Human malignant melanoma is an aggressive skin cancer which has been rising at a greater rate than any other cancers. Although various new therapeutic methods have been developed in previous studies, this disease has properties of high proliferation and metastasis rate which remain obstacles that have lead to a poor prognosis in patients. It has been reported that a specific *Lactobacillus* extract has anti-cancer and metastasis effect in vitro and in vivo. However, previous research has not specified precisely what effect the *Lactobacillus rhamnosus* GG (LGG) extract has had on human malignant melanomas. In this study, we showed that the LGG extract has anti-cancer and metastasis effects on the human malignant melanoma cell lines, A375P and A375SM. At first, it was found that, while the LGG extract affects human neonatal dermal fibroblasts slightly, it induced the dose-dependent anti-cancer effect on A375P and A375SM by a WST-1 proliferation assay. As a result of a real-time PCR analysis, the expression patterns of several genes related to cell cycle, proliferation, and apoptosis were modulating in a manner that inhibited the growth of both malignant melanoma cell lines after the treatment of the LGG extract. Furthermore, genes related to the epithelial-mesenchymal transition were down-regulated, and migration rates were also decreased significantly by the LGG extract. Our study showed that the LGG extract could be used as a potential therapeutic source.

Key words: *Lactobacillus*, Human malignant melanoma, Anti-cancer, Epithelial-mesenchymal transition

Introduction

Malignant melanoma is a growing public health issue because its incidence has grown at a higher rate than other cancers [1-3]. Risk of having invasive melanoma has increased more than 30-fold since 1930s in America [4]. Although melanoma takes a minority place (<10%) of all skin cancers in humans, it has a high mortality rate (>75%) because of its high metastasis and invasion capabilities [5]. Melanoma has various types according to where it develops. Malignant melanoma of oral cavity is an aggressive tumor derived from the mucosal basal layer, which accounts for between 0.2% and 8% of all types of melanoma [6-8]. While the cutaneous melanoma has a relatively well-defined clinical classification, the melanoma of oral cavity does not [9]. The conventional treatment method includes surgery, chemotherapy, and radiotherapy. However, if melanoma has a high metastasis capability spread to other...
organs, it is rarely cured and shows extremely poor prognosis [10]. For these reasons, novel therapeutic methods are urgently required.

Probiotics, which are live microorganisms such as *Lactobacillus* and *Bifidobacteria* derived from foods, are used by humans for their various abilities. For example, the several strains of *Lactobacillus* could enhance immunity by stimulating lymphocyte proliferation [11] and inhibit the inflammation by reducing the cytokine production [12].

Regular consumption of probiotics is known to affect the gut microbiota, which consequently enhances immunity [13]. Several *Lactobacillus* extracts have been reported to prevent gastric and colon cancer by inhibiting mTOR-mediated signaling [14] and ROS-JNK signaling [15], respectively. *Lactobacillus casei* has anti-metastatic effect on a B16 mice melanoma known for its high metastasis capability [16]. In addition, *Lactobacillus rhamnosus* has also been reported to induce the epithelial cell apoptosis and prevent the development of colon cancer [17].

However, there are no preceding studies of the anti-cancer effect of the *Lactobacillus* extract on human melanoma. Therefore, the anti-cancer effect of *Lactobacillus rhamnosus GG* (LGG) extract on two types of melanoma cell lines, A375P (low metastatic) and A375SM (high metastatic) were investigated. To exclude the possibility of the cytotoxicity on non-neoplastic cells, human dermal fibroblasts, neonatal (HDFn) were used [18].

