의학박사 학위논문

고속 대량 스크리닝을 통한 NK 세포매개 췌장암 치료제 개발에 관한 연구

Research on the development of NK cell-mediated pancreatic cancer treatment through high-throughput screening

울 산 대 학 교 대 학 원
의 학 과
마 충 현

고속 대량 스크리닝을 통한 NK 세포매개 췌장암 치료제 개발에 관한 연구

지 도 교 수　　김 송 철

이 논문을 의학박사 학위 논문으로 제출함

2023 년　8 월

울 산 대 학 교 대 학 원
의　　학　　과
마 충 현

마충현의 의학박사학위 논문을 인준함

심사위원 김 송 철 (인)
심사위원 김 지 선 (인)
심사위원 심 인 경 (인)
심사위원 전 은 성 (인)
심사위원 유 상 권 (인)

울 산 대 학 교 대 학 원

2023 년 8 월

영문 요약

Introduction

Pancreatic cancer has a low survival rate and poor treatment effects, highlighting the need for new therapeutic approaches. This study aimed to investigate the effect of a single compound on the activity of NK cells and identify potential candidates for pancreatic cancer treatment.

Methods

Through high-throughput screening (HTS), we identified four single compounds that activate NK cells. Further analysis revealed that Rosiglitazone showed the highest expression of NCRs, which is indicative of increased NK-92MI activity. WST-1 assay was performed to confirm the toxicity of Rosiglitazone to pancreatic cancer cells and NK cells, and western blot was performed to confirm the expression of proteins related to apoptosis and cell proliferation. Using the in vivo imaging system, the inhibition of pancreas cancer cell proliferation was visually confirmed by Rosiglitazone and NK cell combination treatment.

Results

Our results showed that Rosiglitazone had the most potent effect on NK cell activation, as evidenced by increased expression of NKp30, NKp44, and NKp46. Rosiglitazone significantly inhibited the proliferation of pancreatic cancer cells

(PANC-1 and BxPC-3) in a dose-dependent manner, while it did not affect the viability of NK-92MI cells. Rosiglitazone treatment also increased the expression of proteins related to activation in NK-92MI cells in a dose-dependent manner. Co-administration of Rosiglitazone and NK-92MI cells significantly inhibited the proliferation of both PANC-1 and BxPC-3 cells and induced apoptosis more efficiently than Rosiglitazone or NK-92MI cells alone. This was accompanied by increased expression of proteins related to cell death. Using an in vivo imaging system (IVIS), we further confirmed that the combination of Rosiglitazone and NK-92MI cells effectively inhibited the growth of BxPC-3 LUC cells in vitro. Finally, we found that Rosiglitazone increased TNF-α and IFN-γ-expression in NK-92MI cells, suggesting a possible mechanism underlying its effect on NK cell activation.

Conclusion

Our study identified Rosiglitazone as a potent product for activating NK cells and inhibiting the proliferation of pancreatic cancer cells. These findings suggest a potential pancreatic cancer treatment strategy involving enhancing the immune system.

차 례

표 및 그림 차례

Fig. 9 Assessing the viability of BxPC-3 LUC cells treated with a combination of Rosiglitazone and NK-92MI cells using IVIS. (A) Image of NK-92MI activated by rosiglitazone incubated with BxPC-3. (B) NK-92MI activated by rosiglitazone was co-cultured with fluorescent factor-transfected BxPC-3 and confirmed by IVIS.

.

Fig. 10 Proposed mechanism of rosiglitazone activity.

Introduction

Pancreatic cancer is a highly aggressive malignancy that poses a significant challenge to current clinical treatment modalities. (1) In Korea, pancreatic cancer ranks as the eighth most diagnosed cancer, with an estimated annual incidence of 7,000 cases, and it accounts for the fifth leading cause of cancer-related mortality rate. Despite advancements in surgical techniques, chemotherapy, and radiotherapy, the 5-year overall survival rate remains dismally low at around 12%, indicating a very poor prognosis. Disturbingly, both the incidence and mortality rates of pancreatic cancer are on the rise. (2) The development of effective treatment strategies to overcome this deadly disease is imperative.

Recently, there has been increased interest in the tumor microenvironment, and several research has been undertaken on the anticancer effect of immune cells found in it. (3) Meanwhile, immune therapy has emerged as a promising cancer treatment approach, with several studies focusing on cancer treatment using immune target molecules.(4) Despite the recent success of cancer immunotherapy in other tumor types, even the most promising immune checkpoint inhibitors, such as programmed cell death-1 (PD-1)/programmed cell death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), have not demonstrated significant effectiveness in treating PDAC. (5)

This is due to the desmoplastic reaction of the cancer and its surroundings, which is a characteristic of pancreatic cancer. Desmoplastic stroma is a defining feature of pancreatic cancer comprising a large portion of the tumor volume. Computational analysis of PDAC transcriptomic data has revealed two distinct stromal subgroups: a 'normal' subtype resembling myofibroblast or pancreatic stellate cells (PSCs), and an

'activated' subtype characterized by inflammatory signatures. Tumors with the activated stroma subtype have a significantly worse prognosis, emphasizing the crucial role of stromal heterogeneity in defining pancreas cancer biology. (5, 6) Given the recently discovered characteristics of pancreatic cancer, it appears challenging for externally administered drugs to effectively penetrate the dense desmoplastic stroma. Therefore, a promising avenue for investigation involves strategies that stimulate immune cell activity within the tumor microenvironment. Such approaches aim to enhance the immune response against pancreatic cancer cells and overcome the barriers posed by the stromal component.

Natural killer (NK) cells are innate lymphocytes that play a crucial role in immune surveillance by eliminating virus-infected or malignant cells. They do not require antigen pre-sensitization or major histocompatibility complex restriction. (7) NK cells can directly kill target cells and produce cytokines, mobilizing the immune system. Their activity is regulated by surface receptors, and they can induce cell death through various pathways. (8) The number and function of NK cells are associated with pancreatic cancer. In patients with pancreatic cancer, NK cell activity decreases with disease progression, leading to poor clinical outcomes. (9) Studies in mouse models have shown that expanding NK cell numbers enhances their cytotoxicity and antitumor activity. This study has reported that NK cell therapy in pancreatic cancer patients has demonstrated tumor shrinkage and improved survival outcomes. (10) Based on studies showing that the amount and activity of NK cells can affect the survival outcome of pancreas cancer, we tried to find substances that proliferate and activate NK cells.

High-throughput screening (HTS) technology is considered a key enabler for industry drug discovery with new compounds and novel modes of action. HTS is defined as the

number of test compounds in the range of 10,000-100,000 per day. Current drug discovery involves large-scale screening of chemical libraries against a variety of extracellular and intracellular molecular targets to find new substances with desired modes of action. (11) Using this HTS method, it will be possible to determine candidate substances that activate NK cells through mass screening of substances.

This study aimed to screen single compounds including those extracted from various natural products and to identify novel compounds that can activate NK cells and enhance of apoptosis pancreatic cancer cells.

Methods

Materials

HTS (high-throughput screening)

NK92MI cell activity was tested by the HTS method with a total of 1000 samples, 500 natural product single components provided from KIST's natural product library and 500 FDA-approved single component drugs. As an experimental method, a 96-well plate of 100 μl was used, and the concentration of each single substance component was 1, 5, and 10 μM in triplicate to confirm the growth of NK92MI cells. Then, it was confirmed again at concentrations of 5, 10, 20, and 30 μM by the HTS screening method. The HTS equipment used the Integrated Natural Products HTS/HCS System from iHTac-LAB (HTS/HCS System) manufacturer.

