Induction of Triple-negative Breast Cancer Cells to Immunogenic Cell Death and Increase Cross-Presentation by Streptomyces sanyensis

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ABSTRACT

Background: Biomass extract of Streptomyces sanyensis (BE-SS), which is a marine microorganism, has antitumor activity and has white-to-gray aerial mycelium and long chains of long spores in aerial mycelium. Objectives: The anti-cancer effect of BE-SS on triple-negative breast cancer (TNBC) was confirmed and cell death by BE-SS was confirmed to have immunogenicity and whether it could be used for immuno-cancer therapy. Materials and Methods: Human and mouse TNBC cells (MDA-MB-231, HS578T, 4T1-hemagglutinin (HA) and TUBO-P2J-HA cells) were treated with BE-SS at different concentrations for 72 hr and their cytotoxicity was detected using the sulforhodamine B-based (SRB) method. Flow cytometry was used to determine the type of cell death by Annexin V/7-AAD staining and to measure surface exposure to damage-related molecular pattern (DAMP) molecules including calreticulin (CRT), heat shock protein (HSP) 70/90, Carboxyfluorescein succinimidyl ester (CFSE) dilution assay was used to evaluate the immunogenicity of dead cells induced by BE-SS. Results: BE-SS reduced the viability of human (MDA-MB-231 and HS578T-cells) and mouse (4T1-HA and TUBO-P2J-HA cells) breast cancer cells. At 72 h, the IC₅₀ of human and mouse breast cancer cells was 0.02-0.6 μg/ml and 0.005-0.37 μg/ml, respectively. BE-SS-treated breast cancer cells were positively stained with Annexin V. Surface exposure to DAMP molecules increased in a dose-and time-dependent manner. CFSE dilution analysis showed that dendritic cells (DCs) fed with BE-SS treated breast cancer cells successfully stimulated tumor-specific T-cell proliferation without inhibiting DC function and T-cell proliferation.

Conclusion: BE-SS can induce immunogenicity and apoptosis in breast cancer cells and may be a good adjuvant for TNBC immunotherapy.

Key words: Apoptosis, Cross-presentation, DAMPs, Immunogenic cell death, Streptomyces sanyensis, TNBC.

INTRODUCTION

Triple-negative breast cancer (TNBC) is characterized by the lack of expression of progesterone receptor, estrogen receptor and human epidermal growth factor receptor-2. TNBC accounts for approximately 15-20% of breast cancers and its metastasis is strong, disease progression is rapid and survival is relatively poor. In terms of treatment, chemotherapy is currently the only effective systemic therapy for TNBC. However, despite adjuvant chemotherapy, most TNBC patients have a high probability of recurrence within 1-2 years and < 30% of patients survive for 5 years.[1] Notably, TNBC was found to be immunogenic, which makes it a good candidate for immunotherapy.[2] Indeed, immunotherapy has shown great potential in the latest research of TNBC and some clinical studies have shown that immunotherapy has great potential in improving the clinical outcome of patients with this tumor.[3] Although significant clinical progress has been made in the application of immunotherapy, the limitations of this treatment strategy are inevitable;[4] hence, there is an urgent need to identify a substance that can overcome the limitations of immunotherapy and enhance antitumor response.

A recent study reported that compounds extracted from marine microorganisms have potential in drug development, especially mangrove ecosystems, which have attracted increasing attention from the scientific community. In particular, the high biodiversity of mangrove ecosystems and the feasibility of collecting samples have attracted the attention of the scientific community. In more than 200 kinds of endophytic fungi and more than 2,000 kinds of actinomycetes collected from mangroves, many secondary metabolites exhibiting a variety of pharmacological properties including anticancer, antimicrobial and antiviral properties have been found.
identified. Among them, *Streptomyces sanyensis* (*S. sanyensis*) isolated from mangrove soil samples produces staurosoricine, which exerts antiviral, neuroprotective and strong inhibitory effects on many cancer cells. However, there is not much literature on the biomass extract of *S. sanyensis* (BE-SS) and its anti-tumor property is difficult to convince without evidence.

Currently, chemotherapy is the only effective systemic therapy available for TNBC. However, in anti-cancer treatment, chemotherapy or radiotherapy can cause immunogenic cell death (ICD). ICD is a form of cell death that leads to the regulatory activation of the immune response.

In ICD, apoptosis is activated, leading to the release of damage-related molecular patterns (DAMPs) such as calreticulin (CRT), adenosine triphosphate (ATP), heat shock protein (HSP) 70/90 and high-mobility group box 1 (HMGB1), which are exposed or released on the cell surface or extracellular. These DAMPs activate dendritic cells (DCs), thereby stimulating and enhancing the cross-presentation of tumor antigens to cytotoxic T-cells, consequently inhibiting the growth of tumor cells.

