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Effects of *Kimchi* Extracts on Production of Nitric Oxide by Activated Macrophages, Transforming Growth Factor β1 of Tumor Cells and Interleukin-6 in Splenocytes

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Abstract

Methanol extracts from four kinds of *kimchi*, which were differently prepared in kinds and levels of sub-ingredients, were given to Balb/c mice for 3 weeks (0.5 mg/kg/day). Peritoneal macrophages isolated from mice treated with *kimchi* extracts and saline were stimulated by lipopolysaccharide (LPS). K3 and K4 *kimchis*, containing more red pepper powder, garlic, Chinese pepper powder, mustard leaf and organically cultivated Korean cabbage, siginificantly increased NO production by the activated macrophages ($p<0.05$). K1, K2, K3 and K4 *kimchi* extracts (0.01, 0.1, 1.0 μg) significantly reduced the increased TGF-β1 production of *H. pylori* lysate (0.01 μg)-activated human epithelial RPMI 2650 cells (5×10^4 cells/mL) at 24 and 48 hrs of treatment ($p<0.01$). However, the decreased TGF-β1 α production of RPMI 2650 cells by *H. pylori* lysate increased by treatment with *kimchi* extract for 72 hrs. Especially, K4 *kimchi* (containing organically cultivated Korean cabbage and more ingredients, modulated TGF-β1 production of *H. pylori* lysate-activated RPMI 2650 cells to the normal level (control) by treatment for 48 hrs. The treatment of K1 and K4 *kimchi* enhanced the LPS (0.01 μg/mL)-induced IL-6 production of splenocytes. The results suggest that *kimchi* might have an beneficial effect on cancer prevention due in part to the function enhancing NO production of activated macrophages. Our data suggest that *kimchi* could modulate TGF-β1 production by cancer cells and IL-6 production of splenocytes, thereby possibly contributing to control carcinogenesis and the immune system.

Key words: *kimchi*, TGF-β1 production, NO production, antitumor effect

INTRODUCTION

Kimchi is a traditional, fermented vegetable food in Korea. There are about 187 varieties of *kimchi*, depending on the ingredients and processing methods used, which are different in biochemical, microbiological, and nutritional characteristics (1,2).

Kimchi is fermented by the microorganisms which are originally present in the raw vegetable substances, but fermentation is gradually dominated by lactic acid bacteria. Numerous physicochemical and biological factors affect the growth and sequential appearance of principal microorganisms involved in fermentation (1,3). Complex biochemical changes occur depending on the environmental conditions before, during, and after fermentation (4).

Nutritionally, *kimchi* is an important source of vitamins, minerals, dietary fiber, and other nutrients. The vitamin B groups and ascorbic acid are already present in the raw materials and may be synthesized during the fermentation process (4,5). It also contains high levels of organic acid and lactic acid bacteria (3).

Kimchi has typical biochemical and health-related functions. Studies on the antimutagenic and anticarcinogenic properties of *kimchi* have been reported. Carotenoids (6), ascorbic acid (7,8), dietary fiber (9), and flavonoids (10) in yellow-green vegetables used as the major material of *kimchi* showed antimutagenic and anticancer activities. Those are believed to suppress the formation of carcinogenic or mutagenic compounds, the mutagenicity induced by several mutagens and show antitumor effects in mice. Other ingredients, red pepper (11), garlic (12), and lactic acid bacteria (13-15) dominating the fermentation of *kimchi* are believed to have antimutagenic and anticarcinogenic effects. It was reported that properly ripened *kimchi* with 3% salt concentration itself had inhibitory effects on the growth of cancer cells and might have anticarcinogenic activity because of bioactive components or nutrients (1,16). In our previous studies (17), *kimchi* extracts revealed the inhibitory effects on carcinogen-induced cytotoxicity and transformation in C3H/10T1/2 cells. The growth of human cancer cells and [^{3}H] thymidine incorporation in human gastric cancer cells were inhibited by *kimchi*, but normal cells were not affected (18). *Kimchi* extracts enhanced glutathione level, glutathione reductase and glutathione S-transferase, involved in the detoxification of a large group of hydrophobic compounds, in Sarcoma-180 cell transplanted mice (19).

