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RESEARCH ARTICLE

Exosome Encapsulation of Curcumin Blocks TNF-α and IL-2 Dependent Cytokine Storm

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ABSTRACT: Severe acute respiratory syndrome coronavirus 2 (SARS-COV2), a highly contagious virus, has caused a pandemic of a respiratory disease named coronavirus disease 2019 (COVID-19). The disease can be fatal because of a systematic cytokine storm that causes excessive inflammatory responses in the lungs. However, no drugs are available to calm severe inflammatory responses that cause lung injury. Through its antioxidant activity and ability to release different anti-inflammatory cytokines, curcumin could be used as a therapeutic drug for COVID-19. Also, cancer-derived exosomes, which can carry biological molecules inside the cell, induce immunosuppression within the tumor microenvironment. Previous research indicated that curcumin does not inhibit *TNF* and *IL-2* in COVID-19 patients and has a limited effect on inhibiting cytokine storms. To solve this limitation of curcumin treatment, we hypothesized that exosome encapsulated curcumin would enhance the inhibiting effect on cytokine. We tested a total of 12 conditions using curcumin and exosomes in different concentrations and ratios on T-cells (Jurkat), finding the optimal condition for inhibiting *TNF* and *IL-2*. As a result, we found that the 1:3 ratio (curcumin: exosome) treatment most efficiently inhibits the expression of *TNF* and *IL-2* in Jurkat cells. Our result indicates that encapsulating the curcumin with an exosome can be used as a therapeutic application on calming the cytokine storm for COVID-19 patients.

KEYWORDS: Biology; Cell biology; Curcumin; Exosome; Cytokine Storm; COVID-19.

Introduction

At the end of 2019, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, and caused an outbreak of an unusual viral pneumonia.[1] This novel coronavirus disease, known as coronavirus disease 2019 (COVID-19), has spread fast worldwide. The ongoing epidemic of COVID19 still threatens global public health in 2022.[2]

While COVID-19 has caused a million deaths worldwide, approximately 15% of the patients developed respiratory failure from acute respiratory distress syndrome (ARDS), a product of inflammatory cytokine storm.[3] Notably, COVID-19 has a broad spectrum of disease severity for the patients, including ARDS. Previous research showed that ARDS and cytokine storm is a causative syndrome of death in over 70% of fatal COVID-19 cases, where inflammatory responses occur aggressively.[4] As respiratory disorder caused by ARDS is the leading cause of death from COVID-19, many immune cells resulting from the cytokine storm are activated in the lungs.[5]

Curcumin is a polyphenol pigment derived from the perennial plant *Curcuma longa L.*, commonly known as the turmeric spice. It inhibits tumor cell proliferation, regulates anti-inflammation, and induces a neuroprotective effect on brain cells.[6] A previous study aimed to evaluate the efficacy of curcumin (dose- 160 mg in four 40 mg capsules daily for 14 days) on regulating the levels of inflammatory cytokines IL-1β, IL-6, TNFα, and IL-18.7 They found out that decreased expression and secretion of IL-1β and IL-6 were achieved in COVID-19 patients.[7] However, there was no positive effect on TNFα concentration and *IL-2* expression with curcumin treatment.

To solve this limitation of curcumin treatment on inhibiting cytokines, we used cancer-derived exosomes to encapsulate the curcumin. Many research papers indicated that cancer-derived exosomes have an immunosuppressive effect in the tumor microenvironment.[8] Tumor-derived exosomes from glioma (brain cancer) decreased the expression level of TNFα, IL-2, IFN-γ, IL-4, IL-5, IL17, GM-CSF of Jurkat cells.[9]

As TNFα and *IL-2* are present in the blood and disease tissues of patients with COVID-19, it acts as an amplifier of inflammation and is essential in acute inflammatory reactions.[10] Moreover, excessive production of both *TNF* and *IL-2* is involved in inducing cytokine storms, which may cause severe damage to lung tissue.[11] Therefore, in this experiment, we tested a total of 12 conditions using curcumin and exosomes in different concentrations and ratios on T-cells (Jurkat), analyzing its potential effect on inhibiting *TNF* and *IL-2*.

Methods

Cell culture and maintenance:

Jurkat and A172 cells were purchased from Korea Cell Line Bank. The cells were maintained in RPMI 1640 media (Gibco) in a 5 % CO2 incubator at 37 °C (Eppendorf). The cells were subcultured into a new plate every three days to maintain the cells in healthy condition.