In this study, we confirmed the antitumor effect of BE-SS on TNBC. Research data show that BE-SS induces apoptosis in breast cancer cells, increases expression of the ICD markers CRT and HSP70/90 and enhances cross-presentation of tumor antigens.

MATERIALS AND METHODS

Reagents and chemicals

The National Marine Biological Resources Museum of Korea (SNC087) provided BE-SS. The soil samples were heated in an oven at 120°C for 60 min, treated with chloramine-T (1.0%, w/v) solution, and then incubated at 28°C for 20 days. Next, on the separation plate, the glucose-aspartic acid-vitamin medium was separated. The extract was then purified, air-dried and ground with a mortar and pestle. The antibodies we used anti-CRT (Cell Signaling, USA), anti-HSP70 FITC, anti-HSP90 PE (Abcam, United Kingdom), secondary antibody (Alexa flour 750-conjugated anti-mouse Fc antibody, Abcam, United Kingdom) and anti-CD8 APC (Invitrogen, USA). The Bone marrow dendritic cells (BMDC) were differentiated with recombinant Granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, USA). The hemagglutinin (HA) peptide (IYSTVASSL) was obtained from Peptone (Daejeon, Korea). Human breast cancer cell lines (MDA-MB-231 and HS578T-cells) and mouse breast cancer cell lines 4T1 purchased from ATCC (Manassas, Virginia, USA). The TUBO-P2J cell line was established previously. The Dulbecco’s Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased form HyClone (Marlborough, USA). The Cytotoxicity assay kit was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The APC-annexin V apoptosis detection kit from Bio Legend (San Diego, CA, USA).

Cell culture

Human (MDA-MB-231 and HS578T-cells) and mouse (4T1-HA and TUBO-P2J-HA cells) breast cancer cells, which TNBC cells were cultured in DMEM or RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin under a humidified atmosphere containing 5% CO₂ at 37°C and passed every 2 days.

Cytotoxicity assay

Cytotoxicity of BE-SS on human and mouse breast cancer cells were measured with using an in vitro cytotoxicity assay kit that was sulforhodamine B (SRB) assay. Briefly, cells were seeded in 96-well flat-bottom plates at a density of 1-10 × 10⁴ cells/well and treated with various concentrations of BE-SS (MDA-MB-231 cells, 0.1, 0.5 and 1 µg/ml; HS578T-cells, 0.01, 0.05 and 0.5 µg/ml; 4T1-HA cells, 0.05, 0.5 and 2 µg/ml; TUBO-P2J-HA cells, 0.005, 0.01 and 0.1 µg/ml) for 72 hr at 37°C and then subjected to the SRB assay. Absorbance was measured using a multi-mode microplate reader (Spectra Max, USA) at 565 nm.

**APC-Annexin V and 7-AAD assay**

Cell death type (apoptosis or necrosis) was analyzed using the APC-annexin V apoptosis detection kit. Briefly, the cells were seeded in 6-well plates at a density of 1-10 × 10⁴ cells/well and incubated at 37°C overnight. The cells were treated with various concentrations of BE-SS (MDA-MB-231 cells, 0.1, 0.5 and 1 µg/ml; HS578T-cells, 0.01, 0.05 and 0.5 µg/ml; 4T1-HA cells, 0.05, 0.5 and 2 µg/ml; TUBO-P2J-HA cells, 0.005, 0.01 and 0.1 µg/ml) for 72 h at 37°C and stained with APC-annexin V and 7-aminoactinomycin D (7-AAD), after which the cells were incubated for 15 min at room temperature away from light and analyzed using the FACS Canto II flow cytometer (BD, USA).

**Flowcytometry analysis**

The cell surface exposures of CRT, HSP70 and HSP90 were detected using flowcytometry. Briefly, the cells were treated with various concentrations of BE-SS (MDA-MB-231 cells, 0.1, 0.5 and 1 µg/ml; HS578T-cells, 0.01, 0.05 and 0.5 µg/ml; 4T1-HA cells, 0.05, 0.5 and 2 µg/ml; TUBO-P2J-HA cells, 0.005, 0.01 and 0.1 µg/ml) for 24, 48, 72 h at 37°C and stained with primary antibody for anti-CRT (1:100) for 30 min at 4°C and after washing, stained secondary antibody (1:2000). Anti-HSP70 or anti-HSP90 were stained 1:100 for 30 min at 4°C. Fluorescence was analyzed using the FACS Canto II flow cytometer and Flow Jo software (BD, USA).