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Nitric oxide (NO) has been implicated as a key weapon of activated macrophages against bacteria and tumor cells (20,21). Activated macrophages by cytokine produce significant amounts of NO, exerting cytotoxic activities. In tumor development, the synthesis of specific growth factors such as transforming growth factor β1 (TGF-β1) increased (22,23) The resulting autocrine stimulation has been shown to provide a growth advantage to tumor cells and to convert untransformed and nontumorigenic cells into malignant and tumorigenic cells (24,25). Interleukin-6 (IL-6) is another cytokine which is released from monocyte, B lymphocyte, T lymphocyte, vascular endothelial cells, and plays a role in the inflammatory response (26).

In this study, 4 different kinds of *kimchi*s containing varing proportions of ingredients (K1, K2, K3 and K4 *kimchi*s) were prepared. To better understand the anticancer activity and its mechanism of *kimchi*, the extracts from 3 week fermented *kimchi*s and saline were given to Balb/c mice. Three weeks later, peritoneal macrophages were isolated from mice and stimulated by lipopolysaccharide (LPS). And then the produced NO level in the culture supernatant of macrophages was determined. The effects of *kimchi* extracts on TGF-β1 production in *Helicobacter pylori* (*H. pylori*) lysate-activated cancer cells and IL-6 production in LPS-activated splenocytes were also investigated.

MATERIALS AND METHODS

Preparation of *kimchi*

Common Korean cabbage grown in Kimhae, Kyungnam province, was used as major raw ingredient for K1 and K3 *kimchi*. Organically cultivated Korean cabbage was obtained from Milyong, Kyungnam province, for K2 and K4 *kimchi*. Red pepper powder, radish, garlic, ginger, green onion, Chinese pepper powder, mustard leaf, fermented anchovy juice and sugar were purchased from a local market. Common Korean cabbage and organically cultivated Korean cabbage were divided into 8 pieces, brined in 10% salt solution for 10 hours and rinsed with fresh water. Drained Korean cabbages were cut into 4 to 5 cm size pieces. The ratio of ingredients for K1 *kimchi* were 13 of radish, 2 of green onion, 3.5 of red pepper powder, 1.4 of crushed garlic, 0.6 of crushed ginger, 2.2 of fermented anchovy juice and 1 of sugar in the proportion (w/w) of 100 of salted common Korean cabbage. For K2 *kimchi*, the ratio of 7 of red pepper powder and 2.8 of garlic were used in the proportion of 100 of organically cultivated Korean cabbage (100%). 7% of red pepper powder, 2.8% of garlic and 0.1% of Chinese pepper powder versus common Korean cabbage were used for K3 *kimchi*. In K4 *kimchi*, mustard leaf being 5% (w/w) of organically cultivated Korean cabbage was mixed with the same ratio of ingredients as K3 *kimchi*. The final weight percentages of salt in *kimchi*s were adjusted to 2.5%. *Kimchi*s were fermented for 3 weeks at 5°C and then used as test samples.

Preparation of *kimchi* extracts

3 week-fermented K1, K2, K3 and K4 *kimchi* were freeze-dried and minced in a blender. The minced *kimchi* samples (25 g) were extracted with methanol (500 mL), three times, by shaking for 8 hours and then taken as K1, K2, K3 and K4 *kimchi* extracts. The *kimchi* extracts were dried by rotary vaccum evaporator (Buchi 011 & 461, Switzerland) and then dissolved in phosphate buffered solution (PBS).