Cell Culture

The human pancreatic cancer cell lines (Panc-1, BxPC-3), NK-92MI cells were provided by Dr. Kim (Seoul Asan medical center). Pancreatic cancer was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 20 μg/mL penicillin/streptomycin (Lonza). NK-92MI cells were cultured in alpha modification of Eagle's minimum essential medium (a-MEM; Gibco) supplemented with 2 mM l-glutamine, 0.2 mM inositol, 0.02 mM folic acid, 0.01 mM 2-mercaptoethanol, 12.5% fetal bovine serum (FBS), and 12.5% horse serum (Gibco). All cell lines were maintained at 37℃ in a 5% CO_2 incubator.

Reagent and Antibodies

Rosiglitazone was purchased from Sigma-Aldrich. Anti-PARP1, anti-β-Actin, anti-Perforin, anti-Granzyme B, anti-Phospho-AKT, anti-Phospho-p44/42 MAPK (ERK1/2) antibodies were purchased from Cell Signaling Technology. For the secondary antibodies, anti-mouse IgG horseradish peroxidase (HRP) and anti-rabbit IgG HRP were purchased from Cell Signaling Technology. (APC)-conjugated anti-human NKp30, (APC)-conjugated anti-human NKp44, (APC)-conjugated anti-human NKp46 antibodies were purchased from BioLegend.

Cell viability assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well and then cultured at 37 . Cell viability was analyzed using the EZ-Cytox assay purchased from Dozen Bio. EZ-Cytox solution (10 μL) was added to each well, and the plates were incubated for 2 hrs. Absorbance at 450 nm was measured using a microplate reader (Beckman Coulter).

Flow cytometric analysis

Flow cytometry was used for the analysis of surface NCR using (APC)-conjugated anti-human NKp30, (APC)-conjugated anti-human NKp44, (APC)-conjugated anti-human NKp46 antibodies. NK-92MI cells were seeded in 6-well plates at 3.0×10^5 cells per well and cultured overnight. After incubation with Rosiglitazone for 24 h, NK92-MI cells were washed, in ice-cold PBS supplemented, incubated with 5 μL of conjugated antibodies for 30 min at 4°C, washed twice in PBS, and analyzed directly using a flow cytometer.

Western blot

The cells were lysed in a RIPA buffer (50 mM Tris, 150 mM NaCl, 1% X-100, 0.1% SDS, and 1% sodium deoxycholate (pH 7.4)) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured using the bicinchoninic acid (BCA) assay. Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with TBS containing 0.2% Tween 20, and 5% skim milk, incubated with primary antibodies

overnight at 4 , and then incubated with HRP-conjugated secondary antibodies. The protein signals were visualized using an ECL solution on a Chemidoc.

IVIS assay

BxPC-3 LUC cells were seeded in 24-well plates at a density of 1×10^5 cells per well and then cultured at 37 . NK-92MI cells were treated with 30 μM rosiglitazone for 24h. Then, BxPC-3 LUC with NK-92MI were co-cultured for 24h. To confirm fluorescence, D-luciferine (100 ng/ml) was added to co-cultured cells, incubated in the dark room for 10 minutes, and then IVIS was taken.

ELISA assay

To determine the level of TNF-α, IFN-γ in Rosiglitazone treated NK-92MI cell culture media, ELISA was performed. All reagents were obtained from R&D system. Plates and reagents were brought to room temperature, added to each well 50μL assay diluent RD1F. Then, the samples and standards were added to each well 50μL for 2 hrs at room temperature. After washing, 200μL of detection antibody solution was added to each well for 2hr at room temperature. After washing for color development, 200μL substrate solution was added to each well for 30 min at room temperature. The plates where the reaction was stopped by adding 50μL of stop solution to each well. The absorbance was absorbance at 450nm using a microplate reader.

Apoptosis assay

Panc-1 cells were seeded in 6-well plates at 1.5 × 10^5 cells per well and cultured overnight. Then, cells were treated with either 30 μM rosiglitazone, NK-92MI, or both. Thereafter, the apoptosis assay was performed according to the kit protocol. Cells were harvested using trypsin-EDTA and centrifuged at 1000 ×*g* for 5 min. The supernatant was collected and washed with ice-cold PBS at 1000 ×*g* for 5 min. After centrifugation, 500 μL of 1× binding buffer was added to resuspend the cells. Next, the cells were incubated with Annexin V stain (5 μg/mL) at room temperature in a dark room for 15 min. After 15 min, the mixture was centrifuged at 1000 ×*g* for 5 min, and the supernatant was removed; 500 μL of 1× binding buffer and 10 μL PI were added, and cell apoptosis was immediately measured using flow cytometry (Beckman cytoflex). The percentage of viable cells or apoptotic cells was determined by fluorescence-activated cell sorter (FACS) analysis.

Statistical analysis

The data are presented as the mean ± SEM of at least three individual experiments. Student's *t-test* was used to compare the differences between the two groups. A one-way analysis of variance (ANOVA) was applied when more than two groups were compared. The difference was considered significant at $P < 0.05$.

Results

Verification of candidate materials that are not toxic to NK92-MI cells through High-throughput screening

For this study, NK92MI cell cytotoxicity was tested by the HTS with 500 natural product single components and 500 FDA-approved single component drugs, a total of 1000. As a result, a significant effect on NK92MI cell growth was confirmed in 256 cells. Then, as a result of confirming concentrations of 5, 10, 20, and 30 μM by the HTS again, six substances could be selected. Subsequently, as a result of confirming cytotoxicity at the same concentration in the laboratory, four substances were finally selected. (Fig. 1)

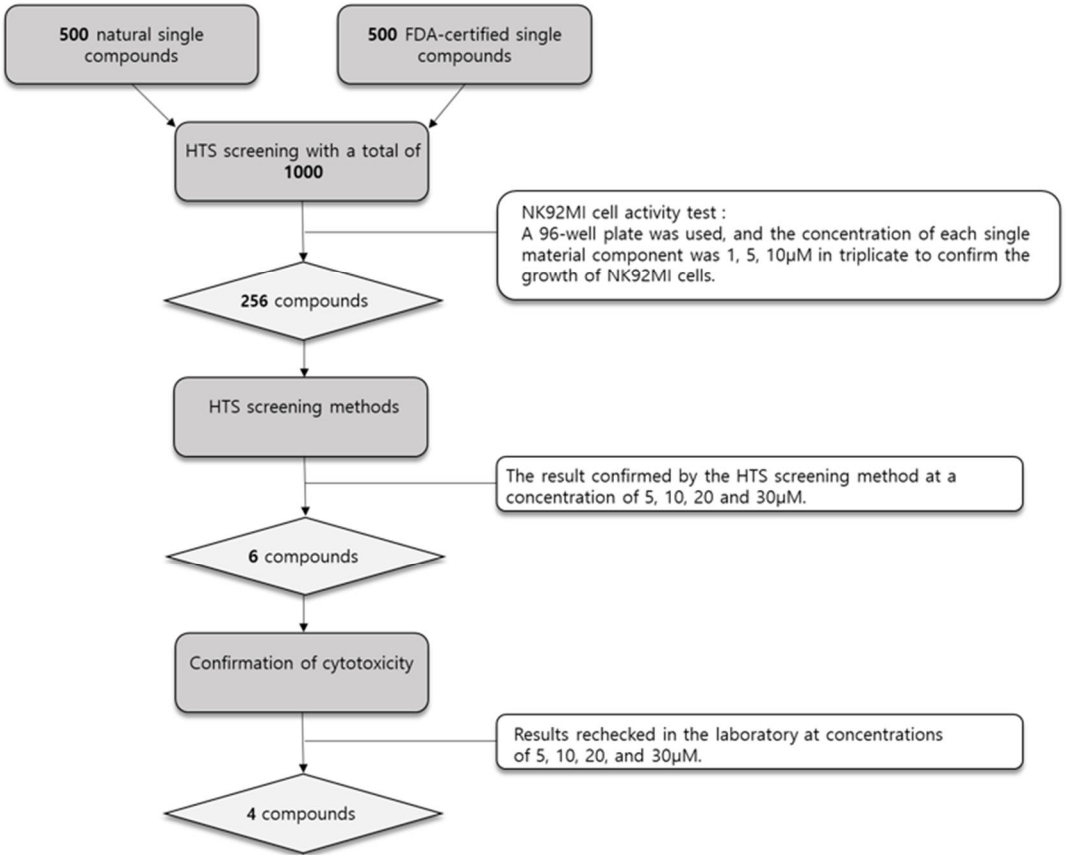

Fig 1. Flow chart for the selection of single-compound based on natural products that are activation of NK92-MI through HTS.

