Application of gene therapy for reversing T-cell dysfunction in cancer

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SUMMARY
T cells are the key players of antitumor function that sense antigens produced by tumor cells and specifically target cancer cells to induce cell death. Therefore, it is widely understood that T cells are critical for cancer immunotherapy. Many recent studies discovered that T cells enter a dysfunctional or inactivated state. Cancer cells induce functional impairment of T cells by sustained expression of inhibitory receptors in T cells. Therefore, reversing T-cell dysfunction is critical for increasing the efficacy of tumor immunotherapy. In this research, we analyzed the cellular inhibition and activation of T cells using two cell line models: Jurkat (T cells) and MDA-MB-231 (breast cancer cells). We hypothesized that the knockdown of T-cell inhibition-causing genes might reverse the T-cell dysfunction caused by cancer cells. Therefore, we targeted Proprotein Convertase Subtilisin/Kexin Type 1 (PCSK1) gene in T cells and programmed death-ligand 1 (CD274) in cancer cells. We used small interference RNA (siRNA) to knock down the target gene. We used four μg/ml of lipopolysaccharide (LPS) to activate Jurkat cells. MDA-MB-231 co-culture with Jurkat cells reduced IL-2 expression level on Jurkat cells. We found that siRNA targeting CD274 in MDA-MB-231 reversed the reduced IL-2 expression level on Jurkat induced by Jurkat and MDA-MB-231 co-culture. Also, when the PCSK1 knockdown Jurkat cells and CD274 knockdown MDA-MB-231 were co-cultured, the most effective recovery of IL-2 expression level in Jurkat cells was observed. Overall, this study provides a novel strategy for reactivating T cells whose activation has been suppressed from cancer cells.

INTRODUCTION
Cancer immunotherapy is a method of cancer treatment in which doctors utilize the body’s immune systems to fight against the cancer cells (1). The main cells used in cancer immunotherapy are T cells (2). T cells play a major role in the human immune system as they attack foreign substances, mainly cancer, and fight against them. In order to do so, they use their receptors to detect and react to antigens related to various tumor cells (3). For example, immunotherapy in which scientists conduct adoptive cell transfers of tumor-infiltrating lymphocytes (TILs), endogenous peripheral blood-derived T cells (ETC), and chimeric antigen receptor-engineered T cells (CAR-T) are undergoing rapid development (4). Likewise, immunotherapy using T cells has shown great promise and has the potential to contribute to the cure of cancer.

Although immunotherapy using T cells has shown great possibilities, the research also includes challenges (5). One major challenge to T-cell immunotherapy is that the T cells when with cancer cells, are inactivated (6). Unlike the ideal response in which T cells recognize and respond to the tumor cells, they are inhibited and unable to defend the body against the rapid growth of tumor cells. Previous research points to the fact that cancer cells inhibit T-cell activity and disable them as a response to the T-cell defense mechanism (7). More specifically, cancer cells contain inhibitory receptors such as PD-1, TIM-3, and LAG-3 that are directly related to the dysfunction of T cells in the tumor microenvironment (8).

In short, the inhibitory receptors of tumor cells disable T-cell activity, thus preventing the T cell from activating its host defense mechanism.

LPS, also known as lipopolysaccharides, are molecules in gram-negative bacteria. According to previous research, it was shown that LPS has the ability to activate T cells. When LPS binds to Toll-like receptor 4 (TLR4), it initiates a signal transduction pathway that leads to the stimulation of T cells (9). Thus, we used LPS to activate the T cells in this experiment.

To check whether or not the T cells were activated, we measured the IL-2, which stands for interleukin-2 expression levels. IL-2 is a pleiotropic cytokine that aids in the control of the immune system by regulating white blood cell activities. This is because other research papers had pointed to the positive correlation between the expression levels of IL-2 and T-cell activation: the higher the IL-2 expression levels, the greater the activation level of T cells (10).

Moreover, in this experiment, we included the MCF7 and the MDA-MB-231 breast cancer cell lines. These cell lines were cell lines that, according to past research articles, showed the most inhibition in T cells (11). These cell lines were used to ensure the inhibiting effects of the cancer cells on the T cells and validate the reactivating effects of the treatment on the T cells.