**Animals**

Adult BALB/c female mice, 6-8 weeks old, were purchased from Nara Biotech (Seoul, Korea). BALB/c Cby. Cg-Thy1a Tg (TcraCl4, TcrbCl4) Shrm/Shrmj mice were purchased from Jackson Laboratory (Bar Harbor, ME), and bred and/or maintained under an individually ventilated cage system and in accordance with the Institutional Animal Care and Use Committee (2019-001), and housed in a specific pathogen-free environment.

**Generation of bone marrow - derived DCs**

Bone marrow (BM) cells were isolated from the femurs and tibias of 6-8 weeks old female BALB/c mice. After sacrifice the BALB/c mouse, BM red blood cells (RBCs) were lysed with lysing buffer for 1 min at room temperature. After washing, the cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and immature bone marrow-derived DCs were differentiated with 10 ng/ml GM-CSF in a humidified 5% CO₂ incubator at 37°C. On day 3, 5, 7 and 9 of cell culture, floating cells were gently removed and fresh, warmed medium with mouse recombinant GM-CSF (10 ng/ml) was added. On day 10, non-adherent cells were harvested and used for T-cell proliferation assay experiment.

**T-cell proliferation assay**

T-cell proliferation was analyzed using the CFSE dilution assay. After sacrifice, splenocytes were isolated from CL4-HA TCR transgenic mice and the cells were labeled with CFSE for 4 min at 37°C. CFSE-labeled splenocytes were cultured in the presence of BMDC fed with BE-SS-treated cancer cells for 72 hr. For T-cell activation, 0.1 µg/ml HA peptide was used. The cells were harvested and stained with anti-CD8 for 30 min at 4°C, analyzed using the FACS Canto II flow cytometer and Flow Jo software.

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Statistical analysis
Between-group differences were analyzed using unpaired t-test. Error bars represent ± standard deviation. Data were analyzed using GraphPad Prism (Version 6 for Windows; GraphPad Software; San Diego, CA). Unless specified, \( p < 0.05 \), \( 0.01 \) and \( 0.001 \) have been noted with *, ** and *** respectively. Differences that are not statistically significant have been left unnoted.

RESULTS
BE-SS exerted cytotoxic effects and induced apoptosis in TNBC cells
To investigate the cytotoxic effect of BE-SS on human and mouse TNBC cells, the cells were treated with various concentrations of BE-SS (0, 0.0005, 0.005, 0.01, 0.02, 0.025, 0.05, 0.1, 0.5, 1 and 2 \( \mu \)g/mL) and SRB analysis was used to assess cell viability (Figure 1a). BE-SS reduced the viability of all tested human (MDA-MB-231 and HS578T-cells) and mouse (4T1-HA and TUBO-P2J-HA cells) breast cancer cell lines. The \( IC_{50} \) (half maximum inhibitory concentration) of BE-SS was 0.55 ± 0.05 \( \mu \)g/mL for MDA-MB-231 cells and 0.03 ± 0.01 \( \mu \)g/mL for HS578T-cells in human breast cancer cells and 0.32 ± 0.05 \( \mu \)g/mL for 4T1-HA cells and 0.006 ± 0.001 \( \mu \)g/mL TUBO-P2J-HA cells in mouse breast cancer cells. Based on the above data, we determined the drug concentration for future experiments. Then, to detect whether the type of cell death induced by BE-SS was apoptosis or necrosis, staining with Annexin V and 7-AAD was performed. The cells were treated with different concentration of BE-SS (MDA-MB-231 cells: 0.1, 0.5, 1 \( \mu \)g/mL, HS578T-cells: 0.01, 0.05, 0.5 \( \mu \)g/mL, 4T1-HA cells: 0.05, 0.5, 2 \( \mu \)g/mL, TUBO-P2J-HA cells: 0.005, 0.01, 0.1 \( \mu \)g/mL) for 72 h and then stained with APC-Annexin V and 7-AAD. The stained cells were analyzed by flow cytometry. Figure 1b shows representative dot blots for Annexin V/7-AAD. The 7-AAD single-stained cells were not detected and most of the stained cells were Annexin V single- or Annexin V and 7-AAD double-stained cells. These data indicate that BE-SS induces apoptosis in human and mouse breast cancer cells. In addition, Annexin V-positive cells increased with the increase in BE-SS dose.