Identification of one single compound that activates NK-92MI cells by confirming NCR expression

When NK-92MI cells were treated with Rosiglitazone, gallic acid, nuciferine, and gingerol, the light source of NCRs, which are indicators of NK cell activity, was confirmed through FACS. As shown in Figure 2, rosiglitazone (30μM) effectively increased the activity index of NK-92MI in NKp30 and NKp46.

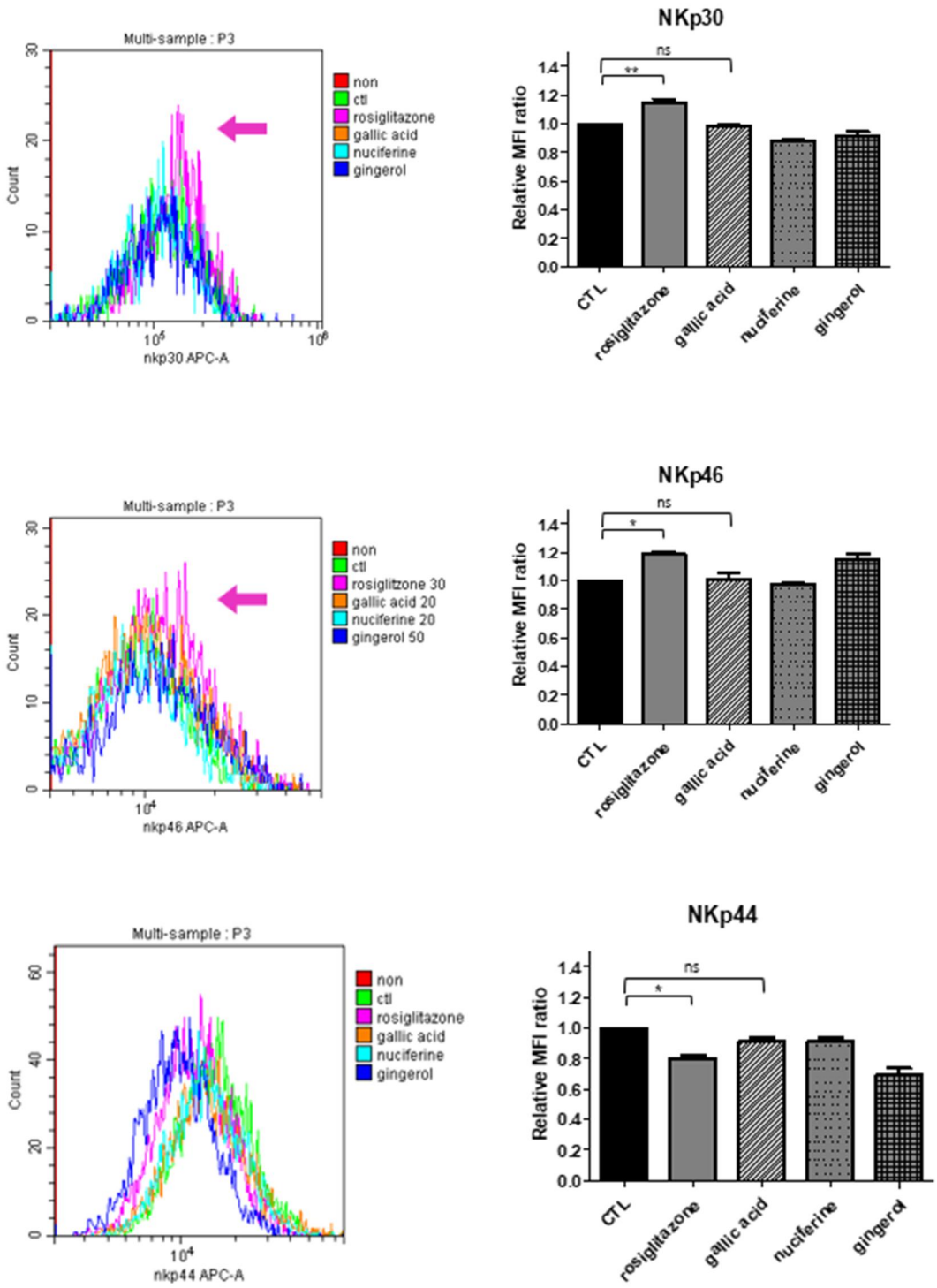

Fig. 2 Cytometry was performed to analyze NKp30, NKp44, NKp46 expression in NK92-MI cells. A. The results for NKp30 and NKp46 confirmed that rosiglitazone effectively increased the activity indicators of NK-92MI cells.

Analysis of cell viability of pancreatic cancer cells and natural killer cells by Rosiglitazone

The WST assay was used to determine cytotoxicity of against pancreatic cancer cells and natural killer cells. As shown in Figure 3, no concentration-dependent cytotoxicity of rosiglitazone (10, 20 and 30μM) was found in Panc-1 cells and BxPC-3 cells. In NK-92MI cells, cell proliferation increased as the concentration of rosiglitazone increased.

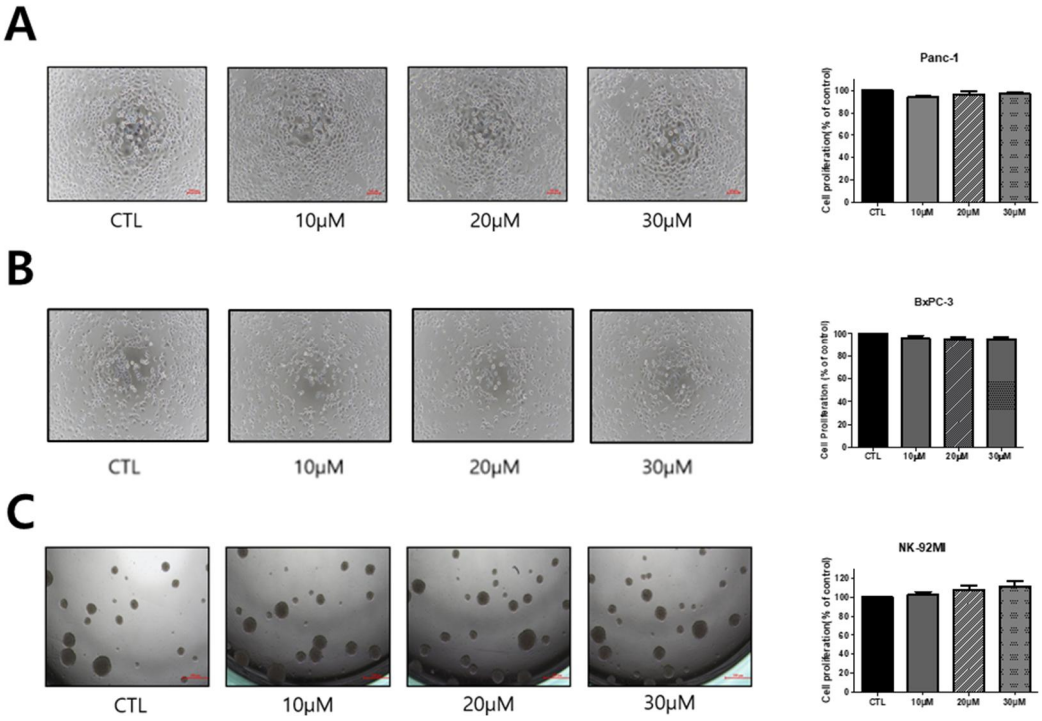

Fig. 3 The relationship between Rosiglitazone and the viability of both pancreatic cancer cells and natural killer cells. (A) Cell proliferation inhibition analysis of rosiglitazone on PANC-1 cells. (B) Cell proliferation inhibition analysis of