RNA Extraction from cultured cell:

After adding 400 µL RB Buffer (lysis buffer) to the cell pellet and 300 µL ethanol (80%) to the cell samples, transfer the samples to the 2 mL collection tubes. The cells were centrifuged at 14,000 rpm for 20 seconds. Repeat the centrifuging process after adding each 700µl RWA1 Buffer (washing buffer 1) and 500 µl RWA2 Buffer (washing buffer 1). The

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ethanol was completely removed from the tube by centrifuging the tube once more at 14,000 rpm for 1 minute. 50~200 µL of ER Buffer (elution buffer) was added to the new tube after transferring the cells. After waiting 1 minute at room temperature (15~25 °C), centrifuge the tubes at 10,000 rpm for 1 minute to elute.

cDNA synthesis:

For each cDNA synthesis reaction sample, we added 1 µL RT Buffer, 0.5 µL RT enzyme, 1 µL dNTP mixture, 0.5 µL T primer, and 7 µL of RNA, with a total of 10 µL. This step was repeated 13 times to make 13 samples for cDNA synthesis. Then, the reaction sample was placed inside a thermocycler machine to synthesize cDNA from extracted RNA. The following temperature and time conditions were used: 1) 25 °C for 10 min 2) 45 °C for 60 min 3) 95 °C for 5 min 4) 4 °C for sample storage.

Polymerase Chain Reaction (PCR):

PCR reaction was performed to amplify cDNA of TNFα, IL-2, and GAPDH. For each PCR reaction sample, we added 0.5 µL synthesized cDNA, 17.5 µL water, 1 µL primer-F, and 1 µL primer-R, with a total of 20 µL. Pre-mixed PCR reaction tubes (Bioneer) were used, and the premix solution contains DNA Polymerase, dNTP, and buffer mixture. The process was repeated 13 times to make 13 samples for PCR reaction. The following primer sequences were used to amplify TNFα, IL-2, and GAPDH. TNFα Forward: CCTCTCTCTAATCAGCCCTCTG, TNFα Reverse: GAGGACCTGGGAGTAGATGAG. IL2- Forward: TCCTGTCTTGCATTGCACTAAG, IL2- Reverse: CATCCTGGTGAGTTTGGGATTC. GAPDH Forward: GGAGCGAGATCCCTCCAAAAT, Reverse: GGCTGTTGTCATACTTCTCATGG. The following temperature and time conditions were used: 1) 95 ˚C for 3 min 2) 95 ˚C for 30 sec 3) 61 ˚C for 30 sec 4) 72 ˚C for 25 sec 5) repeat step 2 to 4 for 34 times 6) 72 ˚C for 5 min 7) 12 ˚C for sample storage.

Agarose gel electrophoresis:

Agarose gel was prepared to visualize the amplified cDNA of TNFα, IL-2, and GAPDH. To prepare the gel, 1.3 g agarose was added to 100 mL of TBE Buffer. After microwaving for 3 minutes until the mixture is completely solubilized, we added 5 µL of Red Safe (Intron) DNA staining solution. For the gel to be completely solidified, we poured the solution into the gel caster and wait for 20 minutes. After the gels were solidified, the gel was placed inside the gel tank. The TBE buffer was poured until the gel was completely submerged. Then, 7 µL of amplified cDNA sample was carefully loaded into the empty well of the agarose gel. The gel was run for 20 minutes and placed on the blue illuminator to visualize the amplified DNA band. Then, the photo was taken to analyze the band intensity. The band intensity was quantified using the ImageJ program.

Statistical test analysis:

An unpaired t-test was used to calculate the statistical significance. Prism 8 program was used to calculate the p-value, and a p-value less than 0.05 was considered to be statistically significant.

Results and Discussion

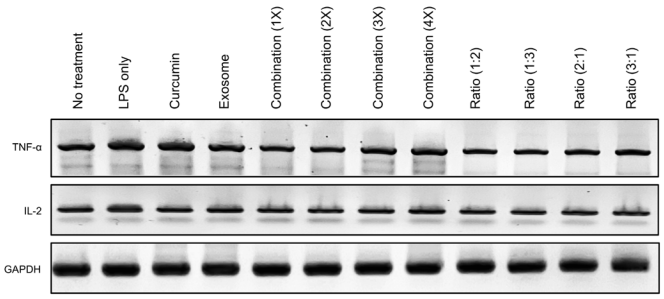

Figure 1: The expression level of TNF-α, IL-2, and GAPDH on Jurkat cells using 12 different treatment conditions: 1) no treatment (negative control), 2) LPS only, 3) LPS+ Curcumin, 4) LPS + Exosome, 5) LPS+ Combination (1X), 6) LPS+ Combination (2X), 7) LPS + Combination (3X), 8) LPS + Combination 4X), 9) LPS + Ratio (1:2), 10) LPS+ Ratio (1:3), 11) LPS + Ratio (2:1), 12) LPS +Ratio (3:1)

