 2006; Chiron and others 2009; Chin and others 2010). Moreover, poly(I:C) directly induces cancer cell apoptosis via both intrinsic and extrinsic apoptotic pathways (Salaun and others 2007; Yoneda and others 2008; Besch and others 2009). However, the effect of poly(I:C) on human gastric adenocarcinoma was not well characterized earlier. We observed that poly(I:C) triggered robust pro-apoptosis in human gastric adenocarcinoma cells (Fig. 1). The detailed mechanism will be well elucidated in the future.

Here, we focused on the changes in NK cell functions. We found that the sensitivities of gastric carcinoma cells to NK cell lysis were increased by poly(I:C) transfection (Fig. 2A). Evidence showed that the interplay between the ligands expressed in target cells and their receptors expressed in NK cells, including inhibitory receptors and activating receptors, was manipulated to regulate the susceptibility of tumor cells to NK cell-mediated lysis and NK cell activity (Lanier 2008; Terme and others 2008). So, we first further confirmed that the expression of NKG2D ligands MICA/B and Fas were increased in poly(I:C)-treated gastric cancer cells; whereas NKG2A Ligand HLA-ABC was decreased, which would be beneficial to NK cell cytotoxicity (Fig. 2B). In addition, we found that NK cell cytolytic activity against BGC-823 cells was augmented by incubation with the supernatant from poly(I:C)-treated BGC-823 cells (Fig. 3A), concomitant with the up-regulation of activating receptors NKG2D and NKp46, and down-regulation of inhibitory receptor NKG2A (Fig. 3B). Meanwhile, the proliferation and migration abilities of

NK cells were enhanced (Fig. 6B, C). These results indicated that some cytokines secreted by poly(I:C)-treated gastric cancer cells contributed to the activation of NK cells.

Inducing the apoptosis of virus-infected cells is one important anti-viral effect of type I IFN, which also contributed to the anti-tumor process and has been validated in many kinds of cancers (Chawla-Sarkar and others 2003). NF-κB was activated immediately in gastric cancer cells after poly(I:C) stimulation (Fig. 4). In some types of cells, NF-κB exhibited a pro-apoptotic function (Bian and others 2001; Chen and others 2003; Campbell and others 2004), and the activation of NF-κB plays key roles in regulating cellular response to IFNs (Pfeffer 2011). In this study, we found that type I IFN, especially IFN-β, was markedly up-regulated by poly(I:C) (Fig. 5A). However, although apoptosis was induced quickly, IFN-β was not the direct factor that triggered tumor cell apoptosis (Fig. 5B, C). This result was in agreement with previous studies, indicating that IFN-β is nearly irrelevant to RIG-I and MDA5-mediated apoptosis of tumor cells stimulated with 5'-pppRNA, poly(I:C) or short viral RNAs (Besch and others 2009; Ishibashi and others 2011). On the other hand, type I IFN also exerted anti-tumor effects through immunomodulation. IFN-α did not increase the survival of STAT1-depleted mice bearing IFN-responsive tumors (Lesinski and others 2003), but IFN-α exhibited an effective anti-tumor response in wild-type animals bearing STAT1-null tumor cells (Badgwell and others 2004), suggesting that IFN exerted an anti-tumor role not only directly on the tumor cell itself but also on the improvement of host immune function. However, NK cells made key contributions in IFN-induced anti-tumor effects, with higher infiltrating ability and cytotoxicity (Biron 2001; Chawla-Sarkar

FIG. 6. Poly(I:C) treatment for gastric cancer enhanced the activation of NK cells. **(A)** NKL cells were incubated with the supernatant from BGC-823 cells treated in the presence or absence of 2.5 µg/mL IFNAR receptor blocking mAb. Then, the cytotoxic activities were measured at an E:T ratio of 2:1. **(B)** BGC-823 cells were transfected with 1 µg/mL poly(I:C) for 4 h; then, poly(I:C) were removed and cells were incubated with fresh medium. After 8 h, the supernatants were collected and used to treat NKL cells for 48 h. Proliferation ability of NKL cells was measured by MTT assay; **(C)** migration assays were performed in transwell culture inserts with 8-µm pore filters. BGC-823 cells were treated as **(B)**, the supernatants were collected and added to the lower chamber, and NKL cells were added to the upper chamber. After 8 h, the migrated NK cells in the lower chamber were counted. Data were represented as the mean ± SD from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus Lipo group.

and others 2003; Chin and others 2010). So, we confirmed whether type I IFN produced by poly(I:C)-treated cancer cells acted as a stimulator of NK cells. In blocking type I IFN receptor assay, the results demonstrated that type I IFN was the major stimulator of NK cells (Fig. 6A), although IFN-β did not directly induce the apoptosis of tumor cells (Fig. 5B, C).

In summary, this present work demonstrated that the treatment with poly(I:C) *in vitro* triggered gastric adenocarcinoma cells apoptosis and enhanced the cytotoxic function of NK cells. In this process, poly(I:C) increased the expression of NKG2D ligands and Fas, which augmented the susceptibility of gastric adenocarcinoma to NK cell lysis. In

addition, poly(I:C) also induced the production of type I IFN, which acted as a critical stimulator for NK cell activation. These findings indicated that if poly(I:C) was intratumorally used for gastric adenocarcinoma therapy, poly(I:C) could promote both gastric adenocarcinoma cell apoptosis and immune surveillance. This clarification will be favorable to develop novel and efficient immunotherapy of poly(I:C) for gastric adenocarcinoma.

Acknowledgment

This work was supported by the Natural Science Foundation of China (grant numbers 81172789, 30972692).

Author Disclosure Statement

All authors declare that no competing financial interests exist.

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Received 1 October 2012 / Accepted 26 June 2013