We hypothesized that the knockdown of T-cell inhibition-causing genes might reverse the T-cell dysfunction caused by cancer cells. To reactivate T cells, we first searched for genes that activate the immunosuppressive response of T cells. In
the breast cancer cell (MDA-MB-231), we selected the CD274 gene that inactivates the T cell (11). A previous study indicated that PD-L1 (CD274) expression levels on tumor cells affect their immunosuppressive activity (11). Additionally, in the T cell (Jurkat), we selected the LAG-3 (PCSK1), which is expressed on inhibited CD4+ and CD8+ tumor-infiltrating T cells that are defective in cytokine production (12). In detail, PCSK1 is a gene known for encoding the proprotein convertase 1, while CD274 encodes the programmed death-ligand 1 (PD-L1). Proprotein convertase 1 is a protease that processes protein and peptide precursors transported through various branches of the secretory pathway. On the other hand, PD-L1 is an immune inhibitory receptor ligand in various types of cells, including T cells and cancer cells (11).

We hypothesize that siRNA targeting PCSK1 and CD274 will reverse the inactivated T cells to an activated state. Since cancer cells inhibit T-cell activity, if the T cells are reversed to normal through gene therapy, then the T cells would become effective once again, fighting cancer cells. Therefore, this experiment was conducted to reverse the T cells back to an activated state and possibly provide a method of treatment for cancer by inhibiting proprotein convertases (PCSK1) in T cells and programmed death-ligand 1 (CD274) in cancer cells. Overall, IL-2 expression level in T cells showed that targeting both genes using siRNA showed the most significant T-cell activating effect compared to the individual treatment.

RESULTS

The main purpose of this experiment was to experimentally demonstrate Jurkat (T cell) activation by LPS (lipopolysaccharide, E. coli cultured). Furthermore, the experiment was also designed in order to inactivate the T cells within breast cancer cells.

There were six independent variables: a control group (with no treatment), LPS (1, 2, 4 µg/ml), LPS (4 µg/ml) + MCF7, and LPS (4 µg/ml) + MDA-MB-231 (Figure 1A). The results of the experiments showed that, when comparing the control group and the LPS (4 µg/ml) samples, the IL-2 expression levels were increased significantly by about threefolds (Figure 1B, \( p < 0.0001 \), one-way ANOVA). The increased levels of IL-2 suggest that the T cells were activated once again (Figure 1B).

Next, to test whether the cancer cells directly affected the T-cell inactivation, we co-cultured LPS (4 µg/ml) + MCF7 and LPS (4 µg/ml) + MDA-MB-231 and compared the IL-2 expression levels. When comparing the IL-2 expression levels of the LPS (4 µg/ml) sample and the LPS (4 µg/ml) + MCF7 sample, there was no significant difference (Figure 1B, \( p = 0.977 \), one-way ANOVA). However, when comparing the LPS (4 µg/ml) sample and the LPS (4 µg/ml) + MDA-MB-231 sample, the IL-2 levels significantly decreased about 20% (Figure 1B, \( p = 0.045 \), one-way ANOVA). From this data, we can conclude that MDA-MB-231 has a greater effect on T-cell inactivation than MCF7.

After selecting two genes, we utilized siRNA to knock down these genes and observed the knockdown efficiency of the target genes (Figure 2). We used siRNA, which are small interfering RNA molecules, to knock down the genes as they can inhibit gene expression by contributing to the RNA-induced silencing complex (RISC) (13).

Furthermore, there were four independent variables in this experiment, including siControl and siCD274 of MDA-MB-231 and the siControl and siPCSK1 of the Jurkat cells. siControl was a negative control in which we used a siRNA that is unable to target a human gene. The results of this experiment showed that each siRNA decreased the target gene expression level in T cells.
expression. To begin, the siRNA targeting CD274 (siCD274) decreased the expression level of CD274 on MDA-MB-231 cells. In addition, the siRNA targeting PCSK1 (siPCSK1) also decreased the expression level of PCSK1 on Jurkat cells. In sum, the experiment confirmed that the siRNA successfully reduced the expression of the target gene. As a result, we optimized the transfection condition.

The next experiment confirmed if the knockdown of genes we selected (CD274 and PCSK1) can recover the immunosuppressive effect induced by cancer cells (Figure 3A). In this experiment, we tested a total of six different variables. First, we prepared Jurkat cells transfected with siControl in three different conditions: Control (Jurkat cells incubated without cancer cells), MDA-MB-231 siControl (Jurkat cells incubated with MDA-MB-231 transfected with siControl), and MDA-MB-231 siCD274 (Jurkat cells incubated with MDA-MB-231 transfected with siCD274). We transfected the next three variables of Jurkat cells with siPCSK1 along with the same three variable conditions mentioned above.

