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Inhibition of Proliferation by Anti-microbial Peptide Isolated from *Pediococcus pentosaceus* and *Lactobacillus* spp. in Colon Cancer Cell Line (HT-29, SW 480 and Caco-2)

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Pediococcus pentosaceus 및 *Lactobacillus* spp. 종의 유산균으로부터 분리한 항균 peptide들 (Safelac and Lactopad)이 인간 결장암 세포주 (HT-29, SW 480 and Caco-2)의 증식 억제에 미치는 효과

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요 약

유산균(Lactic acid bacteria)은 *Escherichia coli*와 *Salmonella typhimurium*과 같은 병원균에 대한 항균활성을 나타낼 뿐만 아니라 면역 증강효과를 나타내는 등 인체내에서 건강에 이로운 다양한 역할을 수행하는 것으로 알려졌다. 특히, *Pediococcus pentosaseus*와 몇몇 *Lactobacillus* 종으로부터 분리한 항균활성을 나타내는 peptide들인 safelac과 lactopad는 몇몇 암세포주의 성장을 억제하는 것으로 나타났다. 이에, 본 연구에서는 HT-29, SW 480 및 Caco-2와 같은 3종류의 인간의 결장암 세포주에 safelac과 lactopad를 투여하여 이들이 항암효과를 나타낼 수 있는 지를 분석하고자 하였다. XTT assay는 safelac과 lactopad가 HT-29, SW 480 및 Caco-2의 성장을 억제하는 것으로 나타났으며, 특히, 이들 peptide들을 72시간동안 처리했을 때 나타나는 항암효과는 3.1~100 mg/mL의 농도범위에서 유의한 결과를 나타내었으며, 분석한 농도 범위에서 용량 의존적인 방식으로 더 강한 효과를 나타내었다. RAW 264.7 세포주는 cytokine인 tumor-necrosis factor (TNF-α)의 생성에 미치는 이들 peptide들의 효과를 조사하기 위한 대식세포의 모델로써 이용되었다. RAW 264.7 세포주에서 TNF-α의 생성은 이들 peptide들에 의해 48시간 배양시 용량에 의존적인 방식으로 영향을 받는 것으로 나타났다. 따라서, 이러한 발견은 safelac과 lactopad와 같은 유산균으로부터 분리한 항균 peptide들이 결장암 세포에 대한 화학적 예방제로서의 잠재성을 갖고 있음을 시사하는 결과로서 주목된다.

Key words : Lactic acid bacteria, Safelac, Lactopad, HT-29, SW 480, Caco-2

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INTRODUCTION

Live microbial feed supplements added to foods in order to beneficially affect the consumers are known as probiotic (Fuller, 1989). The term probiotics, usually refers to highly selected lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Bifidobacterim* spp. and *Streptococcus* spp. with defined gut survival properties and associated biological activities, which can be ingested in fermented milk products or as a supplement (Salminen *et al.*, 1998). LAB are the most common probiotic microorganisms used to exert a given biological function in the host. Several studies have reported the beneficial effects of the consumption of LAB or LAB-fermented products on intestinal health (Gibson *et al.*, 2003).

There is experimental evidence that probiotic microorganisms show an anticancer activity *in vitro* and in animal models. Pool-Zobel *et al.* (1996) reported that *Lactobacillus aciophilus, L. gasseri, L. confusus, Streptococcus thermophilus, Bifidobacterium breve*, and *B. longum* were antigenotoxic toward N′-nitro-N-nitrosoguanidine- or 1, 2-dimethylhydrazine-induced genotoxicities. Other studies have shown that certain strains of LAB prevent putative preneoplastic lesions or tumors induced by carcinogens such as 2-dimethylgydrazine or azoxymethane (Abdelali *et al.*, 1995; Gallaher *et al.*, 1996; Arimochi *et al.*, 1997; Onoue *et al.*, 1997). Many strains such as *L. rhamnosus* GG (Goldin *et al.*, 1996), *L. acidophilus* (Goldin and Gorbach, 1980), *L. casei, B. longum* (Singh *et al.*, 1997; Rowland *et al.*, 1998) *B. infantis, B. adolescentis*, and *B. breve* showed significant suppression of colon tumor incidence in this type of study. In addition, there is direct evidence for antitumor activities of LAB obtained in studies using preimplanted tumor cells in animal models. There are several reports (Kohwi *et al.*, 1978; Kato *et al.*, 1981) that the consumption of fermented milk and/or cultures containing LAB or the intralesional injection of live or dead *Bifidobacterium* cells inhibited the growth of tumor cells injected into mice.