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**Material and Methods**

**Preparation of LGG extract**

LGG obtained from NeoRegen Biotech (Gyeonggi-do, Korea) was cultured in a de Man, Rogosa and Sharp (MRS) broth containing 2% ammonium citrate dibasic, 1% pancreatic digest of gelatin, 0.5% sodium acetate-trihydrate, 0.8% beef extract, 0.1% polysorbate 80, 0.005% MnSO₄-dihydrate, 2% dextrose, 2% KH₂PO₄ at 37°C for 18 h for pre-cultivation. Then, it was 1%-inoculated for main-cultivation in 500 mL MRS broth and cultured at 37°C for 18 h. The cultured LGG was harvested by centrifuge at 10,000 g for 10 min at 4°C and washed three times with distilled water to remove the MRS broth. The LGG resuspended in 20 mL distilled water was sonicated on ice for 30 min by using the sonicator to break the cell wall of bacteria and make the extract homogeneous. To discard the cell wall ingredients and other residues, it was centrifuged at 10,000 g for 20 min at 4°C. The supernatants was filtered (0.45 μm) and frozen at –80°C overnight. It was then freeze-dried and reconstituted with phosphate buffered saline (PBS) before use.

**Cell culture**

HDFn was provided from the American Type Culture Collection (ATCC). A375P, and A375SM were purchased from the Korean Cell line bank (KCLB; Seoul, Korea). HDFn and both melanoma cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; WELGENE, Daegu, Korea) containing 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin; 3.7 g/L sodium bicarbonate, 4 mM L-glutamine, and 1 mM sodium pyruvate. These cells were cultured at 37°C in an incubator containing a humidified atmosphere of 5% CO₂. HDFn, A375P, and A375SM were seeded at a density of 1.0×10⁴ cells per well in 12-well plates in the medium as described above. After 24 h, the medium was replaced with the LGG extract at various concentrations. These cells were maintained for 3 days and observed by an EVOS CL Core microscope (Life technologies, CA, USA) at ×40 magnification.

**Cell viability assay**

HDFn, A375P, and A375SM were also seeded at a density of 1.0×10³ cells per well in 96-well plates. After 1 day, the medium was replaced with the LGG extract at various concentrations and maintained. The cell viability effects of the extract were confirmed by a WST-1 cell viability assay kit (Dongin LS, Seoul, Korea).

**Real-time PCR analysis**

mRNA was isolated from A375P and A375SM, cultured with the LGG extract for 3 days using a PureLink™ RNAminikit (Invitrogen, CA, USA) and reverse-transcribed into cDNA using cDNA kit (Miotech, Seoul, Korea). Then, cDNA was amplified by a SYBR green mix (TAKARA, Shinga, Japan) using T100™ Thermalcycler (BIO-RAD, CA, USA) and analyzed. Primers used for real-time PCR were designed as the listed sequences (Table 1).

**Fluorescence activated cell sorter (FACS)**

A375P and A375SM were seeded at a density of 5.0×10⁴ cells per well in 6-well plates in the medium as described above.
Table 1. Primer sequences used for Real-time PCR

| Genes   | 5' -> 3' Sequences                  |
|---------|------------------------------------|
| c-myc   | Forward ACT CTG AGG AGG AAC AAG AA  |
|         | Reverse TGG AGA CGT GGC ACC TCT T  |
| Mki67   | Forward AGT TTG CGT GGC CTG TAC TAA|
|         | Reverse AGA AGA AGT GGT GCT TCG GAA|
| Gadd45a | Forward GAG AGC AGA AGA CCG AAA GGA|
|         | Reverse CAG TGA TCG TGC GCT GAC T  |
| p21     | Forward TGT CCG TCA GAA CCC ATG C  |
|         | Reverse AAA GTC GAA GTT CCA TCG CTC|
| Vimentin| Forward GAG AAC TTT GCC GTT GAA GC |
|         | Reverse GCT TCC TGT AGG TGG CAA TC |
| N-cadherin | Forward ACA GTG GCC ACC TAC AAA GG |
|         | Reverse CCG AGA TGG GGT TGA TAA TG |

After 1 day, the medium was replaced with 50 and 100 µg/mL of the LGG extract. Three days after the treatment of the extract, the both cell lines were harvested and stained with Annexin V (Cayman Chemical, MI, USA) and 7-Aminoactinomycin D (7-AAD; Life technologies) dyes. These stained cells were analyzed using FACSVerse™ (BD Biosciences, CA, USA).