BE-SS induced TNBC cells surface exposure to CRT, HSP70 and HSP90
To confirm whether the BE-SS-induced cell death was ICD, flow cytometry was used to evaluate the ICD markers CRT, HSP70 and HSP90 exposed on the cell surface. We found that 24 h after drug treatment, surface exposure to CRT significantly increased compared with the untreated control group and it increased from 24 h to 72 h, gradually increasing with time (\( p < 0.01 \) and \( p < 0.001 \)) (Figure 2a). Doxorubicin is a powerful ICD inducer. After treatment with doxorubicin, the positive rate of CRT and HSP70/90 exposed on the surface of human and mouse breast cancer cells was greater than 70% and 25%, respectively, compared with the untreated control group. The aforementioned BE-SS-treated TNBC cells also showed that as the drug concentration gradually increased, surface exposure to CRT, HSP70 and HSP90 also gradually increased (\( p < 0.05 \), \( p < 0.01 \) and \( p < 0.001 \)) (Figure 2b-2d). According to the above data, although the number of positive cells was less than that in the doxorubicin treatment group, BE-SS induced the exposure of ICD markers on the surface of TNBC cells.

Apoptosis induced by BE-SS-treatment cross-presentation with DC, which increased the proliferation of CD8+ T-cells
Although the above experimental data indicate the possibility of ICD induced by BE-SS in TNBC cells, this aspect remains unclear. To confirm that BE-SS can induce ICD, a HA antigen-specific CD8+ T-cell proliferation system was recruited to replace the effect on cross-presentation. The detailed design of this system can be found in previously published literature. BMDCs fed with BE-SS-treated 4T1-HA cells induced the proliferation of CL4 mouse T-cells in a dose-dependent manner (\( p < 0.001 \)) (Figure 3a). We also confirmed whether BE-SS inhibits the function of DC and T-cells because when BE-SS inhibits the function of DC and T-cells, the potential of BE-SS to induce ICD is meaningless. The experimental design was that BE-SS treatment was applied to feed DCs with necrotic cells or that T-cells were co-cultured with DCs fed with necrotic cells. The DCs fed with necrotic cells successfully stimulated the proliferation of CL4 CD8+ T-cells and were not affected by BE-SS treatment (Figure 3b). Upon HA-peptide stimulation, CL4 CD8+ T-cells showed approximately 40% proliferation and they were not affected by BE-SS treatment to change the proliferation rate (Figure 3c). These results indicate that BE-SS successfully induced ICD in human and mouse breast cancer cells and had no effect on the function of DC and T-cell stimulation and the proliferation potential of T-cells.
DISCUSSION

In this study, we confirmed that BE-SS not only induces ICD in TNBC cells but also enhances cross-presentation of antigens. In this study, we repeatedly emphasized the types of cancer cell death because the proliferation of T-cells is different. It is generally believed that apoptosis does not affect the proliferation of T-cells and necrosis will proliferate T-cells (Figure 3a). However, in this experiment, BE-SS not only induced TNBC cells to apoptosis (Figure 1b) but also promoted T-cell proliferation (Figure 3a). In this way, we must suspect that apoptosis is not simply cell death but a special kind of cell death that is related to necrosis. It is not difficult to find this particular type of death, called necroptosis. This type of cell death is a programmed form of necrosis or inflammatory cell death and has the characteristics of apoptotic and necrotic cell death. This explains our experimental results well; hence, we drew a conclusion that BE-SS-induced breast cancer cell death is not true apoptosis, but necroptosis.