Various types of LAB preparations showed antitumor activities. Sekine *et al.* (1995) found antitumor activity in peptidoglycans isolated from *B. infantis* strains ATCC 15697, and Oda *et al.* (1983) reported antitumor polysaccharide fractions originating from *Lactobacillus* cultures.

The precise mechanisms by which LAB inhibit colon cancer are presently unknown. However, based on experimental and epidemiological studies (Shahani and Ayebo, 1980; Maclennan and Jensen, 1997; Malhotra, 1997), several mechanisms have been proposed, including (1) enhancing the host's immune response, (2) binding and degrading potential carcinogens, (3) qualitative alterations in the intestinal microflora incriminated in producing putative carcinogens and promoter (e.g., bile-acid-degrading bacteria), (4) production of antitumorigenic or antimutagenic compounds in the colon, and (5) alteration of metabolic activities of intestinal microflora (Hirayama and Rafter, 2000; You *et al.*, 2004).

One explanation for tumor suppression by LAB may be mediated through an immune response of the host. In addition, there are studies to suggest that LAB play an important role and function in the host's immunoprotective system by increasing specific and nonspecific mechanisms to have an antitumor effect (Kato *et al.*, 1983; de Simone *et al.*, 1993; Schiffrin *et al.*, 1995). *Lactobaillus casei* Shirota (LcS) has been shown to have potent antitumor and antimetastatic effects on trasplantable tumor and antimetastatic effects on transplantable tumor cells and to suppress chemically induced carcinogenesis in rodents. Also, intrapleural administration of LcS into tumor-bearing mice has been shown to induce the production of several cytokines, such as IFN-γ, IL-1 and TNF-α, in the thoracic cavity of mice, resulting in the inhibition of tumor growth and increased survival (Matsuzaki, 1998).

The present study investigated the effect of safelac and lactopad which are antimicrobial peptide isolated from *pediococcus pentoseseus* and *lactobacillus* spp. of LAB on human colon cancer cell line.

Table 1. The characteristics of cell lines used in this study (KCLB , Korean Cell Line Bank)

Cell lines	Tissue	Species	Growth property	KCLB(ATCC)no
HT-29	Colon, adenocarcinoma	Human	Adherent	KCLB 30038
SW480	Colon, adenocarcinoma	Human	Adherent	KCLB 10228
Caco-2	Colon, adenocarcinoma	Human	Adherent	KCLB 3003

Table 2. Anti-microbial peptides

Product name	Strains	Specification	Main target bacteria/use
Lactopad	*Lactobacillus*	Anti-microbial peptides	*P. acnes*. Health food supplements, Cosmetic
Safelac	*Pediococcus*	Anti-microbial peptides	*H. pylori*. health food supplements

MATERIALS AND METHODS

1. Cell cultures

This study used three human colon cancer cell lines (Table 1): HT-29, SW 480 and Caco-2 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). They were grown in RPMI culture media supplemented with 10% fetal bovine serum (FBS), and 1% (v/v) penicillin (10,000 U/mL)/streptomycin (10,000 U/mL) (P/S). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. After they were grown to confluence in 75 cm^2 tissue culture flask (nunk, Denmark), cells were detached and transferred to in new cell culture dishes using trypsin -versene mixture (Cambrex Bio Science, USA) for each experiment. Cell number and viability were assessed by trypan blue dye exclusion (Strober *et al.*, 1991) on a Neubauer hemacytometer (American Optical, Buffalo, NY, USA).