Cell migration assay

Migration of A375P and A375SM were assessed by wound-healing assay. Both cell lines were seeded at a density of 1.5×10⁵ cells per well in 12-well plates in the medium as described above until they reached 80% confluence. The monolayers of both melanoma cells were scratched by the pipette tip, and the medium was replaced with 50 and 100 µg/mL of the LGG extract. The cell migration capabilities were analyzed using the microscope at ×100 magnification.

Statistics

Data is presented with mean ± standard deviation (S.D). Statistical analysis was determined using ANOVA. Significance was defined as *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

LGG extract affects A375P and A375SM on morphology and growth

To investigate how the extract affects HDFn, A375P, and A375SM, these cell lines were seeded in 12-well plates. After 1 day, the medium was replaced and treated with the various range of 50, 75 and 100 µg/mL of the LGG extract. After 3 days, the morphologies of HDFn, A375P, and A375SM were observed and photographed by microscope. The LGG extract had anti-cancer effects on both melanoma cell lines in a dose-dependent manner (Fig. 1).

LGG extract inhibits cell viabilities of A375P and A375SM significantly

For quantitative analysis, HDFn, A375P, and A375SM were seeded in 96-well plates. In our previous study, ED50 value of HDFn was 250-300 µg/mL (data not shown). After 1 day, thus, the medium was replaced with the various concentration ranges of 50, 75, 100, 125 and 150 µg/mL of the LGG extract, which were under the ED50 value of HDFn. The viabilities over time were determined by the WST-1 cell viability assay. The cell viability rates of melanoma cell lines cultured with the extract were significantly decreased in a dose-dependent manner compared to control group (Fig. 2A and B), while HDFn was less influenced by the extract (Fig. 2C). Interestingly, although the A375SM is known for having more aggressive and metastatic properties, it was more susceptible to the LGG extract than A375P (Fig. 1 and 2). When A375P and A375SM were treated with 50 and 100 µg/mL of the LGG extract for 72 h, the proliferation of A375SM was inhibited by 52% and
80% and that of A375P was inhibited by 22% and 61%, respectively. On the other hand, the HDFn was less vulnerable to the LGG extract. To investigate the anti-cancer effect of the LGG extract as time passed, the viabilities at the specific concentrations of the LGG extract were determined each day (Fig. 2E and F). The results showed that the viabilities rates of A375P and A375SM over time were decreased significantly compared to the Control groups at the 100 µg/mL of the LGG extract, while HDFn maintained the increasing trend. LGG extract regulates gene expressions related with cell cycle, proliferation, and apoptosis

To investigate how the LGG extract affects A375P and A375SM, real-time PCR was performed. The mRNAs isolated from A375P and A375SM cultured with the LGG extract for 3 days were reverse-transcribed to cDNA and the mRNA levels were analyzed. The expressions of c-Myc, which was known as a cancer-associated gene and proliferation marker gene, was significantly decreased in both A375P and A375SM in a dose-dependent manner (Fig. 3) Although the expression of proliferation marker gene, Mki67 was down-regulated by the LGG extract, there was the significant difference only in the A375SM. Furthermore, the expressions of Gadd45a known as cell cycle arrest marker and cyclin-dependent kinase inhibitor, p21 were increased greatly in A375SM. To investigate whether the LGG extract induced the anti-cancer effect through apoptosis, the FACS was performed. The staining dye, Annexin V was used to detect cells which have been in process of apoptosis and as a result, express phosphatidylserine on the cell surfaces. The data showed that the control groups were almost not stained for both Annexin V and 7-AAD (Fig. 4). As the concentration of the LGG went up, the cells with higher Annexin V staining intensity were observed, which were considered apoptosis in accordance with real-time PCR data. However, the LGG extract also induced necrosis, especially about the A375P. These results showed the LGG extract regulated the gene expressions related with cell cycle and consequently, induced cell cycle arrest, apoptosis, and necrosis.