Chemicals

Lipopolysaccharide (LPS) and H. *pylori* lysate were obtained from Sigma Chemical Co. (St. Louis MO, USA) and used at concentrations which did not affect the cells viability in the culture system. Sulfanilamide, naphthylethylene diamine dihydrochloride and phosphoric acid were also purchased from Sigma Chemical Co. (St. Louis MO, USA).

Minimum essential medium (MEM), fetal calf serum (FCS), 0.05% trysin-0.02 EDTA, penicillin-streptomycin were obtained from Gibco Chemical Co. (Grand Island, NY, USA). PBS (pH 7.2) and 96 wells microplate were purchased from Sigma Chemical Co. (St. Louis MO, USA) and Corning Co. (NY, USA), respectively. All chemicals used in the present experiment were sterilized through millipore membrane filtration or autoclaved.

To determine TGF-β1 and IL-6 levels, human TGF-β1 Duoset ELISA kit and Mouse IL-6 quantikine M ELISA kit were obtained from Genzyme (Cambrige, MA) and R & D (Miniapolis, USA).

Nitric oxide (NO) assay

Animals

Male Balb/c mice at 4 weeks of age were used. A basal diet and drinking water were available ad libitum. Mice were housed in polycarbonate cages with a 12 hr light/dark cycle in the temperature (21±2°C) controlled room.

K1, K2, K3 and K4 *kimchi* extracts or saline solution as control were orally injected to mice with 0.5 mg/kg per day. After 3 weeks, peritoneal macrophages were isolated.

NO production

Peritoneal macrophages (1×10^6 cells/mL) were exposed to 10 μg/mL of LPS for 72 hours at 37°C. The concentration of NO in culture supernatant was determined by Griess reaction as described previously (27,28). The macrophage supernatant (0.1 mL) was mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5 phosphoric acid) and incubated at room temperature for 10 min. Optical density was measured with microplate reader (Model 550, Bio-Rad, Richmond, USA) at 540 nm using sodium nitrite asstandard.

TGF-β1 assay

Cell treatment

RPMI 2650 cells, human epithelial cells, were obtained

from ATCC (CCL 30, Maryland, USA). The cells were cultured in MEM supplemented with 10% FCS and 100 unit/mL of penicillin-streptomycin at a humidified atmosphere of 5% CO_2 at 37°C. A medium change is made on the 2nd day after seeding. The cells were transferred every 6~7 days, using PBS and 0.05% trysin-0.02% EDTA.

RPMI 2650 cells (5×10^4 cells/mL) were treated with 0.01 μg *Helicobacter pylori* lysate and 0.01, 0.1, 1.0 μg of various *kimchi* (K1, K2, K3 and K4) extracts for 24, 48 and 72 hrs, respectively. Culture supernatants were harvested for TGF-β1 bioassay.

TGF-β1 immunoassay

The concentration of produced TGF-β1 in the test system was quantitated by TGF-β1 immunoassay, using Human TGF-β1 Duoset ELISA kit and Human TGF-β1 as standard. Before immunoassay, 20 μL of standard (100 ng/mL) and 200 μL of culture supernatant were acid activated with 1 N HCl followed by neutralization with 1 N NaOH. TGF-β1 ELISA was performed according to the manufacturer's instructions (Genzyme Corp., Cambridge, MA).

IL-6 assay

Splenocytes (2×10^6 cells/mL, 1 mL) isolated from Balb/c mouse were treated with K1, K2, K3 and K4 *kimchi* extracts or saline solution and 1 μg/mL of LPS. After incubation for 24 hours at 37°C, the concentrations of produced IL-6 in culture supernatants were measured by IL-6 immunoassay, using Mouse IL-6 quantikine M ELISA kit. IL-6 ELISA was performed according to the manufacturer's instructions (R&D, Miniapolis, USA).

Statistical analysis

The statistical analysis of the test data from the experiments, repeated three times was performed by analysis of variance. Significant differences between treatment means were determined by Student's *t*-test.