Doxorubicin is a strong ICD inducer and our data show the release of high percentage of CRT, HSP70 and HSP90 (Figure 2b-2d). The ICD maker induced by BE-SS was obviously lower than that induced by doxorubicin (Figure 2). However, doxorubicin is a chemotherapeutic drug that shows great side effects and great damage to the human body. Cross-presentation in immunotherapy directly affects the proliferation of CD8$^+$ T-cells, and the concept of ICD is closely related to cross-presentation. Therefore, DAMPs as ICD maker, such as CRT, HSP70 and HSP90, are released from dead cells, which promote the enhancement of cross-presentation, to enhance the proliferation of T-cells. Our experimental data can also be verified. The CD8$^+$ T-cell proliferation rate of doxorubicin was higher than 70% (Figure 3a). On the contrary, compared with doxorubicin, BE-SS reached only half of it. However, judging from the results, CD8$^+$ T-cell proliferation rate is still very optimistic. However, for drugs with small side effects and small effects on the body, the results are very optimistic. Our results indicate that the immunogenic cell death induced by BE-SS meets the criteria for inducing ICD. The method of confirming whether to induce ICD has been upgraded from the original gold standard to a faster and easier method of confirming DAMPs. Although it cannot be used as the main anti-cancer drug, it has a high potential for use as an auxiliary anticancer drug. At present, the monotherapy of chemical drugs cannot meet the requirement for effective treatment of cancer. They are efficient and eliminate tumors completely, but severe cytotoxicity and side effects make it difficult for patients to receive these therapies. But as an emerging adjuvant therapy, ICD can now be an induced artificially now. Although its pharmacological mechanism is not clear, it still has a great research value.
Figure 3: Biomass extract of Streptomyces sanyensis (BE-SS)-treated mouse breast cancer cells can enhance T cell proliferation without inhibiting the function of DCs and T-cell proliferation response.
(a) Representative determination of T-cell proliferation using flow cytometry. CL4 CD8+ T-cell proliferation stimulated by DCs treated with BE-SS dose-dependently on 4T1-HA cells. (b) BM-derived DCs were fed with necrotic cells that were prepared by freezing three times and then thawing. DCs were treated with BE-SS during feeding of necrotic cells. After feeding, DCs were co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled CL4 splenocytes. (c) CFSE-labelled CL4 splenocytes were stimulated with HA-peptide (0.2 µg/ml) with or without BE-SS. T-cell proliferation was evaluated using flow cytometry. Data are presented as mean ± standard deviation (SD) for triple replicates. Data are presented as mean ± standard deviation (SD) for triple replicates. ***p<0.001 compared with control.
CONCLUSION
In summary, BE-SS inhibits the growth of TNBC (human and mouse breast cancer) cells, promotes apoptosis and successfully induces ICD. With the increase in time and drug concentration, the CRT, HSP70 and HSP90, indicated by cell surface exposure, also gradually increased, which is an important feature of ICD. We also proved that after BE-SS induces cell death, the dead cell is immunogenic through in vitro cross-presentation experiments. According to our experimental data, BE-SS is a good adjuvant and anticancer drug for TNBC with poor prognosis. However, the pharmacological mechanism and constituents of BE-SS are not clear and the substance that is responsible for the main anti-cancer effect remains to be determined. However, the immunotherapy for TNBC has much potential for clinical application.

ACKNOWLEDGEMENT
This work was supported by the 2018 Inje University research grant (Grant numbers 2018-0199).

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest regarding the publication of this paper.

Author Contributions
Xingguo Quan, Illhwan Kim, Anbok Lee, Il-Whan Choi and SaeGwang Park designed the study. Xingguo Quan, Ji-Young Lee, Jin Hee Park, Md. Masudul Haque and Hee Yeon Kim collected the data. Xingguo Quan, Park designed the study. Xingguo Quan, Ji-Young Lee, Jin Hee Park, Md. Xingguo Quan, Ilhwan Kim, Anbok Lee, Il-Whan Choi and SaeGwang Park drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

ABBREVIATIONS
BE-SS: Biomass extract of Streptomyces sanyensis; TNBC: Triple-negative breast cancer; BMDCs: Bone marrow dendritic cells; ICD: immunogenic cell death; DAMP: Damage-related molecular pattern; CRT: Calreticulin; ATP: Adenosine triphosphate; HSP: Heat shock protein; HMGB1: High-mobility group box 1; HA: Hemagglutinin; SRB: Sulfurhodamine B-based method; CFSE: Carboxyfluorescein succinimidyl ester; 7-AAD: 7-aminoactinomycin D; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

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**SUMMARY**

- Biomass extract of *Streptomyces sanyensis* (BE-SS) extracted from marine microorganisms and produces staurosporine which exerts antiviral, neuroprotective and strong inhibitory effects on many cancer cells.
- The IC$_{50}$ (half maximum inhibitory concentration) of BE-SS was 0.02-0.6 µg/ml for human (MDA-MB-231 and HS578T-cells), 0.005-0.27 µg/ml for mouse (4T1-HA and TUBO-P2J-HA cells) triple-negative breast cancer (TNBC) cells and induced apoptosis.
- Immunogenic cell death (ICD) maker (E.g., calreticulin and heat shock protein 70/90) exposed on the surface of TNBC cells treated by BE-SS.
- TNBC cells treated with BE-SS enhanced dendritic cell (DC) cross-presentation.
- In this study, we confirmed that BE-SS is an ICD inducer.
Quan, et al.: Induction of TNBCs to Immunogenic Cell Death by S. sanyensis

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Cite this article: Quan X, Lee J, Park JH, Haque Md. M, Kim HY, Kim I, Lee A, Choi I, Park S. Induction of Triple-negative Breast Cancer Cells to Immunogenic Cell Death and Increase Cross-Presentation by Streptomyces sanyensis. Pharmacog Res. 2021;13(3):165-72.