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Antitumor metastasis activity of pectic polysaccharide purified from the peels of Korean *Citrus* Hallabong

Eun Hye Lee[a,1], Hye-Ryung Park[b,1], Myoung-Sook Shin[a], Sun Young Cho[a], Hyuk-Joon Choi[c], Kwang-Soon Shin[a,*]

[a] Department of Food Science and Biotechnology, Kyonggi University, San 94-6, Ieui-dong, Youngtong-gu, Suwon, Gyeonggi-do 443-760, Republic of Korea
[b] Department of Integrated Biomedical and Life Science, Korea University, Seoul 136-703, Republic of Korea
[c] BK-Bio Co. Ltd., 5439-1, Sangdaewon-dong, Jungwon-gu, Sungnam, Gyeonggi-do 462-819, Republic of Korea

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ABSTRACT

A polysaccharide fraction, HBE-III, was successfully purified in a high yield (40.4%) from its crude polysaccharide (HBE-0) which was prepared from pectinase hydrolysates of the peels of the Korean *Citrus* Hallabong. In experimental lung metastasis studies of Colon 26-M3.1 carcinoma cells, prophylactic administration of HBE-III significantly inhibited lung metastasis in a dose-dependent manner. In an *in vitro* cytotoxicity analysis, HBE-III (up to 1000 μg/mL) did not affect the growth of Colon 26-M3.1 cells and normal cells. HBE-III enhanced production of IL-6 and IL-12 by murine peritoneal macrophages. In an assay for natural killer (NK) cell activity, HBE-III (1000 μg/mouse, *i.v.*) significantly augmented NK cytotoxicity against Yac-1 tumor cells. The depletion of NK cells by injection of mouse anti-asialo GM1 serum abolished the inhibitory effect of HBE-III on lung metastasis of Colon 26-M3.1 cells. These data suggest that HBE-III may inhibit tumor metastasis *via* activation of macrophages and NK cells.

1. Introduction

Cancer is one of the most common diseases that pose a threat to human life. The >90% mortality rate for cancer patients is not because of primary tumors but rather owing to the development of metastases (Sporn, 1996). Progressive metastasis of tumor cells in the host results in biological heterogeneity for immunogenicity factors such as growth rate, cell markers, and sensitivity to chemotherapeutic drugs (Fidler, 1987). Therefore, preventing metastasis is one of the most crucial problems in cancer treatment. Many experimental studies and clinical trials have shown that natural immunity plays an important role in blocking the metastasis of primary tumors (Leyon & Kuttan, 2004). It is well recognized that innate immune system activation plays a critical role in the defense against foreign antigens and malignant self-cells including tumors (Jakóbisiak, Lasek, & Gołąb, 2003). The primary mechanisms in the immune-stimulating activities relate to their abilities to activate phagocytes such as macrophages and dendritic cells; in addition, various cytokines (*e.g.*, IL-6, IL-12, TNF-α, or IL-1β) produced from macrophages and phagocytes elicit natural killer (NK) cell cytotoxicity to tumors (Azuma & Seya, 2001). These mechanisms indicate that the functional activation of NK cells and macrophages suppress tumor growth as well as metastasis.

Polysaccharides are an interesting class of additives used in the food and drug industries. They have been widely studied because of their broad spectrum of therapeutic properties, relatively low toxicity, few side effects, and unique biological, chemical, and physical properties (Schepetkin & Quinn, 2006). In particular, their immunomodulatory, anti-inflammatory, and antitumor effects have recently attracted more and more attention in the biochemical and medical fields (Fan, Ding, Ai, & Deng, 2012).

Hallabong is a new hybrid citrus crop in South Korea and has been regarded as a citrus fruit with potential commercial value because of its attractive and pleasant aroma (Choi, 2003). The total production of Hallabong in South Korea was about 45,000 tons in 2011 (Jeju special self-governing province Agricultural Research), and it is now widely cultivated in South Korea. Hallabong fruits are used primarily for juice manufacture; the peels are the main byproduct and are usually discarded. If they go unprocessed, the peels become waste and a possible source of environmental pollution. However, citrus peels are an attractive source of phytochemicals (*e.g.*, pectin, flavonoids, and carotenoids) with potential health benefits (Mandalari et al., 2006). In some regions of the

* Corresponding author. Tel.: +82 31 2499655; fax: +82 31 2499655.
E-mail address: ksshin@kyonggi.ac.kr (K.-S. Shin).
[1] These authors contributed equally to this study.

