Original Article
Nattokinase produced by natto fermentation with Bacillus subtilis inhibits breast cancer growth

Bin Zhang1,2, Jing Chai2, Lamei He2, Mahebaer Dusanbieke2, Aihua Gong2

1Department of Laboratory Medicine, Affiliated Hospital of Jining Medical University, Jining Medical University, Jining, Shandong, P. R. China; 2Key Laboratory of Laboratory Medicine of Jiangsu Province, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu, P. R. China

Received April 29, 2019; Accepted October 7, 2019; Epub December 15, 2019; Published December 30, 2019

Abstract: Breast cancer remains a foremost health problem in women. Nattokinase, produced by Bacillus subtilis, performs therapeutic effects on various diseases including hypertension, vitreoretinal disorders, and Alzheimer disease. Whether nattokinase can inhibit breast cancer growth is unclear. We therefore investigated its inhibitory effect on a murine breast carcinoma. We isolated the cultured supernatant, bacterial suspension, and bacterial pellet of Bacillus subtilis. The supernatant and suspension had higher nattokinase activity than the pellet. Tumor growth was measured by tumor volume and weight. We found that the cultured supernatant and bacterial suspension could inhibit breast cancer growth. The blood vessels in the tumor were distributed with treatment with supernatant and bacterial suspension by contrast-enhanced ultrasound. The supernatant and suspension also reduced the expressions of FOXM1 and MMP2 in the tumor tissue. This study revealed a new potential role of nattokinase in tumor therapy and demonstrated that NCE extended the application of nattokinase.

Keywords: Nattokinase, Bacillus subtilis natto, breast cancer, cancer stem cells, angiogenesis

Introduction

Breast cancer has the highest incidence of malignancy in women all over the world, and represents nearly 25% of all cancers in females [1]. During the last decade, breast cancer incidence rates and death rates have generally increased worldwide [2]. The extensive highly heterogeneity of breast cancer and distant organ metastases is the primary reason for these therapeutic failures. The molecular biological mechanism involved in breast cancer is complicated, including multistep, multistage, and multigene synergy and gradual development [3]. Factors, such as alcohol consumption, inactivity, obesity, and hormone replacement therapy (HRT), are the chief culprits for increased the risk of breast cancer [2, 4]. Therefore, it is necessary to look for “natural substances” that can control the development of breast cancer.

The current therapeutic strategies for breast cancer include surgery, radiation therapy, chemotherapy, and endocrine therapy [5]. However, all these treatments have shortcomings, such as a high rate of relapse after surgery [6] and the toxic side effects of radiotherapy and chemotherapy [7, 8]. Therefore, anti-breast cancer drugs with high efficiency and low toxicity are required.

Natto is a traditional food that has been eaten for thousands of years in Japan and is fermented by Bacillus subtilis. Nattokinase attracts the most attention among the enzymes arising from Bacillus subtilis natto. Numerous studies have confirmed that nattokinase has a direct fibrinolytic activity in vivo and in vitro [9]. Nattokinase has a strong thrombolytic effect [10], which has paved the way for thrombolytic drug studies [11]. Nattokinase has excellent therapeutic effects in other diseases, such as vitreoretinal disorders, Alzheimer’s disease, and hypertension [10]. Clinical trials have shown that increased intake of nattokinase helps prevent and treat hypertension [12]. However, it remains unclear whether it has an anti-tumor effect in breast cancer.

The inhibiting effects of soybeans and soy foods on carcinogenesis was attributed to sev-
Nattokinase inhibits breast cancer growth

Several compounds, such as protease inhibitors [13], phytates [14, 15], and isoflavones [16, 17]. Therefore, we suspected that nattokinase, a Bacillus subtilis soybean metabolite, may hamper the aggressive nature of breast cancer. Here, we explored the inhibitory function of nattokinase on breast cancer, in order to provide an experimental basis for clinical research and to explore a new effective anti-breast cancer drug.

Materials and methods

Preparation of supernatant through the activation of the fermentation broth of a Bacillus subtilis strain

Recovery of Bacillus subtilis strain: The Luria-Bertani (LB) slant medium used in this study included the following composition: 1 g of sodium chloride, 1.5 g of agar, 0.5 g of yeast extract, 1 g of peptone, and 100 mL of deionized water (pH 7.0). The medium was sterilized at 121°C for 20 min, then plated on a super clean bench. Bacillus subtilis was inoculated on LB slant medium and cultivated in a thermostat at 37°C for 24 h (relative humidity, 60%-70%).