LGG extract inhibit cell migration by reducing epithelial–mesenchymal transition

To determine the effect of the LGG extract on epithelial-
Anti-cancer and -metastatic effects of *Lactobacillus rhamnosus* GG extract on human malignant melanoma cells, A375P and A375SM

**Figure 3.** Gene regulation effects of LGG extract on A375P and A375SM. (A-B) The mRNAs isolated from A375P and A375SM cultured with the LGG extract for 3 days were reverse-transcribed to cDNA and the expressions of several genes related with cell cycle, proliferation, and apoptosis levels were analyzed. Results are the means of three independent experiments (mean + S.D). *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control group.

**Figure 4.** Apoptosis and necrosis induced by LGG extract. A375P and A375SM cultured with the LGG extract for 3 days were harvested. These cells were stained with Annexin V and 7-AAD, respectively, and analyzed by FACS. Control groups of A375P (A) and A375SM (B) were rarely stained for both Annexin V and 7-AAD. However, the higher concentration the LGG extract was treated at, the more apoptosis was induced in both cell lines.
Figure 5. Down-regulation of epithelial-mesenchymal transition (EMT) markers by LGG extract. (A-B) The mRNAs isolated from A375P and A375SM cultured with the LGG extract for 3 days were reverse-transcribed to cDNA and the expressions of EMT marker genes related with cell cycle, proliferation, and apoptosis levels were analyzed. Results are the means of three independent experiments (mean ± S.D). *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control group.

Figure 6. Anti-migration effect of the LGG extract on A375P and A375SM. The migration capabilities of A375P and A375SM were assessed by wound-healing assay. When the cells reached to 80% confluence, the monolayers of both melanoma cells were scratched by the pipette tip and the medium was replaced with 50 µg/mL and 100 µg/mL of the LGG extract. The cell migration capabilities were analyzed using the microscope at ×100 magnification. At 72 h post-wounding, almost full migrations of the A375P (A) and A375SM (B) cells were observed in the control group, while the extract-treated groups show delayed migration capabilities in a dose-dependent manner, scale bar = 200 µm.
mesenchymal transition (EMT) of melanomas, the mRNA expression levels of Vimentin and N-cadherin, which are known as EMT marker, were quantified using real-time PCR. The expression level of both genes were significantly decreased in the A375P and A375SM (Fig. 5) and it suggested that the extract made the migration and invasion capabilities of both melanoma cell lines down-regulated. To confirm this hypothesis, the migration assay was performed. Both serum-starved cells were used as negative control, inducing the lack of cell proliferation and cell migration, while the positive control and experiment groups were cultured with 10% FBS and the extract. At 72 h post-wounding, almost full migrations of the A375P and A375SM were observed in the control group, while the extract-treated groups showed delayed migration capabilities in a dose-dependent manner (Fig. 6). These results supported that the LGG extract could suppress the EMT by regulating genes.

Discussion

Malignant melanoma is pointed out as the most aggressive cancer and having extremely unfavorable prognosis according to the Centers for Disease Control and Prevention [19], and its incidence is also consistently growing [20]. The fact remains that out of the various skin cancers, malignant melanoma remains the highest cause of death [21,22]. Although various therapeutic methods have been developed so far, its high metastasis capabilities and frequency of intrinsic and obtained resistance to therapies in patients imply the serious need for new therapeutic methods [23].

*Lactobacillus* has been used as probiotics due to the several enzymes and metabolites it produces [14]. It also has anti-pathogenic bacteria effects, which could decrease the incidence of gastrointestinal diseases [24]. Recently, several strains of *Lactobacillus* have been reported to have anti-cancer activities, affecting cancer cell growth and metastasis directly or indirectly [15]. *Lactobacillus* has low or no pathogenic activities, and therefore it is regarded as a potential therapeutic source [25].