2. Preparation of antimicrobial peptide

Pediococcus pentosaseus and *lactobacillus* spp. belong to LAB (Yu *et al.*, 2003). Safelac and lactopad were isolated from *pediococcus pentosaseus* and *lactobacillus* spp., respectively, and they are all antimicrobial peptides (Table 2). We obtained these antimicrobial peptides from Cellbiotech Co. Ltd., Korea.

3. Cell proliferation by XTT assay

Cell proliferation was quantified using XTT assay (sodium 3-[1-(phenylaminocarbonyl)-3, 4-tetrazolium] -bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate). Cell seeded on 96-well microplates at 3,000 cells/well were incubated with the test compounds for 72 hr, respectively. Then incubated with 50 μL of XTT solution (1 mg/mL) for 8 h and was measured on ELISA reader at 490 nm.

4. TNF-α production

LPS, cells only (1×10^5 cells/mL), safelac and lactopad of 3.1, 6.25, 12.5, 25, 50, 100 mg/mL) were prepared as the treated groups and incubated for 2 days. After incubation, TNF-α quantification was performed using TNF-α immunoassay kit (BioSource International. Inc., Camarillo). Briefly, 1×10^5 cells/mL RAW 264.7, the cells were treated with each safelac and lactopad at the concentration of 3.1, 6.25, 12.5, 25, 50, 100 mg/mL in 5.5% CO_2 humidified air for 2 days at 37°C.

After incubation, 100 μL of culture fluid and standard solution were added to each well, respectively. After then, add 50 μL of biotin conjugate and incubate for 90 minutes at RT and aspirate and wash four times. Add 100 μL of streptavidin-HRP working solution and incubate for 30 minutes at RT and aspirate and wash four times. And then, add 100 μL of stabilized chromogen and incubate for 30 minutes at RT. Add 100 μL stop solution and used ELISA reader at

450 nm.

5. Statistical analysis

The effects of safelac and lactopad on the growth inhibition of colon cancer cell line and cytokine production of RAW 264.7 cell were analyzed by unpaired t-test.

RESULTS AND DISCUSSION

1. The effects of safelac and lactopad on the growth inhibition of HT-29, SW480 and Caco-2 Cells

Colon cancer is a serious health problem in most developed countries and the third leading cause of cancer mortality throughout the world (Pisani *et al.*, 1993). LAB or a soluble compound produced by the bacteria may interact directly with tumor cells in culture and inhibit their growth (Reddy *et al.*, 1973; Reddy *et al.*, 1983). This study examined the effect of safelac and lactopad on the growth of three types of human colorectal adenocarcinoma cell lines using a XTT assay.

Fig. 1 shows the growth inhibition caused by safelac and lactopad in HT-29. Safelac and lactopad tend to decrease the number of HT-29 cells even at a concentration of 3.1 mg/mL. Safelac at 6.25 mg/mL that low concentration inhibited the growth of HT-29 cell by 11.9% and lactopad at 3.1 mg/mL inhibited the growth of HT-29 cell by 20.3%. Effect of growth inhibition of HT-29 cell was increased in dose-dependent manner (with increasing concentration from 3.1 to 100 mg/mL). HT-29 cells were noticeably inhibited at concentrations up to 100 mg/mL by safelac and lactopad to 72.83% and 83.25%, respectively.

As shown in Fig. 2, the addition of safelac and lactopad reduced SW480 viable cell number in a dose -dependent manner (with increasing concentration from 3.1 to 100 mg/mL). Compared with SW480 cells treated with nothing, incubation with 100 mg/mL safelac and lactopad for 72 h induced 91% and 86.85