rosiglitazone on BxPC-3 cells. (C) Cell proliferation inhibition analysis of rosiglitazone on NK-92MI cells. Cell proliferation was determined using the WST assay.

Expression of perforin and Granzyme B in NK92-MI cells by Rosiglitazone

Western blot was performed to determine the effects of rosiglitazone on expressions of proteins in NK-92MI cells. As shown in Figure 4, rosiglitazone (10, 20 and 30μM) could significantly upregulate expressions of granzyme B and ERK in NK92-MI cells, but had no effect on perforin and TRAIL(tumor necrosis factor-related apoptosis-inducing ligand). As a result of confirming perforin and granzyme b, which are granules that are released when NK cells are activated, it was confirmed that the protein expression level in granzyme b was increased, and ERK related to cell growth was also increased.

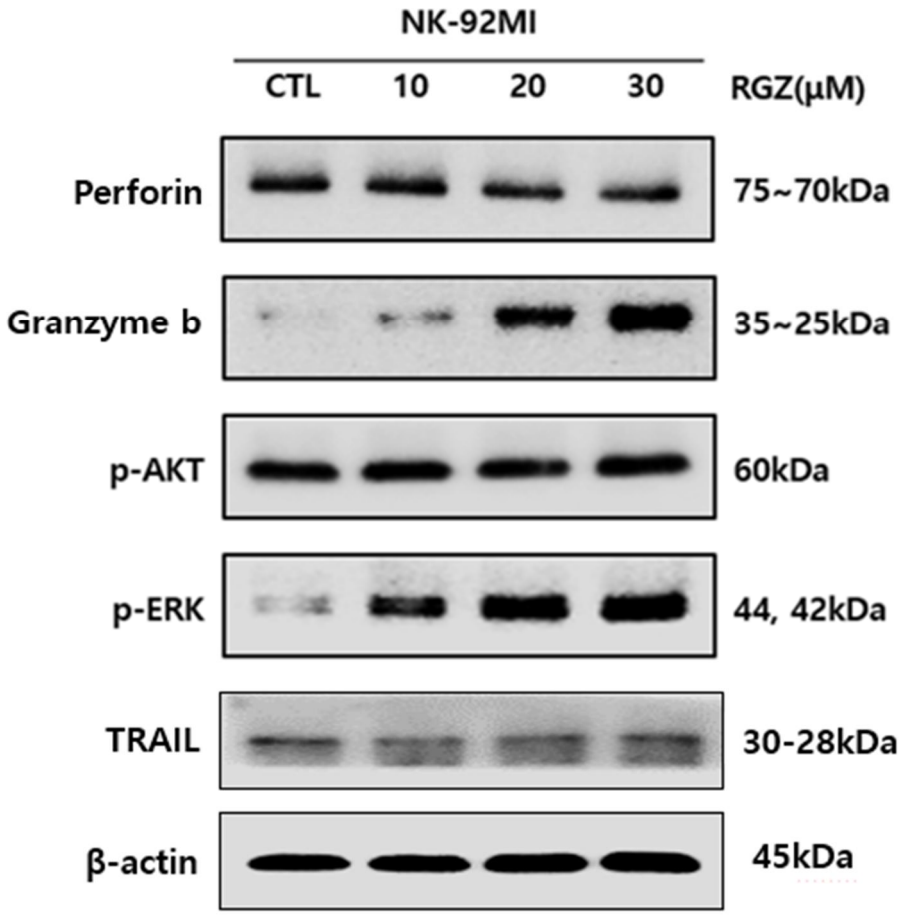

Fig. 4 Analysis of the impact of Rosiglitazone on perforin and granzyme B expression in NK92-MI cells.

Cytokine secretion of NK92-MI cells by Rosiglitazone

Expression of cytokines in NK-92MI cells activated by rosiglitazone was confirmed using ELISA assay. As shown in Figure 5, the ELISA assay of the supernatants derived from NK92-MI cells showed that rosiglitazone (10, 20 and 30μM) could upregulate expressions of TNF-α. While compared with the control group, IFN-γ downregulated in the rosiglitazone-treated groups.

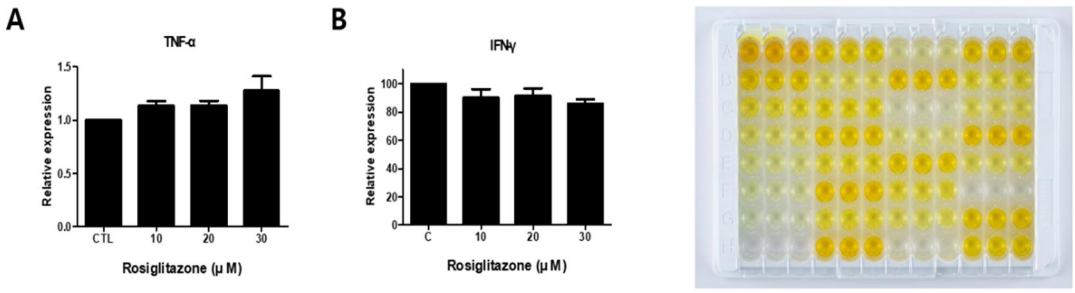

Fig. 5 ELISA assay. Culture supernatants from NK92-MI cells were analyzed by ELISA assay.

Effect of co-administration of Rosiglitazone and NK-92MI cells on the cells viability of pancreatic cancer cells.

The WST assay was used to determine the cytotoxicity of NK92-MI cells against pancreatic cancer cells. As shown in Figure 6, rosiglitazone (30μM) could significantly enhance NK92-MI cell cytotoxicity against pancreatic cancer cells ($p < 0.05$).

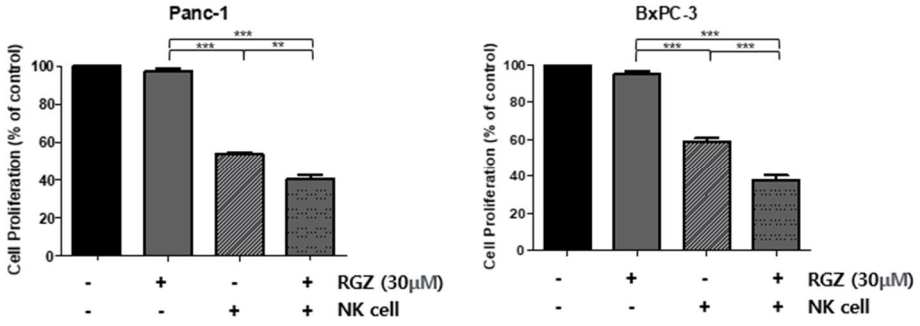

Fig. 6 Cell viability assay. Analysis of cell proliferation inhibition analysis in combination with rosiglitazone and NK-92MI in Panc-1 and BxPC-3 cells.