Culture of Bacillus subtilis: The seed fluid medium included the following composition: 1% glucose, 0.1% yeast extract, 0.5% sodium chloride, 1% peptone, and 0.5% beef extract (pH 6.8-7.3). This medium was sterilized at 121°C for 20 min. An inoculation ring was used to select a single healthy colony. We inoculated the colony into the seed fluid medium and cultivated it in a table concentrator at 37°C for approximately 12 h at 225 rpm.

Isolation of fermented liquid: The fermentation medium included the following reagents: 5% red bean powder, 0.5% sodium chloride, 2% glucose, 0.1% potassium dihydrogen phosphate, 0.01% calcium chloride, 0.1% dipotassium hydrogen phosphate, and 0.05% magnesium sulfate. The seed fluid was inoculated in a cone of liquid medium at a dose of 0.5% and cultured at 37°C, at for 225 rpm with shock for 24 h to obtain the brewed liquid (suspension), then centrifuged the suspension at 12,000 rpm for 8 min to gain the supernatant and Bacillus subtilis mud (precipitation).

Cell culture

The murine breast carcinoma cell line 4T1 was acquired from the Institute of Biochemistry and Cell Biology in the Chinese Academy of Sciences (Shanghai, China). 4T1 breast cancer cells were cultured in a high-glucose Dulbecco’s modified Eagle’s medium (Gibco, USA), which was mixed with 10% fetal bovine serum. The 4T1 cells were cultured at 37°C in humidified air with 5% CO₂.

Animal model

4T1 cell suspension with a concentration of 10⁵/mL was injected into the second nipple fat pad on the left side of mouse at a dose of 0.1 mL with a sterile medical syringe. After a breast cancer model was successfully established, BALB/C female mice were divided into four groups at random: saline (control) (15 ml/kg), suspension (750 U/kg), supernatant (750 U/kg), and precipitation (15 ml/kg). All mice were treated orally with saline, suspension, supernatant, or precipitation once every 2 days for 1 month. Tumor growth was analyzed by evaluating the length and width of the tumor with calipers, and the tumor volume was obtained by the modified ellipsoidal pattern: (length × width²)/2.

Tumor inspection using B-mode ultrasound

Two-dimensional ultrasonic inspection: After being anesthetized with 1% pentobarbital sodium, mice were placed on their back with legs pointing upward to fully expose the tumors. Before inspection, the skin surface of the tumor was coated with a coupling agent. An inspection probe was placed on the surface of the tumor, and tumor size, shape, and presence of calcification were noted.

Ultrasonography: 15-µL contrast media was injected into the venae angularis; the distribution of contrast media near the tumor was observed, and the tumor size was assessed.

Grade of malignancy: Grade 0: No reinforcement or uniform reinforcement; grade 1: Edge of the tumor or circular enhancement; grade 2: Tumor-rim ring reinforcement and internal point or fine line like reinforcement; grade 3: Tumor-rim ring enhancement and internal stripe enhancement; grade 4: Nonuniform tumor enhancement.

Histopathological staining

Tumor tissues were treated for paraffin embedding, then 4-µm-thick sections were stained.
Nattokinase inhibits breast cancer growth

with hematoxylin-eosin and Masson trichrome in line with standard protocols.

**Immunohistochemistry**

The expression of forkhead box M1 (FOXM1) (Cell Signal Technology, USA) and matrix metalloproteinase (MMP2) (Cell Signal Technology, USA) were detected by immunohistochemistry in vivo. The horseradish peroxidase-labeled secondary antibody was supplied by Bostor. The procedure was carried out according to the immunohistochemical staining kit (Bostor, Wuhan, China). The reaction time of the horseradish peroxidase substrate was approximately 5 min. The cell and morphological tissue sections were visualized under a high-power light microscope (Nikon, Japan).

**Statistical analysis**

All data were shown as mean ± standard deviation. Statistical analysis was performed with GraphPad Prism 5.0. Comparisons between groups were analyzed using one-way analysis of variance. A P < 0.05 was considered statistically significant.

**Results**

**Supernatant from Bacillus subtilis fermentation had nattokinase activity**

To test whether our culture system enabled Bacillus subtilis to produce nattokinase, we examined nattokinase activity in the suspension, supernatant, and precipitation from the Bacillus subtilis culture media. The suspension, supernatant, and precipitation had enzyme activities of approximately 10,000, 12,500, and 0 U/mL, respectively (Figure 1). Therefore, the active ingredient of nattokinase was mainly derived from the culture supernatant of Bacillus subtilis.