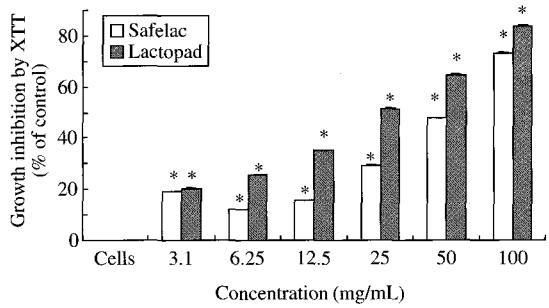

Fig. 1. Effect of growth inhibition of HT-29 cell line by anti-microbial peptide (safelac, Lactopad). The cells were treated with anti-microbial peptide (safelac-3.1, 6.25, 12.5, 25, 50, 100 mg/mL, Lactopad-3.1, 6.25, 12.5, 25, 50, 100 mg/mL) and incubated for 48 hr at 37°C and 5.5% CO_2. After adding 50 μL of the XTT labeling mixture. They ere then incubated for 6 hours at 37°C in 5.5% CO_2. The absorbance was measured using an ELISA reader at 490 nm.

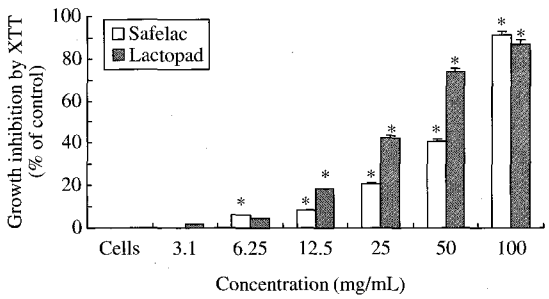

Fig. 2. Effect of growth inhibition of SW 480 cell line by anti-microbial peptide (safelac, Lactopad). The cells were treated with anti-microbial peptide (safelac-3.1, 6.25, 12.5, 25, 50, 100 mg/mL, Lactopad-3.1, 6.25, 12.5, 25, 50, 100 mg/mL) and incubated for 48 hr at 37°C and 5.5% CO_2. After adding 50 μL of the XTT labeling mixture. They ere then incubated for 6 hr at 37°C in 5.5% CO_2. The absorbance was measured using an ELISA reader at 490 nm.

% reductions in cell number, respectively.

Treatments of safelac and lactopad indicated the significant effect of growth inhibition of SW480 cells even at a concentration of 50 mg/mL. Caco-2 cells were not noticeably inhibited at concentration to 6.25 mg/mL. But safelac at 50 mg/mL concentration inhibited the growth of Caco-2 cells by about 30%, and

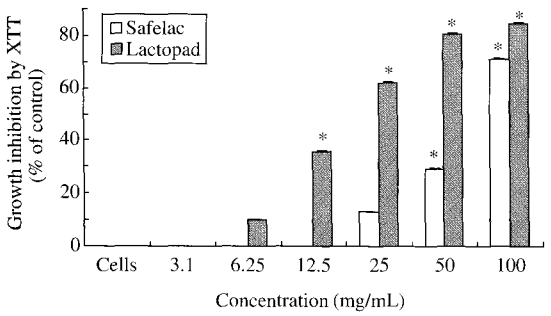

Fig. 3. Effect of growth inhibition of Caco-2 cell line by anti-microbial peptide (safelac, Lactopad). The cells were treated with Lactic acid bacteria product (safelac-3.1, 6.25, 12.5, 25, 50, 100 mg/mL, Lactopad-3.1, 6.25, 12.5, 25, 50, 100 mg/mL) and incubated for 48 hr at 37°C and 5.5% CO_2. After adding 50 μL of the XTT labeling mixture. They ere then incubated for 6hours at 37°C in 5.5% CO_2. The absorbance was measured using an ELISA reader at 490 nm.