RESULTS AND DISCUSSION

Effects on NO production

Nitric oxide (NO) is a reactive nitrogen intermediate released by macrophages, endothelial and vascular smooth muscle cells after immunological activation in response to endotoxin, tumor necrosis factor α or γ-interferon (29-31). NO is synthesized from L-arginine by the NO synthase (29,32). Nearby, circulating macrophages activated by cytokines enzymatically produce a significant amout of NO which can be related to cytotoxic activities. It has been reported that the produced NO acted as a molecule controlling immunity and contributed to defense against infections and cancer.

To study the effects of *kimchi* extracts on NO production by macrophages, various *kimchi* extracts (K1, K2, K3 and K4) or saline solution as control were orally injected at a concentration of 0.5 mg/kg per day. After 3 weeks, isolated peritoneal macrophages were exposed to 10 μg/mL of LPS. Following 72 hours incubation at 37°C, NO productions of supernatants were measured.

As shown as in Fig. 1, NO productions of K-1 and K-2 treated group were lower than those of controls. However, K-3 and K-4 extracts siginificantly increased NO productions of peritoneal macrophages compared to controls ($p<0.05$).

These results show that *kimchis* (K3 and K4) containing more ingredients, red pepper powder, garlic, Chinese pepper powder, mustard leaf and organically cultivated Korean cabbage, increased NO productions more than other *kimchis* (K1 and K2).

It has been reported that NO released by activated macrophages functioned as a cytotoxic effector molecule for tumor target calls and exerted antimicrobial effects of pathogenic intracellular microorganisms (33,34).

With above results, we thought that *kimchi* might have the function enhancing NO production by activated macrophage, thereby be involved in the cancer prevention system. And this effect of *kimchi* was different, depending on the ingredients.

Effects on TGF-β1 production

TGF-β1 is a ubiquitous peptide produced by both normal and neoplastic cells, with production increased when a cell undergoes malignant transformation (22-24,35). Macrophages and periperal blood monocytes activated with toxin secrete TGF-β1. However, TGF-β1 has different biological properties

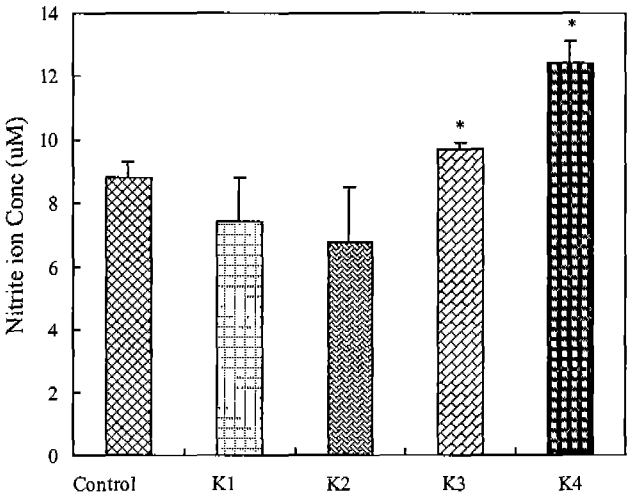

Fig. 1. Nitrite ion concentration in peritoneal macrophages of mice treated with *kimchi* extract. Mice were treated orally with 0.5 mg/kg/day of *kimchi* extracts or saline solution as control for 3 weeks. Peritoneal macrophages were isolated and then exposed with 10 μg/mL of LPS. Following 72 hours, NO productions of supernatants were measured.

*Significantly different compared with control at the $p<0.05$ level

K1: standardized *kimchi* using common Korean cabbage (SKC)

K2: standardized *kimchi* using organically cultivated Korean cabbage (SKO)

K3: SKC with 7% of red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt

K4: SKO with 7% red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt

in several cells. Recently, TGF-β1 might be regarded as both inhibitor and stimulator in tumorigenesis, depending on the stage of tumor development and cooperation with various oncogenes and growth factors (36-38).