Effect of co-administration of Rosiglitazone and NK-92MI cells on the apoptosis of pancreatic cancer cells.

The flow cytometry was used to determine cytotoxicity of NK92-MI cells against pancreatic cancer cells. As shown in Figure 7, rosiglitazone (30μM) could significantly enhance NK92-MI cell cytotoxicity against pancreatic cancer cells ($p < 0.05$).

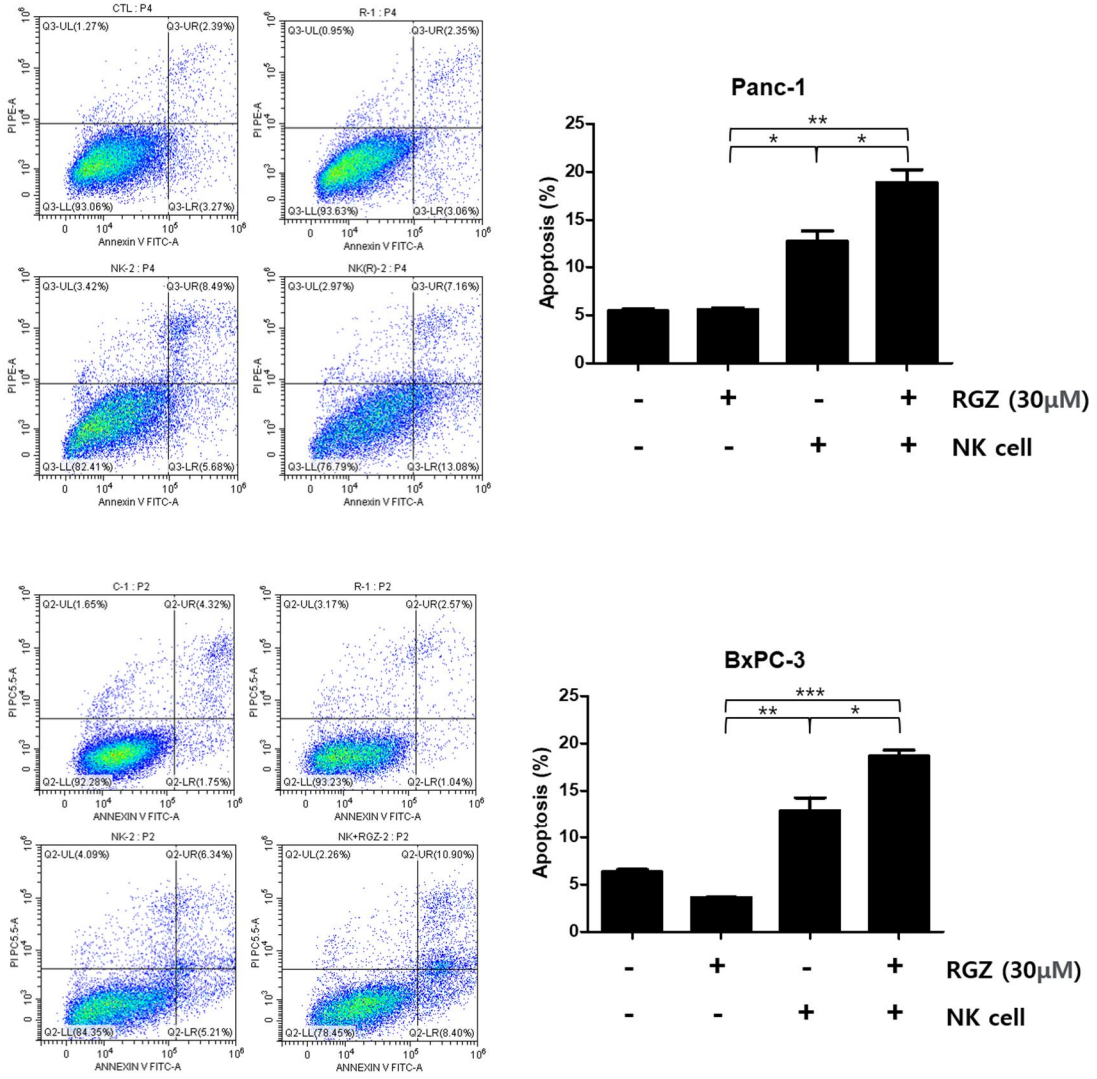

Fig. 7 Apoptosis assay. Analysis of cell apoptosis effect in combination with rosiglitazone and NK-92MI in Panc-1 and BxPC-3 cells.

Effects of co-administration of Rosiglitazone and NK-92MI cells on the expression of apoptosis-related proteins in pancreatic cancer cells.

The Western blot was performed to determine the cytotoxicity of NK92-MI cells against pancreatic cancer cells. As shown in Figure 8, NK92-MI cells treated with rosiglitazone (30μM) can significantly upregulate the expression of apoptosis-related proteins and decrease cell growth-related proteins in Panc-1 and BxPC-3 cells.

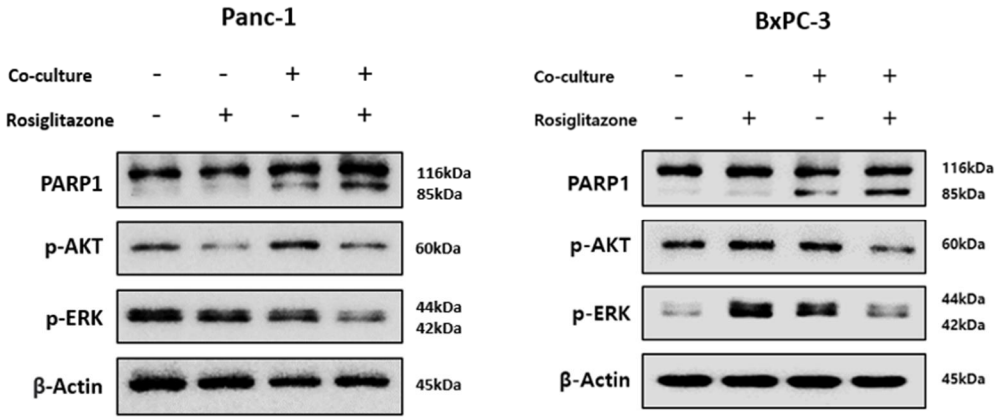

Fig. 8 Analysis of apoptotic protein expression in combination with rosiglitazone and NK-92MI in Panc-1 and BxPC-3 cells.

IVIS analysis of BxPC-3 LUC cells viability by co-administration of Rosiglitazone and NK-92MI cells

The IVIS analysis was performed to determine cytotoxicity of NK92-MI cells against pancreatic cancer cells. As shown in Figure 9, co-culture of BxPC-3 cells transfected with fluorescent factors and NK-92MI activated by rosiglitazone (30μM) and confirmed through IVIS, it was visually confirmed that cell growth was inhibited in the rosiglitazone-treated group ($p < 0.05$).