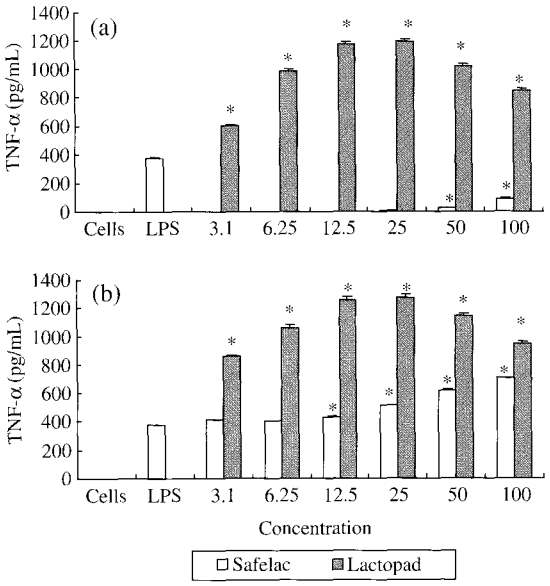

Fig. 4. Effect of anti-microbial peptide (safelac and lactopad). TNF-α production in the LPS (lipopolysaccharide)-stimulatd RAW 264.7 cells. (a) without LPS *$p < 0.05$, compared with cells, (b) with LPS *$p < 0.05$, compared with LPS.

at 100 mg/mL inhibited the growth of Caco-2 cells by 70.81% (Fig. 3). Lactopad significantly inhibited the growth of Caco-2 cells from 12.5 mg/mL concentration. Lactopad was inhibited at 100 mg/mL concentration by 84.25%. Data showed that the induction of antiproliferative effect by safelac and lactopad treatment for 72 hr was significant only at high concentrations ranging from 50 to 100 mg/mL in a dose-dependent manner within the tested concentration range.

2. TNF-α production

The potential mechanisms of probiotic-induced immune suppression of carcinogenesis are complex. An inflammatory immune response produces cytokine-activated monocytes and macrophage, which release cytotoxic molecule capable of lysing tumor cells *in vitro* (Philip *et al.*, 1986). The inflammatory cytokine interleukin-1 (IL-1) and tumor necrosis factor-α (NF-α) exert cytotoxic and cytostatic effects on neoplastic cells *in vitro* models (Onazaki *et al.*, 1985; Raitano and Korc, 1993).

In the present study, exposure of RAW 264.7 cell line to safelac and lactopad isolates resulted in marked increase of TNF-production

To assess the effects of safelac and lactopad on TNF-α production by macrophages, RAW 264.7 cells were incubated with a range of bacteria concentrations in the absence or the presence of LPS, and cytokine secretion in culture supernatant was monitored by ELISA.

The patterns observed for safelac and lactopad on TNF-α stimulation are displayed in Fig. 4. In non-LPS and LPS treated cells, exposure to antimicrobial peptide (safelad and lactopad) affected TNF-α production in dose-dependent manner. When co-stimulated with LPS (10 ng/mL) and low concentration of safelac and lactopad, TNF-α levels were higher than LPS (10 ng/mL) alone, and this aspact is more prominent in the treatment of safelac than lactopad. When co-stimulated with LPS (10 ng/mL), the production of TNF-α increased to a much greater lever than either LPS or antimicrobial pepetide alone.

Especially, production of TNF-α by lactopad was

significantly increased. Production of TNF-α by lactopad (596.40 pg/mL) compared to the control group of LPS-treated (10 ng/mL) cultures (376.75 pg/mL) at a concentration of 3.1 mg/mL was increased in a dose dependent manner up to 25 mg/mL of lactopad which gave 1,191.818 pg/mL of TNF-α.

In conclusion, many health- promoting effects are attributed to the LAB, some of these effects having more scientific support than the anticancer effect. Anticancer effect was measured in growth inhibitory effect against HT-29, SW480 and Caco-2 cell of antimicrobial peptide (safelac and lactopad) isolated from *Pediococcus pentosaseus* and *Lactobacillus* spp. which are LAB. The ability to show similar activity against HT-29, SW480 and Caco-2 were looked for. Safelac and lactopad at 100 mg/mL inhibited the growth of three colon cancer cells (HT-29, SW480 and Caco-2) over 70%. In these results, we have found that safelac and lactopad decreased cell viability in concentration-dependent manner. The physiological significance of LAB-induced cytokine secretion to human health remains to be clarified TNF-α exerts cytotoxic effects on tumor cells. In this study, we have shown the significant effect of safelac and lactopad on the production of TNF-α. Therefore, our data suggest that two kinds of anti-microbial peptides isolated from LAB effectively inhibited some colon cancer cells, but further studies will be required to clarify the precise mechanism for this inhibition.

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