In this study, the effects of *kimchi* extracts on TGF-β1 production of human epithelial cells by *H. pylori* lysate were investigated. RPMI 2650 cells (5×10^4 cells/mL) were treated with 0.01 μg *H. pylori* lysate and 0.01, 0.1, 1.0 μg of *kimchi* extracts for 24, 48 and 72 hrs, respectively.

Fig. 2 shows TGF-β1 production of human epithelial cells activated by *H. pylori* lysate. TGF-β1 production of *H. pylori* lysate-activated RPMI 2650 cells was significantly ($p<0.01$) higher at 48 hrs. But, with 72 hrs of treatment, the TGF-β1 production was lower than those of unactivated cells.

By treatment with K1 *kimchi* extracts for 48 hrs, TGF-β1 production of RPMI 2650 cells activated by *H. pylori* lysate were significantly inhibited ($p<0.01$) (Fig. 3). However, the decreased TGF-β1 production of RPMI 2650 cells by *H. pylori* lysate increased at 72 hrs of incubation with K1 *kimchi* extracts (Fig. 3).

K2, K3 and K4 *kimchi* extracts also reduced the increased TGF-β1 production of *H. pylori* lysate-activated RPMI 2650 cells at 24 and 48hrs of treatment, significantly ($p<0.01$) (Fig. 4~6). The decreased TGF-β1 production of RPMI 2650 cells by *H. pylori* lysate increased by the treatment with K2, K3 and K4 *kimchi* extracts for 72 hrs.

The effect of K2 *kimchi*, modulating the changes of TGF-β1 production of *H. pylori* lysate-activated RPMI 2650 cells, in the highest concentration was higher than that in other concentration (Fig. 4). But, in K3 and K4 *kimchi*, the modulating effects of a concentration of 0.01 μg were higher than those of a concentrations of 0.1 or 1.0 μg (Fig. 5, 6).

Fig. 7 shows how 0.1 μg of K1, K2, K3 and K4 *kimchi*

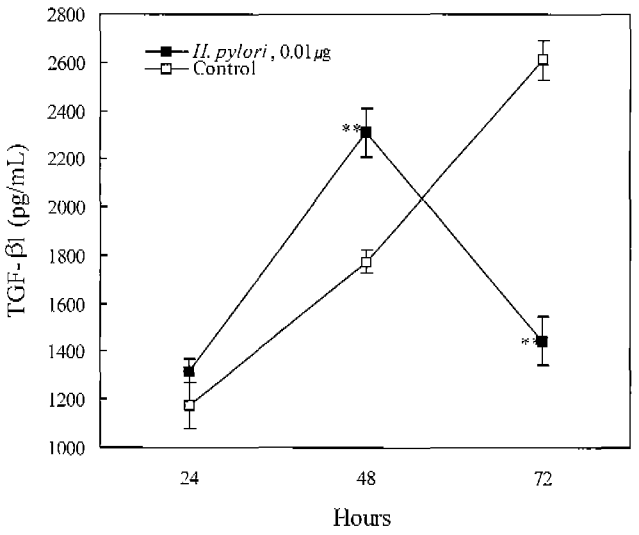

Fig. 2. Production of TGF-β1 by epithelial cells with 0.01 μg of *H. pylori* lysate for 24, 48 and 72 hours, respectively, in MEM. Culture supernatants were harvested after each incubation time and TGF-β1 was assayed.
**Significantly different compared with control at the $p<0.01$ level.