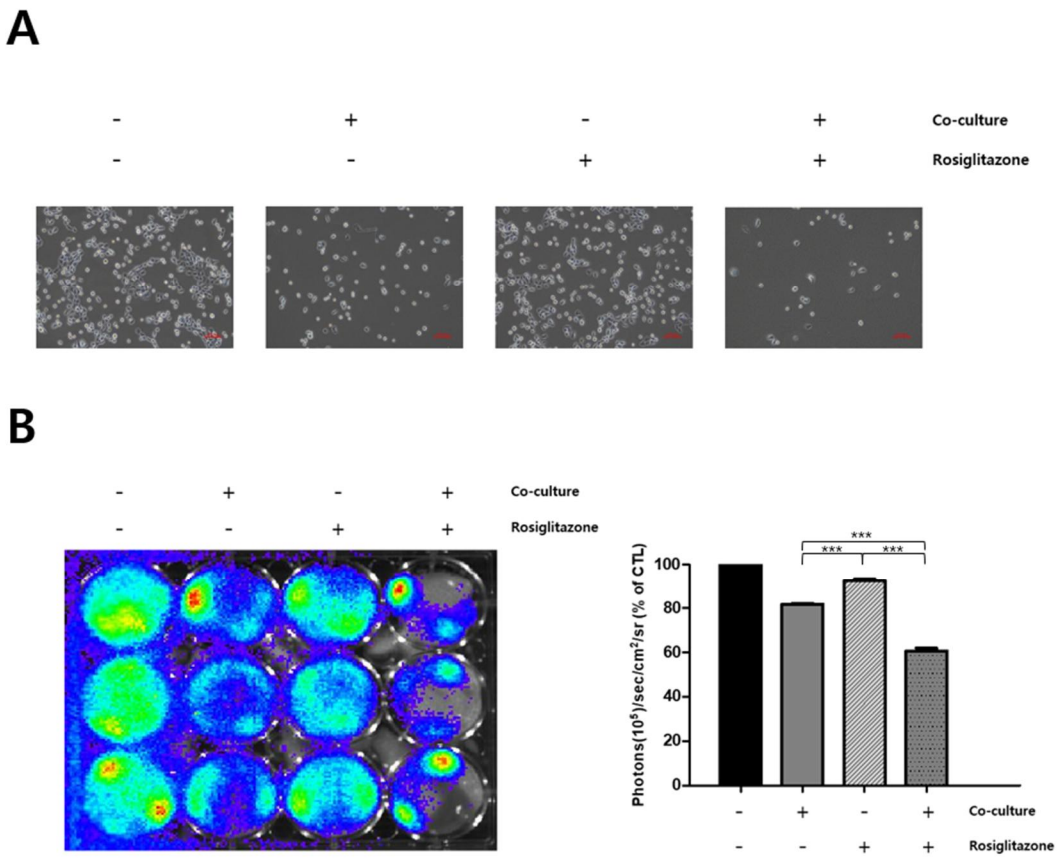

Fig. 9 Assessing the viability of BxPC-3 LUC cells treated with a combination of Rosiglitazone and NK-92MI cells using IVIS. (A) Image of NK-92MI activated by rosiglitazone incubated with BxPC-3. (B) NK-92MI activated by rosiglitazone was co-cultured with fluorescent factor-transfected BxPC-3 and confirmed by IVIS.

The mechanism by which Rosiglitazone activates NK cells, causes pancreas cancer cell apoptosis, and inhibits growth.

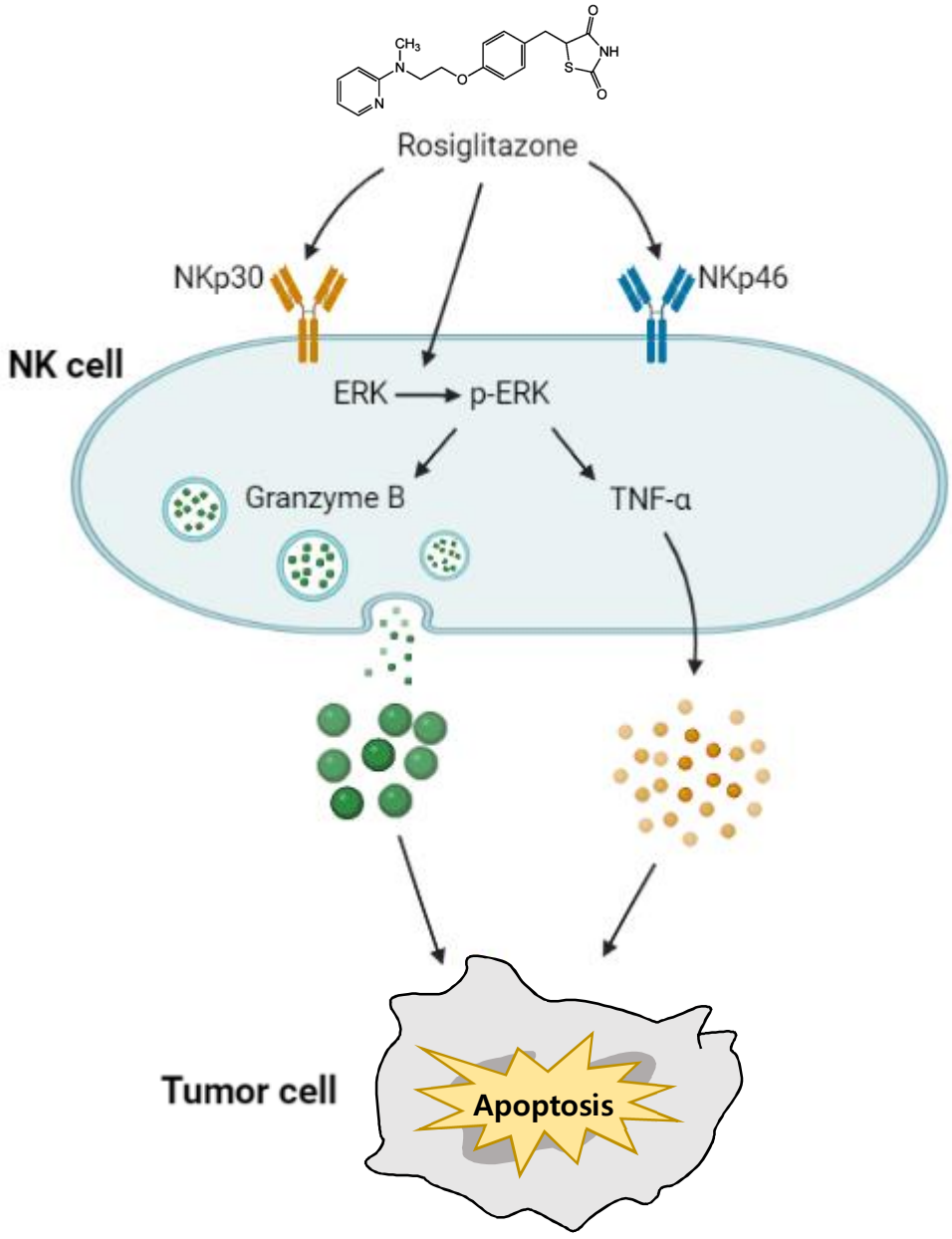

Fig. 10 Proposed mechanism of rosiglitazone activity.

Discussion

We identified four candidate substances from a pool of 1000 compounds using HTS to activate NK cells. Among them, Rosiglitazone was finally selected as a substance due to its minimal toxicity and effective activation of NK cells. Our experiments showed that Rosiglitazone effectively activated NK cells to induce inhibiting proliferation and increasing apoptosis of pancreas cancer cells. Specifically, Rosiglitazone significantly increased the expression of NKp30 and NKp46 receptors indicating enhanced NK cell activity of NK-92MI cells, and ERK pathway related to cell proliferation and differentiation. In NK-92MI cells activated by Rosiglitazone, the increased secretion of granzyme B and TNF-α was identified. When co-cultured with a pancreatic cancer cell line, NK-92MI cells treated with Rosiglitazone not only showed a greater effect of cell death compared to NK-92MI cells not treated with Rosiglitazone, but also demonstrated a further decrease in the expression of P-ERK, which is associated with cell growth, and an increase in the expression of RARP1, which is involved in cell death, in PANC-1 and BxPC-3 cells.