We prepared 12 different treatment conditions in this experiment. We used lipopolysaccharide (LPS), which is a natural adjuvant synthesized by gram-negative bacteria to activate the human T-cell (Jurkat). The high expression level of TNF-α and IL-2 represents the cytokine storm phenotype. GAPDH was used to normalize the expression level of TNF-α and IL-2. This experiment aims to find the optimal condition for inhibiting the expression level of both TNF-α and IL-2. As we expected, when we compared the band intensity of TNF-α and IL-2 between no treatment and LPS only condition, LPS treatment increased the band intensity of both TNF-α and IL-2 on Jurkat cells (Figure 1). Also, the individual treatment of both curcumin and exosome decreased the band intensity of TNF-α and IL-2 compared to the LPS only condition. This result indicates that both curcumin and exosome inhibit TNF-α and IL-2 dependent cytokine storms.

Next, we tested the combination treatment of curcumin and exosomes. We investigated four different combination conditions by increasing the concentration of both curcumin and exosomes up to four times. The combination treatment of 1X and 2X showed lower band intensity of both TNF-α and IL-2. However, combination treatment of 3X and 4X showed increased band intensity compared to 1X and 2X. In addition, we also tested four different ratios of curcumin to exosome. All four combination treatments with different ratios decreased the band intensity of both TNF-α and IL-2 compared to LPS only condition. In conclusion, our result indicates that combination treatment effectively inhibits the expression level of both TNF-α and IL-2, which may block the cytokine storm on T-cells.

To find the optimized condition that efficiently inhibits TNF-α dependent cytokine storm, we quantified the gene expression level of TNF-α in all twelve conditions. Compared to LPS only condition, we found that Ratio (1:3) significantly decreased the expression level of TNF-α in Jurkat cells (Figure 2). Since an increased expression level of TNF-α is commonly found in the COVID-19 patient's blood with severe cytokine storm, this treatment condition may calm the overexpression of TNF-α, inhibiting inflammation in patients' lung tissue.

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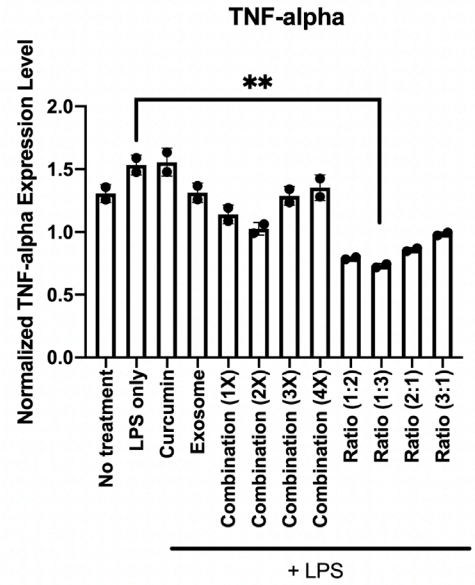

Figure 2: The ratio (1:3) treatment condition significantly decreased the expression level of TNF-α compared to LPS only condition. Normalized TNF-α expression level was calculated using the following equation: (band intensity of TNF-α) / (band intensity of GAPDH). The mean and standard deviation is graphed. (n= 2), Unpaired t-test, ** P< 0.01.

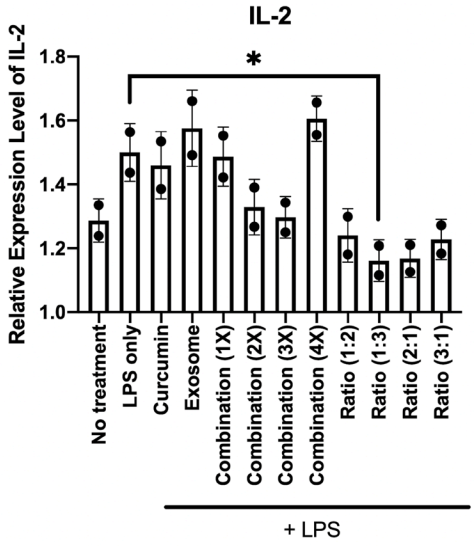

Figure 3: The ratio (1:3) treatment condition significantly decreased the expression level of IL-2 compared to LPS only condition. Normalized TNF-α expression level was calculated using the following equation: (band intensity of IL-2) / (band intensity of GAPDH). The mean and standard deviation is graphed. (n= 2), Unpaired t-test, * P< 0.05.