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world, the peels of *Citrus* species are used in traditional medicine to treat upset stomachs, cough, skin inflammation, muscle pain, ringworm infections, and high blood pressure (Li et al., 2009). Furthermore, citrus peels contain high amounts of polysaccharides (Mandalari et al., 2006). However, there are no reports on the polysaccharides of *Citrus* Hallabong peel that refer to chemical properties or biological activities.

Accordingly, this study reports the purification and chemical characterization of a polysaccharide from the peels of Korean *Citrus* Hallabong, the antimetastatic effect of the purified polysaccharide against tumors, and its role in the activation of NK cells and macrophages.

2. Materials and methods

2.1. Materials and chemicals

The peels of *Citrus* Hallabong fruits were cultured and collected (December 2011) from Gashi-ri, South jeju-gun, Jeju-do, Korea. They were then dried and used as samples. Pectinase from *Aspergillus niger* was purchased from Sigma (St. Louis, MO, USA). Sephadex G-75 was obtained from GE Healthcare Bio-Science (Piscataway, NJ, USA) and Sep-Pak C18 cartridges were from Waters Co. (Milford, MA, USA). RPMI 1640 medium, Hank's balanced salt solution (HBSS), and fetal bovine serum (FBS) were purchased from Gibco BRL Co. (Grand Island, NY, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Gaithersburg, MD, USA).

2.2. Isolation and purification of polysaccharides from Hallabong peels

Dried Hallabong peels (2 kg) were suspended in water (20 L) and hydrolyzed with pectinase (40 mL) from *Aspergillus niger* for 24 h at 40 °C. Pectinase was then deactivated by boiling for 10 min at 100 °C. Enzyme-treated *Citrus* Hallabong peel was centrifuged at 6000 rpm for 20 min to obtain the supernatant. Crude polysaccharides were precipitated by the addition of four volumes of 95% cold ethanol containing 60 mM HCl to the supernatant samples. The precipitate was then dissolved in water and dialyzed using Spectra/Por 2 (molecular weight cut off [MWCO]: 12,000–14,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The salt-free solution was finally lyophilized to yield the crude polysaccharide fraction (HBE-0) from *Citrus* Hallabong peel. 100 mg of HBE-0 was re-dissolved in water of 5 mL applied to a column (4 cm × 95 cm) of Sephadex G-75 equilibrated with 50 mM acetate buffer (pH 5.2), and was eluted with the same buffer at 50 mL/h flow rate. Four major purified polysaccharides (HBE-I, HBE-II, HBE-III, and HBE-IV) were obtained, and were lyophilized after desalting by dialysis (MWCO: 2000; Spectrum Laboratories Inc.). TBA-positive material was found in the retarded fraction (HBE-III).

2.3. General methods

The total neutral sugar content was determined by means of the phenol-sulfuric acid reaction with galactose (Gal) as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined by measuring the absorbance at 525 nm using the *m*-hydroxybiphenyl colorimetric procedure and with galacturonic acid (GalA) as the standard (Blumenkrantz & Asboe-Hansen, 1973). Protein contents were analyzed using Bradford (Bradford, 1976) assays with bovine serum albumin (BSA) as the standards. The contents of Kdo (2-keto-3-deoxy-D-*manno*-2-octulosonic acid) and Dha (2-keto-3-deoxy-D-*lyxo*-2-heptulosaric acid) were determined colorimetrically by the modified thiobarbituric acid (TBA) method (Karkhanis, Zeltner, Jackson, & Carlo, 1978). The sugar composition of the polysaccharide samples was determined by gas chromatography (GC; ACME-6100, Young-Lin Co., Anyang, Korea) analysis of their alditol acetates (Jones & Albersheim, 1972). The samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1.5 h at 121 °C, and converted into the corresponding alditol acetates using the procedure of Zhao, Kiyohara, Yamada, Takemoto, and Kawamura (1991). The composition of Kdo, Dha and uronic acids were determined through GC as alditol acetates according to the modified methods of York, Darvill, McNeil, and Albersheim (1985) and Stevenson, Darvill, and Albersheim (1988). The resulting alditol acetates were analyzed through GC using an SP-2380 capillary column (0.2-μm film thickness, 0.25 mm i.d. × 30 m; Supelco, Bellefonte, PA) at 60 °C for 1 min, 60 → 220 °C (30 °C/min), 220 °C for 12 min, 220 → 250 °C (8 °C/min), and 250 °C for 15 min. The molar percentage was calculated from the peak areas and response factors using the flame-ionization detector (FID). High performance size-exclusion chromatography (HPSEC) of HBE-III was performed on an Agilent 1260 Infinity LC system (Agilent Technologies Co., Ltd., Palo Alto, CA, USA) equipped with a Superdex 75 GL column (GE Healthcare, 1.0 cm × 30 cm) and refractive index (RI) detector (Agilent 1200 series). A total of 10 μL of each polysaccharide solution were analyzed using an isocratic mobile phase (50 mM ammonium formate buffer, pH 5.5) at a flow rate of 0.5 mL/min at room temperature. MWs of the polysaccharides were estimated from the calibration curve constructed for standard pullulans (P-200, 100, 50, 20, 10, and 5; Showa Denko Co. Ltd., Tokyo, Japan).