Rosiglitazone is a medication used for the treatment of type 2 diabetes mellitus that reduces insulin resistance and enhances the sensitivity of cells to insulin. It works by activating PPARγ, which leads to the transcriptional regulation of genes involved in glucose metabolism, lipid metabolism, and insulin sensitivity. (12) PPARγ also contributes to the suppression of inflammatory signaling pathways, apoptosis, and the inhibition of angiogenesis. (13, 14) Several studies have suggested the potential of Rosiglitazone as an anticancer agent due to its impact of PPARγ activated by

Rosiglitazone in various types of cancer. (15, 16, 17, 18) In a colorectal cancer cell line HT-29 cells, PPARγ was expressed and PPARγ activation treated with Rosiglitazone inhibits cell growth through inducing apoptosis and suppressing the cell cycle. (19) Kumar et al. observed that tumor selective downregulated NHE1 which is associated with carcinogenesis in breast cancer activated by PPARγ in vitro and in pathologic specimens from breast cancer patients. (20) Furthermore, Rosiglitazone may have antitumor effects in a PPARγ-independent way via inhibition of NF-κB, Prostaglandin E2, increasing MAPK phosphatase 1, and regulation of other apoptosis-related cell factors. (21, 22, 23) Han et al. suggested that Rosiglitazone inhibits non-small cell lung cancer growth partially through phosphorylation of tuberous-sclerosis complex-2 via a PPARγ-independent way. (24) Consistent with previous studies, our study results also showed that Rosiglitazone has a therapeutic effect on pancreatic cancer by NK cells activation. Accordingly, Rosiglitazone may have promising potential as a therapeutic agent for medical treatment of cancer.

NK cells are an important component of the innate immune system and play a critical role in recognizing and eliminating infected or malignant cells. (7) Numerous studies have demonstrated their anticancer effect in various animal models as well as their value in human studies. (25, 26, 27) NK cells can recognize and kill tumor cells while sparing normal cells by employing a well-defined set of activating and inhibitory receptors, more specifically because they sense a lack of major histocompatibility complex (MHC)- molecules. (28) Moreover, once activated, NK cells can secret a vast number of cytokines and chemokines such as IFN-γ and TNF-α. (8) These unique features make NK cells ideal targets for cancer immunotherapy. In the field of

hematologic oncology such as AML, treatment for NK cells has been carried out in the past. Miller et al. reported that The administration of haploidentical natural killer (NK) cells following non-myeloablative chemotherapy has been observed to induce complete remissions (CRs) in individuals with acute myeloid leukemia (AML), independent of hematopoietic stem cell transplantation. (27)

The activation of NK cells requires a complex signaling pathway, which involves the engagement of multiple activating and inhibitory receptors on the surface of NK cells. One of the mechanisms of NK cell activation is through the engagement of activating receptors such as NKG2D, DNAM-1, and NKp46, which recognize stress-induced ligands on the surface of target cells. The engagement of these receptors leads to the activation of downstream signaling pathways, including the phosphorylation of the adaptor protein DAP10 and the activation of the PI3K-Akt and MAPK signaling pathways. (29) Pancreas cancer has the capacity to alter the balance of signaling in NK cells by downregulating the expression of several activating receptors. Peng et al. have shown a decreased expression and frequency for NKp46 and NKp30 and correlated with the pathological state and histological grade of pancreas cancer. (30, 31) We found that Rosiglitazone can enhance the anticancer effect of NK cells by activating NKp46 and NKp30, which are downregulated in the microenvironment of pancreatic cancer.

The tumor microenvironment (TME) of pancreatic ductal adenocarcinoma (PDAC) is characterized by strong desmoplastic reaction, resulting in extensive fibrosis, hypoxia, and an immunosuppressive environment, all of which contribute to disease progression, chemotherapy resistance, and evasion of immune-mediated surveillance. (6) Despite the difficulty of treatment due to the Pancreas cancer tumor environment,

various studies are being attempted to apply NK cells clinically. Ongoing research into the clinical application of natural killer (NK) cells is progressing steadily, with a notable emphasis on exploring the potential of chimeric antigen receptor (CAR)-engineered NK cells, which are currently under active investigation in comparison to CAR- engineered T cell therapy. (29, 30)Similarly, Lee et al. reported that an apoptosis-inducing death ligand was added to the CAR-NK to increase anti-tumor cytotoxicity. In sub-cutaneous models of pancreatic cancer, treatment with the FR/DR4 targeting NK cells greatly boosted tumor-selective apoptosis and NK cell infiltration in tumor tissue. (31) These studies are thought to be good examples of clinical application through technical manipulation of NK cells and showing NK cell infiltration into tumor tissue. In our study, we showed evidence of activation of NK cells and subsequent induction of apoptosis in pancreatic cancer cells, but the clinical significance of these findings has yet to be established. As in the previous study, it is thought that additional research is needed to apply NK cell activation clinically and explore the possibility of overcoming barriers in the tumor microenvironment.

Conclusion

We screened several single component through the HTS method and found the final substance called Rosiglitazone, and investigated the potential of rosiglitazone to activate NK cells and its therapeutic implications in pancreatic cancer treatment. Our results showed that Rosiglitazone effectively activated NK-92MI cells, induced inhibition of proliferation and increased apoptosis of pancreatic cancer cells. The findings of this study are significant as they indicate a potential therapeutic approach that can be translated into clinical practice for the treatment of pancreatic cancer. However, additional research is needed to validate our findings and further optimize the efficacy and safety of NK cell-based therapies for clinical use.

References

1. Mizrahi JD, Surana R, Valle JW, Shroff RT. Pancreatic cancer. The Lancet. 2020;395(10242):2008-20.

2. Hong S, Won Y-J, Park YR, Jung K-W, Kong H-J, Lee ES. Cancer statistics in Korea: incidence, mortality, survival, and prevalence in 2017. Cancer research and treatment: official journal of Korean Cancer Association. 2020;52(2):335-50.

3. Valsecchi ME, Diaz-Canton E, de la Vega M, Littman SJ. Recent treatment advances and novel therapies in pancreas cancer: a review. J Gastrointest Cancer. 2014;45(2):190-201.

4. Tseng C-H. Rosiglitazone reduces breast cancer risk in Taiwanese female patients with type 2 diabetes mellitus. Oncotarget. 2017;8(2):3042.

5. Yao W, Maitra A, Ying H. Recent insights into the biology of pancreatic cancer. EBioMedicine. 2020;53:102655.

6. Fincham REA, Delvecchio FR, Goulart MR, Yeong JPS, Kocher HM. Natural killer cells in pancreatic cancer stroma. World J Gastroenterol. 2021;27(24):3483-501.

7. Van Audenaerde JRM, Roeyen G, Darcy PK, Kershaw MH, Peeters M, Smits ELJ. Natural killer cells and their therapeutic role in pancreatic cancer: A systematic review. Pharmacol Ther. 2018;189:31-44.

8. Paul S, Lal G. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. Front Immunol. 2017;8:1124.

9. Lee HS, Leem G, Kang H, Jo JH, Chung MJ, Jang SJ, et al. Peripheral natural killer cell activity is associated with poor clinical outcomes in pancreatic ductal adenocarcinoma. Journal of Gastroenterology and Hepatology. 2021;36(2):516-22.

10. Masuyama J-i, Murakami T, Iwamoto S, Fujita S. Ex vivo expansion of natural killer cells from human peripheral blood mononuclear cells co-stimulated with anti-CD3 and anti-CD52 monoclonal antibodies. Cytotherapy. 2016;18(1):80-90.