When comparing the results between the Control group and the MDA-MB-231 siControl sample, MDA-MB231 significantly decreased the expression level of IL-2, meaning that this cancer cell line induces T-cell dysfunction (Figure 3B, \( p = 0.004 \), one-way ANOVA). Moreover, when comparing the results between MDA-MB-231 siControl and MDA-MB-231 siCD274, the IL-2 expression is recovered up to \( \sim 80\% \) in MDA-MB-231 siCD274, meaning that CD274 knockdown on MDA-MB-231 reactivated dysfunctional T cell induced by cancer cells (Figure 3B, \( p = 0.006 \), one-way ANOVA).

The three samples that were explained above depict the expression pattern of Jurkat cells that were transfected with siControl. Next, the three samples analyze the IL-2 expression patterns in each condition of Jurkat cells with the siPCSK1 knockdown.

When comparing the results between the Control group and the MDA-MB-231 siControl samples of the Jurkat cells with siPCSK1 knockdown, the IL-2 expression levels decreased significantly for the MDA-MB-231 siControl sample (Figure 3B, \( p = 0.003 \), one-way ANOVA). This decrease in the IL-2 expression levels was due to cancer cells. Furthermore, when we incubated both MDA-MB-231 cells with and without the CD274 knockdown, the data displayed that Jurkat cells with MDA-MB-231 cells with CD274 knockdown showed significantly increased expression of its IL-2 levels compared to MDA-MB-231 siControl sample (Figure 3B, \( p = 0.004 \), one-way ANOVA).

Also, the IL-2 expression level of the MDA-MB-231 siCD274 sample was about 90\% of the IL-2 expression levels of the Control group. In conclusion, the results of this experiment support the claim that the downregulation of the PCSK1 of Jurkat cells aids the recovery of T-cell dysfunction due to cancer cells.
DISCUSSION

In this study, we engineered T cells and breast cancer cells to reverse T-cell dysfunction induced by cancer cells. We targeted two genes, PCSK1 in T cells and CD274 in breast cancer cells, by siRNA to inhibit the target gene expression. We hypothesized that siRNA treatment targeting both genes in each cell showed the significant reversing effect of T-cell dysfunction induced by cancer cells. IL-2 expression level in T cells indicated that targeting both genes showed the most significant T-cell activating effect compared to the individual treatment. A previous research paper indicated that MCF7 down-regulated PD-L1 (CD274) expression level (11). Since the IL-2 gene is a different cytokine gene, MCF7 may not be able to inhibit IL-2 by the same molecular mechanism as PD-L1 (CD274).

The limitation of this research is that we only tested one type of T cell and one type of breast cancer cell. Other types of cell models should be tested to verify the effect of reversing T-cell dysfunction induced by cancer cells. Also, in vivo experiments such as mouse models should be used to verify this siRNA gene silencing strategy. We also have not defined the molecular mechanism behind this phenotype. Therefore, future studies need to identify the detailed molecular interactions between T cells and cancer cells. For example, the genetic or molecular interaction of PCSK1 and CD274 is unknown. Therefore, RNA-sequencing should be performed to investigate the gene expression patterns of T cell and breast cancer cells. Also, protein interactions translated from PCSK1 and CD274 should be tested further.

Previously, many people believed that it was essentially impossible to change gene expression in the tumor cells in patients. However, recent research indicates that siRNA-loaded nanoparticles targeted several gene expressions of tumor cells in patients (14). Even though many siRNA-targeted genes are known for cellular growth and survival, targeting genes associated with T-cell interaction could be vital. Since tumor cells evade the T-cell mediated apoptosis, increasing the sensitivity of T cell detecting tumor cells may decrease the tumor progression (15). However, the side effects of T-cell immunotherapy should be further investigated in the future.

The results of this study yielded many significant conclusions. First, our investigation identified that knockdown of the two target genes, PCSK1 in T cells and CD274 in breast cancer cells, reversed T-cell dysfunction. Additionally, we demonstrated that T cell dysfunction depends on the expression level of PCSK1 in T cell and CD274 in breast cancer. Therefore, targeting the gene expression level on both types of cells may efficiently recover the T-cell dysfunction caused by cancer cells.

MATERIALS AND METHODS

Cell Culture and Maintenance

Jurkat, MCF7, and MDA-MB-231 cells were purchased by Korea Cell Line Bank. The cells were maintained in the cell media RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The cells were incubated at 37°C on a 5% CO2 incubator (Eppendorf). Cells were subcultured every three days with 0.25% Trypsin-EDTA (Gibco).