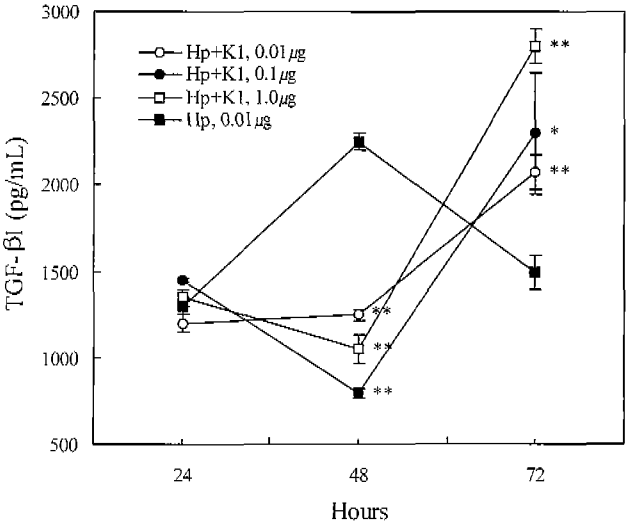

Fig. 3. Production of TGF-β1 by epithelial cells with 0.01 μg of *H. pylori* (Hp) lysate and 0.01, 0.1, 1.0 μg of K-1 *kimchi* extract for 24, 48 and 72 hours, respectively, in MEM. Culture supernatants were harvested after each incubation time and TGF-β1 was assayed.
K-1: standarzied *kimchi*
*, **Significantly different compared with *H. pylori* lysate treated at the level of $p<0.05$ and $p<0.01$, respectively.

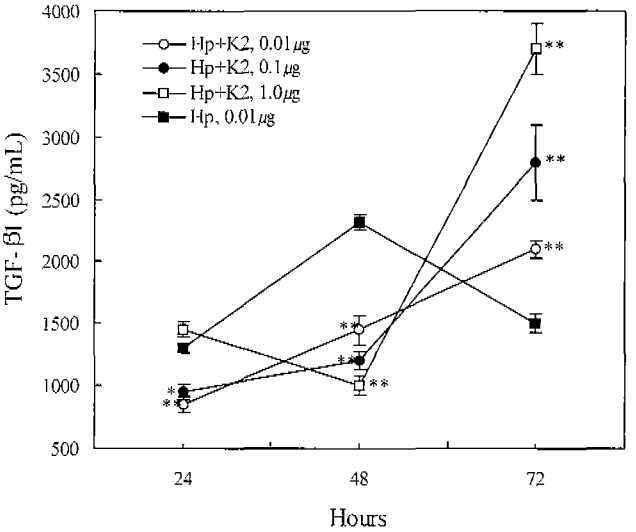

Fig. 4. Production of TGF-β1 by epithelial cells with 0.01 μg of *H. pylori* (Hp) lysate and 0.01, 0.1, 1.0 μg of K-2 *kimchi* extract for 24, 48 and 72 hours, respectively, in MEM. Culture supernatants were harvested after each incubation time and TGF-β1 was assayed.
K2: standardized *kimchi* using organically cultivated Korean cabbage
**Significantly different compared with *H. pylori* lysate treated at the level of $p<0.01$.

extracts affect the TGF-β1 production of RPMI 2650 cells activated by *H. pylori* lysate with control. K4 *kimchi* containing organically cultivated Korean cabbage and more ingredients modulated TGF-β1 production of *H. pylori* lysate-activated RPMI 2650 cells to the normal level (control) by the treatment for 48 hrs.