**Supernatant inhibited breast cancer growth in vivo**

According to these results, the relative tumor volumes of the suspension, supernatant, and precipitation groups were compared with those of the control group. The tumors in the supernatant and suspension groups grew significantly slower than the control and precipitation groups (Figure 2A). As indicated in Figure 2B, the relative volume of the tumor in the supernatant group was the smallest, followed by the suspension group. Moreover, only the supernatant and suspension from natto fermentation with Bacillus subtilis had nattokinase activity (Figure 1). However, the relative body weight of the tumor-bearing mice did not differ significantly between the groups (Figure 2D).

**Supernatant inhibited the growth of tumor vessels**

According to the ultrasound results, the mice in all four groups had liquefaction necrosis in the tumor (Figure 3). The results of contrast-enhanced ultrasound were interpreted as follows: the more internal contrast agent entered the tumor, the higher the vascularity of the tumor. The supernatant of Bacillus subtilis impeded malignant angiogenesis, and limited the growth of malignant vascularization of tumors by inducing the growth of normal blood vessels in breast cancer (Figure 3).

**Supernatant inhibited the tumor tissue malignancy of breast cancer**

The hematoxylin-eosin staining of nuclear in breast cancer cells is deeper, there are more complicated arrangements of cells and higher malignancy of the tumor [18]. Compared with the control group, the tumors in the supernatant and suspension groups showed shallow nuclear staining, internal necrosis of tumor cells by several packages, incomplete internal necrosis, and low malignancy (Figure 4). Control
Nattokinase inhibits breast cancer growth

Figure 2. The effect of natto cultured supernatant of Bacillus subtilis on tumor growth in a murine model. A: The tumor volume in a mammary mouse model is measured and calculated once every two days while mammary tumor mice are treated with Normal saline, suspension, supernatant and precipitation for about one month; B: Representative tumors from mammary tumor mice after being treated with Normal saline (Control group), suspension, supernatant and precipitation form Bacillus subtilis cultured mixture for one month (supernatant group or supernatant group compared with control group, *P < 0.05; supernatant group or supernatant group compared with precipitation group, #P < 0.05); C: Tumor weight was evaluated in mice (*P < 0.05; **P < 0.01); D: Tumor bearing mouse body weight is detected.

and precipitation group tumor cells showed nuclear staining that was shallow, coated huge damage between the malignant degree between the control group, and high malignancy (Figure 4). The results indicated that the supernatant from Bacillus subtilis fermentation could impede the malignant transformation in breast cancer.

Supernatant reduced the expression of FOXM1 in tumor tissue

FOXM1 as an oncogenic transcription factor participated in various of cellular processes including cell cycle progression, migration, proliferation, metabolism, differentiation, and DNA damage response [19]. FOXM1-positive cells have yellow or brownish-yellow particles, and their staining is localized in the nucleus (Figure 5A). The FOXM1-positive nuclear staining rate of the suspension-group cells was approximately 33.8% of those of the control group (Figure 5A and 5B). The amount of FOXM1-positive cells was the lowest (approximately 15.5%) in supernatant-treated tumors (Figure 5A and 5B). Accumulating evidence have indicated that MMPs are associated with the prognosis of breast cancer. A lot of research has revealed the relationship between MMPs and tumor growth, metastasis, and invasion in breast cancer [20]. MMP2-positive cells, which were yellow or brownish-yellow, were mainly located in the matrix of breast cancer tissue; several were localized in the intercellular stroma (Figure 6A). Histological staining of MMP2 indicated that the expression of MMP2 was inhibited by suspension and supernatant in tumor tissues (Figure 6A); the positive cell number that expressed MMP2 was the lowest in the tumors treated with supernatant (Figure 6A).
Nattokinase inhibits breast cancer growth

Two-dimensional ultrasound          Radiography

Control group

Suspension group

Supernatant group

Precipitation group

Figure 3. The supernatant from Bacillus subtilis extract could inhibit the growth of malignant blood vessels in vivo. Representative two-dimensional ultrasound and contrast-enhanced ultrasound images of mammary tumors after treatment with Normal saline (Control group), suspension, supernatant and precipitation for one month.

6B). Thus, the initial supernatant extract of Bacillus subtilis fermentation could decrease MMP2 activity, which dominated the invasion and metastasis of breast cancer.

Discussion

Research has indicated the progress of breast cancer is related to vasculature [21, 22]. Accumulating evidence has also illustrated angiogenesis of tumors to play a critical role in tumor growth, metastasis, and progression [23]. A study indicated tumor growth remains quiescent within a 1-2 mm diameter with lack of blood vessels. They found tumor cells could produce chemical compounds that promoted tumor angiogenesis, and endothelial cells from the existing blood vessels that could migrate toward the tumors, and sprout to shape new blood vessels [24]. Therefore, neoangiogenesis plays a necessary role in tumor growth. Nattokinase has the ability to dissolve thrombus, improve blood circulation, decrease blood viscosity, and promote blood vessel elasticity [25]. However, no study has proved that nattokinase affects the regeneration of tumor blood vessels. In this study, we accurately determined the size of the tumor and the distribution of
Nattokinase inhibits breast cancer growth

Figure 4. The supernatant of Bacillus subtilis natto cultured mixture restricted the malignant degree of tumors in vivo. Representative micrographs of tumor histological images (H&E stain) at 30 days after treatment. Scale bar = 100 μm.