To find the optimized conditions that efficiently inhibit IL-2 dependent cytokine storm, we quantified the gene expression level of IL-2 in all twelve conditions. Compared to LPS only condition, we found that Ratio (1:3) significantly decreased the expression level of IL-2 in Jurkat cells (Figure 3). A high expression level of IL-2 is found in COVID-19 patients since it induces a severe cytokine storm in COVID-19 patients' lungs. Previous studies indicated that curcumin treatment did not effectively suppress IL-2 in COVID-19 patients. However, our result showed that this Ratio (1:3) treatment would efficiently prevent the cytokine storm induced by IL-2.

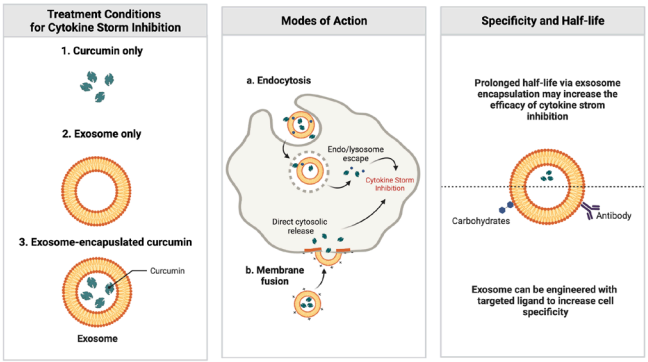

Figure 4: The proposed model for exosome encapsulated curcumin delivery that inhibits cytokine storm. Three treatment conditions were listed: 1) curcumin only, 2) exosome only, 3) Exosome-encapsulated curcumin.

Previous research indicated that curcumin alone did not inhibit TNF-α and IL-2 dependent cytokine storm. Therefore, our experiment aimed to find an optimal condition that efficiently inhibits TNF-α and IL-2 dependent cytokine storm on T-cells. In this study, we tested three treatment conditions with varying curcumin and exosome concentrations and ratios; a total of twelve conditions were investigated in this study. Since exosomes efficiently deliver the cargo into target cells, we expected that exosome encapsulated curcumin would be delivered more efficiently rather than curcumin treatment alone. As a result, combination treatment with a ratio (1:3) demonstrated the most efficiency in inhibiting both TNF-α and IL-2 expression on T-cells. The prolonged half-life and increased delivery efficiency via exosome encapsulation may enhance the stability and activity of curcumin to block the cytokine storm (Figure 4).

Conclusion

COVID-19, which is caused by SARS-CoV-2, is expressed by diverse symptoms ranging from moderate fatigue to life-threatening problems, including cytokine storm and multiorgan failure.[12] Importantly, cytokine storm was also found in patients with SARS and often led to poor outcomes. Previous studies indicated that cytokine blockades improved the survival rate of patients with COVID-19 who are at risk of respiratory failure.[13] However, since many cytokines induce inflammation in the lungs, current cytokine blocking treatment is limited. Also, previous studies showed that high serum IL-2, IL-6, IL-8, and TNF-α levels at the time of hospitalization were strong and independent predictors of low patient survival rates.[14] Therefore, our study aims to find the effect on blocking TNF-α and IL-2.

We focused on curcumin treatment, which has shown blocking inflammation effects on COVID-19 patients. However, curcumin alone cannot block IL-2 and TNF-α dependent inflammation.[15] To increase the efficacy of blocking both IL-2 and TNF-α, we encapsulate the curcumin with human brain cancer cell exosomes, which are known to change the cytokine molecules in T-cell. As we hypothesized, the combination treatment of curcumin and exosomes with 1:3 ratio showed the most effective blocking of cytokines of IL-2 and TNF-α. This result indicates that our new method can be applied to patients with COVID-19 dependent

inflammations for calming the cytokine storm. We speculate that 1:3 ratio (curcumin:exosome) efficiently delivers the curcumin to the T-cell and may exhibit the most effective blockage of cytokine storm-related genes. Also, there is a possibility that the exosome itself has the blocking effect of the cytokine storm. However, further experiments are needed to confirm how 1:3 ratio efficiently blocks the cytokine storm.

One of the limitations of our study is that only Jurkat T-cell was used during the experiment. More types of immune cells (T-cells) should be used to validate our experimental results. Moreover, a mouse model should also be tested to confirm our results, while our study only performed cell experiments. In the future, clinical studies comparing control groups to exosome encapsulated curcumin treatment groups should be performed. Another limitation is that our experiment only investigated blocking IL-2 and TNF-α. As cytokine storms can be induced by hundreds of genes depending on growth factors and hormones, we should explore more types of cytokine genes that can be controlled by exosome encapsulated curcumin.

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