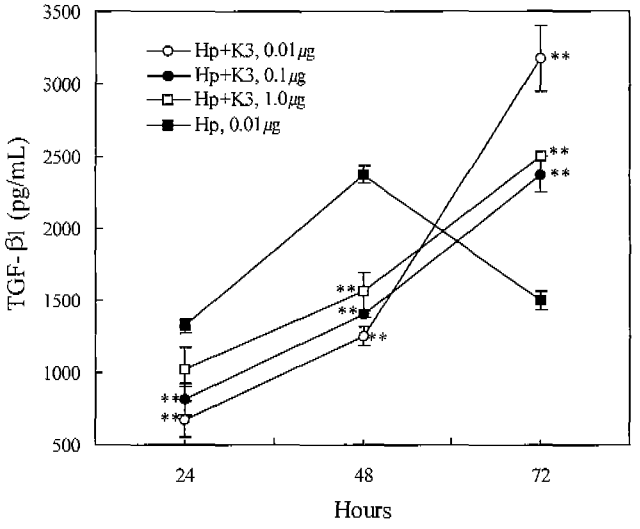

Fig. 5. Production of TGF-β1 by epithelial cells with 0.01 μg of *H. pylori* (Hp) lysate and 0.01, 0.1, 1.0 μg of K-3 *kimchi* extract for 24, 48 and 72 hours, respectively, in MEM. Culture supernatants were harvested after each incubation time and TGF-β1 was assayed.
K3: SKC with 7% of red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt
**Significantly different compared with *H. pylori* lysate treated at the level of $p<0.01$.

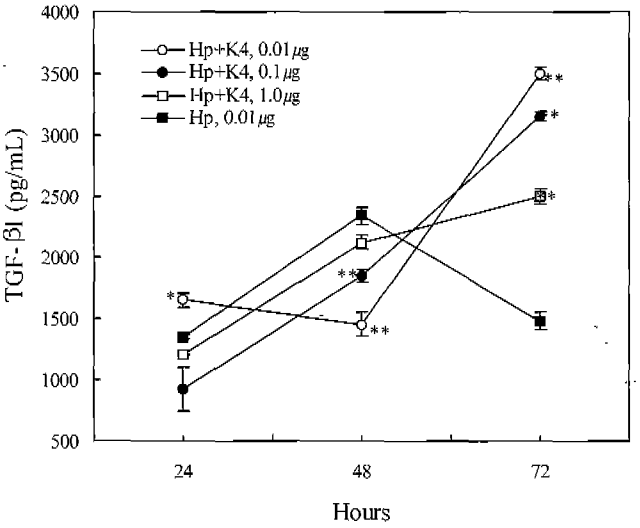

Fig. 6. Production of TGF-β1 by epithelial cells with 0.01 μg of *H. pylori* (Hp) lysate and 0.01, 0.1, 1.0 μg of K-4 *kimchi* extract for 24, 48 and 72 hours, respectively, in MEM. Culture supernatants were harvested after each incubation time and TGF-β1 was assayed.
K4: SKO with 7% red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt
*,**Significantly different compared with *H. pylori* lysate treated at the level of $p<0.05$ and $p<0.01$, respectively.

Chang et al. (36) reported that, in tumor formation, TGF-β1 could function as a growth regulator either in a positive or negative way, depending on the study and model system used. Whereas TGF-β1 stimulated proliferation of mesenchymal cell types *in vitro*, it revealed potent antiproliferative effects on other cell types such as epithelial and endothelial cells

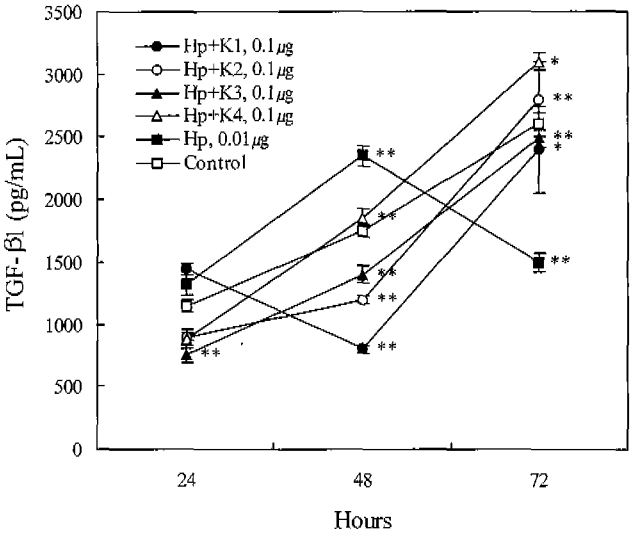

Fig. 7. Production of TGF-β1 by epithelial cells with 0.01 μg of *H. pylori* (Hp) lysate and 0.1 μg of *kimchi* extracts (K1, K2, K3, K4) for 24, 48 and 72 hours, respectively, in MEM. Culture supernatants were harvested after each incubation time and TGF-β1 was assayed.
K1: standardized *kimchi* using common Korean cabbage (SKC)
K2: standardized *kimchi* using organically cultivated Korean cabbage (SKO)
K3: SKC with 7% of red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt
K4: SKO with 7% red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt
* **Significantly different compared with *H. pylori* lysate treated at the level of $p<0.05$ and $p<0.01$, respectively.