Our study showed that the LGG extract induced the dose-dependent anti-cancer effect on human melanoma cells, A375P and A375SM. Both cell lines are generally known for their aggressive growth and high metastasis capabilities. On the other hand, the HDFn was less vulnerable to the LGG extract. These results showed that the LGG extract could be used to cancer therapy with lesser side-effects. However, in high-dose treatment of the LGG extract, the proliferation of the HDFn also decreased, which might result from the inflammation responses induced by LGG. The several fractions with different molecular weight were separated from the LGG extract, and it was reported that the use of the fraction with specific molecular weight induced less inflammation significantly [26]. It was reported that the probiotics could improve the skin natural defense and benefit immune responses [27]. Thus, the LGG extract could be used as a new therapeutic method such as an ointment for the malignant melanoma.

To understand the mechanisms underlying the effect of the LGG extract, several genes related to cell cycles were evaluated by real-time PCR. The data showed the expressions of c-Myc and Mki67 were down-regulated in a dose-dependent manner. The oncogene, c-Myc is the master regulator, which is related to many aspects of the cell growth process [28] and the MKi-67, also known as Ki-67, represents a mitotic index, which could be used for tumor grading due to being observed in proliferating cells [29,30]. Moreover, the expressions of Gadd45a and p21 were up-regulated in a dose-dependent manner. The Gadd45a and p21 were related in negatively regulating the cell cycle and inducing cell cycle arrest [31]. These changes of gene expressions suggested that LGG extract could induce apoptosis and, consequently, down-regulate the growth of the malignant melanoma cell lines. Our Annexin V and 7-AAD double staining assay using the FACS supported the fact that the apoptosis was induced by the LGG extract by regulating the gene expressions. However, the LGG extract also induced necrosis as well as apoptosis. In our previous study, it was elucidated that the effect molecules were not proteins by using the autoclaved and boiled LGG extract, but it still remained unclear what exactly the effect molecules were. Therefore, further studies are required to investigate the effect molecule to maximize the anti-cancer effect of the LGG extract and reduce the unexpected side-effect [26].

One of the reasons why malignant melanomas have the worst prognosis is due to its aggressive metastasis capability, and therefore, the development of therapies to inhibit the metastasis of malignant melanomas is urgently required. It was reported that the extract of *Lactobacillus casei* had the anti-metastatic capability [16,32,33]. Our gene expression analysis and cell migration assay showed that the EMT and cell migration were significantly decreased by the LGG extract [34,35], which
suggested that the LGG extract could be used as the agent to inhibit the metastasis in vivo.

Surprisingly, A375SM was more susceptible to the LGG extract than A375P overall, although A375SM had the higher proliferation and metastasis rate than A375P. There are several differences of gene expression patterns between the A375P and A375SM, and these differences would be the key factors for the anti-cancer mechanism of the LGG extract. For example, the chemokine CXCL-8, also known as Interleukin-8, is the key regulator of human malignant melanomas and regulates the proliferation, metastasis and angiogenesis by autocrine and paracrine. The gene expression pattern of CXCL-8 is totally different between A375P and A375SM and as a result, it makes different properties on the proliferation and metastasis [36]. The capabilities of CXCL-8 on human malignant melanomas are the results of the interaction with the CXCL-8 receptors, CXCR-1 and CXCR-2. The CXCR-1 and CXCR-2 are up-regulated in A375SM and it also corresponds with the higher proliferation and metastasis rate of A375SM than A375P [37]. Therefore, the mRNA and the protein levels of CXCL-1, CXCR-1, and CXCR-2 should be evaluated in further studies. In conclusion, our study showed the anti-cancer effect of the LGG extract on human malignant melanoma cells by modulating the several genes related to cell cycle, proliferation, and apoptosis. EMT-related genes were also decreased after the treatment of the LGG extract significantly. Thus, this data suggested that the LGG extract could be used as a new therapeutic method with lesser side-effects. To investigate its anti-cancer and -metastasis effects in vivo and accurate mechanism, further studies will be required.

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Conflict of interest

The authors declare no conflict of interest.

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