Figure 5. Expression of FOXM1 in breast cancer tissues of mice in each group. A: Representative immunohistochemistry images of FOXM1 expression showed further deterioration in mice tumor after treating with Normal saline, suspension, supernatant and precipitation for one month. Scale bar = 100 μm; B: The positive rate of the expression of FOXM1 in breast cancer tissue (Control group compared with suspension or supernatant group, ***P < 0.001 and **P < 0.01).

Figure 6. Expression of MMP2 in breast cancer tissues of mice in each group. A: Representative immunohistochemistry images of MMP2 expression showed further inhibition of the invasion and metastasis of breast cancer after treating with Normal saline, suspension, supernatant and precipitation for one month. Scale bar = 100 μm. B: The positive rate of the expression of MMP2 in breast cancer tissue (Control group compared with suspension or supernatant group, ***P < 0.001 and *P < 0.05).
Nattokinase inhibits breast cancer growth

tumor blood vessels by B-mode ultrasound with and without contrast. Our results showed that nattokinase, a Bacillus subtilis extract, can treat early mouse subcutaneous breast tumor by promoting normal breast cancer peripheral vascular regeneration, inhibiting tumor angiogenesis, which may effectively inhibit the occurrence or development of breast cancer.

FOXM1, as a novel biomarker, is a widely researched transcription factor which is also used as a hallmark of various of malignant cancers [26]. The expression of FOXM1 in many human cancers is increased, including breast, stomach, liver, prostate, colon, pancreas, lung, and nervous system cancers [27]. Our previous study confirmed that, as with β-catenin, FOXM1 performed important roles in promoting the proliferation of cancer stem cells (CSCs) [28], and is also associated with the self-renewal of pancreatic cancer stem cells [29]. Thus, FOXM1 plays an indispensable role in the formation and spread of CSC. CSCs, as tumor-initiating cells, have the abilities of self-renewal, which enhance tumor aggressiveness, metastasis, drug resistance, and recurrence [30]. In the present study, Bacillus subtilis suspension and supernatant, which have nattokinase activity, inhibited the expression of FOXM1, thereby indicating that nattokinase may regulate CSC formation.

MMPs is a multigene family comprised over 23 secreted and membrane related enzymes which are responsible for cleaving or degrading various pericellular substrates [31]. MMP2 is an important target of tumor regulation [32, 33]. MMP2 not only promoted tumor invasiveness by degrading the extracellular matrix, but also increased tumor growth by regulating vessel maturation and function [34]. In our study, bacterial suspension and supernatant, which have nattokinase activity, reduced MMP2 expression, suggesting that nattokinase regulates tumor angiogenesis and metastasis.

In conclusion, the extract of Bacillus subtilis fermentation can prevent breast cancer development and inhibit the expressions of FOXM1 and MMP2 in the tumor tissue. Our findings may provide new strategies for the treatment of breast cancer in future.

Acknowledgements

National Natural Science Foundation of China (81702439, 81802446), the Scientific Research Foundation for PhDs, the affiliated hospital of Jining Medical University (Nos. 2018-BS-001, 2018-BS-013), Shandong Provincial Natural Science Foundation (No. ZR201807-080357) and Tai Shan Young Scholar Foundation of Shandong Province. This study was approved by the Ethics Committee of Jiangsu University (2012258).

Disclosure of conflict of interest
None.

Address correspondence to: Aihua Gong, Key Laboratory of Laboratory Medicine of Jiangsu Province, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, Jiangsu, P. R. China. Tel: +86-511-85038334; Fax: +86-511-85038483; E-mail: ahg5@ujs.edu.cn; Bin Zhang, Department of Laboratory Medicine, Affiliated Hospital of Jining Medical University, 89 Guhuai Road, Jining 272000, Shandong, P. R. China. Tel: +86-0537-3616505; Fax: +86-0537-2903223; E-mail: zhb861109@163.com

References


[8] Rodgers L, Peer CJ and Figg WD. Diagnosis, staging, and risk stratification in prostate cancer: utilizing diagnostic tools to avoid unneces-
Nattokinase inhibits breast cancer growth