(36,39). TGF-β1 could provide an advantage in tumor formation by its function for localized immunosuppression (40). However, this study shows that *kimchi* might function to modulate the TGF-β1 production of activated cancer cells to a level similar to that in unactivated cells.

Effects on IL-6 production

IL-6 is a multifunctional cytokine which is produced by monocyte, B lymphocyte, T lymphocyte and epithelial cells (41). It has been believed that Il-6 played a central role in the response to injury and infection in animals (42).

Table 1 shows the effects of *kimchi* extracts on the production of IL-6 from splenocytes in the presence or absence of LPS. The IL-6 production of splenocytes increased by the expose of LPS above 10 times compared to without endotoxin. The treatment of K1 and K4 *kimchi* enhanced the IL-6 production of splenocytes increased by the expose of LPS at the concentration of 0.01 μg/mL. However, K1 and K4 *kimchi* significantly decreased the IL-6 production of splenocytes in higher concentration (0.1,1 μg/mL) ($p<0.01$). With the treatment of K2 and K3 *kimchi*, the increased IL-6 production of splenocytes by LPS were reduced. The higher concentration of *kimchi* extracts were, the lower IL-6 produced. These shows that *kimchi* can also modulate IL-6 production in spleen.

Based on above results, we suggested that *kimchi* could have

Table 1. Interleukin-6 (IL-6) production in mice splenocytes exposed with *kimchi* extracts (unit: pg/mL)

Samples		0.01 μg/mL	0.1 μg/mL	1 μg/mL
Control	5.55±0.98			
LPS (1.0 μg/mL)	79.06±2.94			
LPS + K1[1)]		94.66±7.36**	70.74±1.96**	55.83±1.47**
LPS + K2[2)]		78.02±2.45	65.53±1.47**	62.07±1.47**
LPS + K3[3)]		70.44±2.94**	70.44±2.94**	60.68±2.45**
LPS + K4[4)]		88.42±4.41**	72.13±0.98**	65.88±1.97**

[1)]K1: standardized *kimchi* using common Korean cabbage (SKC)
[2)]K2: standardized *kimchi* using organically cultivated Korean cabbage (SKO)
[3)]K3: SKC with 7% of red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt
[4)]K4: SKO with 7% red pepper powder, 2.8% garlic, 0.1% Chinese pepper powder, 5% mustard leaf and guwoon salt
**Significantly different compared with control at the $p<0.01$ level.

an beneficial effect in the prevention of cancer by enhancing NO production in macrophages. And the modulating effect of *kimchi* on the changes of TGF-β1 production by cancer cells and IL-6 production of sprenocytes seems to control the carcinogenesis and immune system. Especially, the modulating effects of organically cultivated Korean cabbage *kimchi* containing more ingredients, red pepper powder, garlic, Chinese pepper powder and mustard leaf, was strong. It seems that the ingredients of *kimchi* also effect NO, TGF-β1 and IL-6 production in the anticancer and immune system.

But the question about how *kimchi* increased NO production in macrophages and modulated TGF-β1 production in cancer cells still remains. How the ingredients of *kimchi*, organically cultivated Korean cabbage, red pepper powder, garlic, Chinese pepper powder and mustard leaf act in cancer prevention and the immune system should be investigated continuously. We expect that the active compounds of *kimchi* and its ingredients showing antitumor effects will be identified in further investigations.

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