11. Mayr LM, Bojanic D. Novel trends in high-throughput screening. Current opinion in pharmacology. 2009;9(5):580-8.

12. Lau MF, Chua KH, Sabaratnam V, Kuppusamy UR. Rosiglitazone enhances the apoptotic effect of 5-fluorouracil in colorectal cancer cells with high-glucose-induced glutathione. Sci Prog. 2020;103(1):36850419886448.

13. Dang YF, Jiang XN, Gong FL, Guo XL. New insights into molecular mechanisms of rosiglitazone in monotherapy or combination therapy against cancers. Chem Biol Interact. 2018;296:162-70.

14. Bunt SK, Mohr AM, Bailey JM, Grandgenett PM, Hollingsworth MA. Rosiglitazone and Gemcitabine in combination reduces immune suppression and modulates T cell populations in pancreatic cancer. Cancer Immunol Immunother. 2013;62(2):225-36.

15. Lin MS, Chen WC, Bai X, Wang YD. Activation of peroxisome proliferator-activated receptor gamma inhibits cell growth via apoptosis and arrest of the cell cycle in human colorectal cancer. J Dig Dis. 2007;8(2):82-8.

16. Hazra S, Batra RK, Tai HH, Sharma S, Cui X, Dubinett SM. Pioglitazone and rosiglitazone decrease prostaglandin E2 in non–small-cell lung cancer cells by up-regulating 15-hydroxyprostaglandin dehydrogenase. Molecular Pharmacology. 2007;71(6):1715-20.

17. Kumar AP, Quake AL, Chang MKX, Zhou T, Lim KSY, Singh R, et al. Repression of NHE1 Expression by PPARγ Activation Is a Potential New Approach for Specific

Inhibition of the Growth of Tumor Cells In vitro and In vivoPPARγ-Induced NHE1 Repression. Cancer research. 2009;69(22):8636-44.

18. Leung. Rosiglitazone suppresses gastric carcinogenesis by up-regulating HCaRG expression. Oncology Reports. 1994.

19. Tai C-J, Wu AT, Chiou J-F, Jan H-J, Wei H-J, Hsu C-H, et al. The investigation of mitogen-activated protein kinase phosphatase-1 as a potential pharmacological target in non-small cell lung carcinomas, assisted by non-invasive molecular imaging. BMC cancer. 2010;10:1-14.

20. Han S, Zheng Y, Roman J. Rosiglitazone, an Agonist of PPARgamma, Inhibits Non-Small Cell Carcinoma Cell Proliferation In Part through Activation of Tumor Sclerosis Complex-2. PPAR Res. 2007;2007:29632.

21. Terren I, Orrantia A, Vitalle J, Astarloa-Pando G, Zenarruzabeitia O, Borrego F. Modulating NK cell metabolism for cancer immunotherapy. Semin Hematol. 2020;57(4):213-24.

22. Fang F, Xiao W, Tian Z. NK cell-based immunotherapy for cancer. Semin Immunol. 2017;31:37-54.

23. Pahl J, Cerwenka A. Tricking the balance: NK cells in anti-cancer immunity. Immunobiology. 2017;222(1):11-20.

24. Lopez-Soto A, Gonzalez S, Smyth MJ, Galluzzi L. Control of Metastasis by NK Cells. Cancer Cell. 2017;32(2):135-54.

25. Peng Y-P, Zhang J-J, Liang W-b, Tu M, Lu Z-P, Wei J-S, et al. Elevation of MMP-9 and IDO induced by pancreatic cancer cells mediates natural killer cell dysfunction. BMC cancer. 2014;14:1-12.

26. Chiossone L, Vienne M, Kerdiles YM, Vivier E, editors. Natural killer cell

immunotherapies against cancer: checkpoint inhibitors and more. Seminars in immunology; 2017: Elsevier.

27. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105(8):3051-7.

28. Peng Y-P, Zhu Y, Zhang J-J, Xu Z-K, Qian Z-Y, Dai C-C, et al. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. Journal of translational medicine. 2013;11:1-10.

29. Myers JA, Miller JS. Exploring the NK cell platform for cancer immunotherapy. Nat Rev Clin Oncol. 2021;18(2):85-100.

30. Xie G, Dong H, Liang Y, Ham JD, Rizwan R, Chen J. CAR-NK cells: A promising cellular immunotherapy for cancer. EBioMedicine. 2020;59:102975.

31. Lee YE, Ju A, Choi HW, Kim J-C, Kim EE, Kim TS, et al. Rationally designed redirection of natural killer cells anchoring a cytotoxic ligand for pancreatic cancer treatment. Journal of Controlled Release. 2020;326:310-23.

국문 요약

배경 및 목적

췌장암은 생존율이 낮고 현재 알려진 치료의 효과가 나쁘기 때문에 새로운 치료방법에 대한 접근 필요한 상태이다. 본 연구의 목적은 단일성분 물질이 NK 세포의 활성에 미치는 영향을 조사하고 췌장암 치료의 잠재적 후보를 선별하는 것이다.

방법

고속 스크리닝을 통해 NK92-MI 세포에 독성이 없는 4 가지 후보 물질을 선별하였다. 추가 실험을 통해 Rosiglitazone 에서 증강된 NK-92MI 활동을 나타내는 NCR 의 가장 높은 발현을 보여주어 최종 물질로 선택하였다. WST-1 분석을 통해 Rosiglitazone 의 췌장암 세포 및 NK 세포에 대한 독성을 확인하고, apoptosis 및 세포 증식과 관련된 단백질 발현을 확인하기 위해 western blot 을 수행하였다. in vivo imaging system 을 이용하여 Rosiglitazone 과 NK 세포의 병용 요법에 의한 췌장암 세포 증식 억제를 시각적으로 확인하였다.

결과

본 연구 결과는 Rosiglitazone이 NK 세포의 활성화에 가장 강한 영향을 미친다는 것을 보여주었다. 이는 NKp30, NKp44 및 NKp46의 발현 증가로 입증할 수 있었다. Rosiglitazone은 췌장암 세포(PANC-1 및 BxPC-3)의 증식을 억제하는 반면, NK-92MI 세포의 생존율에는 영향을 미치지 않았다. 또한 Rosiglitazone은 NK-92MI 세포에서 활성화와 관련된 단백질의 발현을 증가시켰다. Rosiglitazone과 NK-92MI 세포의 동반 투여는 PANC-1 및 BxPC-3 세포의 증식을 유의하게 억제하며, Rosiglitazone 또는 NK-92MI 세포 단독 치료보다 더 효과적으로 세포 자살을 유도했다. 이와 더불어 세포사와 관련된 단백질의 발현 증가 또한 함께 관찰되었다. 이를 시각적으로 확인 하기위해 in vivo imaging system(IVIS)을 사용하였는데, Rosiglitazone과 NK-92MI 세포의 결합 치료가 BxPC-3 LUC 세포의 성장을 효과적으로 억제한다는 것을 확인할 수 있었다. 마지막으로, Rosiglitazone은 NK-92MI 세포에서 TNF-α 및 Granzyme B 발현을 증가시켰으며, 이를 통해 NK 세포 활성화의 가능한 기전을 알 수 있었다.

결론

우리의 연구는 대량 스크리닝 및 실험을 통해 Rosiglitazone이 NK 세포를 활성화시키고 췌장암 세포의 증식을 억제하는 강력한 단일성분임을 밝혀냈다. 이러한 결과는 단일 성분 물질을 사용하여 면역 체계를 강화시키는 것이 췌장암 치료에 유망한 전략이 될 수 있음을 시사한다.