T-Cell Activation Test

For testing Jurkat activation, we prepared four cell culture conditions in a six-well cell culture plate: 1) Jurkat and water, 2) Jurkat and LPS (1 µg/ml), 3) Jurkat and LPS (2 µg/ml), 4) Jurkat and LPS (4 µg/ml). Cells were incubated for 24 h and harvested. Then, the RNA was extracted using Total RNA extraction kit (Bioneer), and the IL-2 gene expression level was quantified using gel-based RT-PCR.

siRNA Transfection

After seeding the 0.3 x 10^6 cells in six-well culture plates, two types of samples were prepared. Jurkat cells were transfected with 20 nM siRNA targeting PCSK1 (siPSK1) and 20 nM siRNA for negative control (siCon). After siRNA was mixed with the Lipofectamine RNAiMax transfection reagent for 5 min (Invitrogen), the cells were incubated for 24 hours. The same condition was used for cell seeding on MDA-MB-231. Then two types of samples were prepared with the MDA-MB-231 cell line. MDA-MB-231 was transfected with siRNA targeting CD274 (siCD244) and siCon. After incubating for 24 hours, Jurkat and MDA-MB-231 cells were incubated together for 48 hours. The following siRNA was purchased from bioneer for PCSK1 (Cat # 5122-1, HPLC) and (Cat # 29126-1HPLC).

RNA Extraction

Total RNA was extracted using a Total RNA Extraction Kit (Bioneer). The protocol provided by the RNA extraction kit was used to isolate RNA from the cell-cultured samples. For each sample, 350 µl of lysis buffer supplemented with 1% 2-mercaptoethanol were used to lysis the cells. After 5 minutes of incubation, 350 µl of 70% ethanol was used to precipitate the RNA. After the solution was mixed well, the solution was moved to the RNA binding column. After the centrifugation, the flow-through was discarded, and two washing buffer was used to increase the purification of the RNA. Finally, 50 µl of elution buffer was used to extract the RNA from the RNA binding column.

cDNA synthesis

Using the extracted RNA, cDNA was synthesized by reverse transcriptase enzyme (Enzymomics). The 10 µl of cDNA was synthesized by mixing 1 µl of 10x RT-buffer, 1 µl of dNTP, 0.5 µl of oligo dT, 0.5 µl of reverse transcriptase enzyme, and 7 µl of extracted RNA. The following condition was used in the thermocycler (Bioneer): 25 °C for 15 min, 45 °C for 60 min, 95 °C for 5 min, 4 °C for infinite.

RT-PCR

The primer pairs targeting IL-2, PCSK1, CD274, and...
GAPDH were used to amplify synthesized cDNA. For each sample, 20 µl reaction was prepared with the PCR kit (Bioneer). The following PCR reaction was used: denaturing step (95 °C), primer annealing step (60 °C), and the extension step (72 °C). Thirty-four cycles were used to amplify target cDNA. The following primers were used in this study. For IL-2, F: 5′-TCCTGTTCTGGCATGGCTAA-3′ and R: 5′-CATCTGGTGAAGTTGGATCC-3′. For PCSK1, F: 5′-GGACCTCTGGATAGCCCG-3′ and R: 5′-AGCTTTGGCATTTAGCACG-3′. For CD274, F: 5′-TGGCATTGCTGAAACGATT-3′ and R: 5′-TGCAGGCGGTCAATTGTGT-3′. For GAPDH, F: 5′-GGAGCGAGATCCCTCCAAAAT-3′ and R: 5′-GGCTGTGTGTCATGCTTCAAAAT-3′.

**Agarose Gel Electrophoresis**

After preparing 1.3% agarose gel, the Redsafe DNA staining solution (Intron) was loaded on the gel. The gel-electrophoresis device (Mupid) was set to run for 25 minutes. After running the gel, the gel was illuminated on the blue light gel documentation (Biobox). The image was taken for band analysis.

**Quantification of Gene Expression Level**

The band image from the electrophoresis was analyzed using the ImageJ program. After selecting the rectangle region of the band, the band intensity was plotted. The area of the plotted graph was used to calculate the band intensity of each gene. The GAPDH expression level was used to normalize the target gene expression level. After GAPDH normalization by dividing the target gene intensity by GAPDH intensity, the bar graph was created.

**Statistical Test**

We used Prism 8 program to calculate the p-value. We used One-way ANOVA with Tukey’s post hoc test. A p-value less than 0.05 was considered to be statistically significant.

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