2.4. Methylation analysis

Methylation analysis was performed according to the Hakomori method (Hakomori, 1964). For the methylation of HBE-III which contained 2-methylfucose and 2-methylxylose, trideutriomethyl iodide (CD_3I) instead of CH_3I was used (Darvill, McNeil, & Albersheim, 1978). HBE-III which contained Kdo or Dha residues was prereduced with $NaBD_4$ and permethylated according to the method of York et al. (1985) in a separete experiment. Uronic acids in the methylated polysaccharides were reduced by $Li(C_2H_5)_3BH$ in tetrahydrofuran (Super-Hydride; Sigma) (York, Darvill, McNeil, Stevenson, & Albersheim, 1986). Methylated polysaccharide was hydrolyzed with 2 M TFA for 1.5 h at 121 °C, and the products were reduced with $NaBH_4$ or $NaBD_4$ followed by acetylation. The resulting partially methylated alditol acetates were analyzed *via* GC–mass spectrometry (GC–MS) using an SP-2380 capillary column (Supelco). GC–MS was performed on an Agilent 6890 gas chromatograph/5973 mass selective detector. Helium was used as the carrier gas at a constant flow rate of 0.5 mL/min. The temperature programs were 60 °C for 1 min, 60 °C → 150 °C (30 °C/min), 150 °C → 250 °C (1.5 °C/min), and 250 °C for 5 min. Methylated alditol acetates were identified by their fragment ions and relative retention times. Molar percentages were estimated from the peak areas and response factors (Sweet, Shapiro, & Albersheim, 1975).

2.5. Mice and cell cultures

Specific pathogen-free (SPF), 6-week-old female BALB/c mice were purchased from G-Bio Animal, Inc. (Seoul, Korea). The mice were maintained in a clean rack in an SPF room at Kyonggi University. Water and a diet of pellets were supplied *ad libitum*. All animal experiments were carried out according to the instructions of the Ethics Committee for Use of Experimental Animals at Kyonggi University (2011–2003). A lung metastatic sub-line of a highly metastatic cell line of Colon 26-M3.1 carcinoma cells was maintained as monolayer cultures in Eagle's MEM supplemented with 7.5% FBS, vitamin solution, sodium pyruvate, non-essential amino acids, and L-glutamine. The mouse peritoneal macrophages

and Yac-1 cells were cultured in RPMI-1640 supplemented with 7.5% FBS and L-glutamine.

2.6. Experimental lung metastasis

Experimental lung metastasis was induced by *i.v.* inoculation of Colon 26-M3.1 carcinoma cells (3×10^4 cells) into BALB/c mice (Ha et al., 2004). To study the antitumor metastasis activity by sample, the mice were injected *i.v.* with the purified *Citrus* Hallabong peel polysaccharide (10–1000 μg/mouse, 5 mice/group) 2 days before tumor cell inoculation. The mice were killed 14 days after tumor inoculation, and their lungs were removed and fixed in Bouin's solution. Lung tumor colonies were counted microscopically.

2.7. Cytotoxicity assay

For the mitogenic response assay, peritoneal macrophages (2.5×10^5 cells/well) from BALB/c mice were co-cultured with or without the indicated doses of HBE-III in 96-well culture plates for 72 h. In the cytotoxicity test, various doses of HBE-III in the culture medium were added to each well of the culture plate of Colon 26-M3.1 cells (2.5×10^5 cells/well), and incubated for 48 h. Cytotoxicity to tumor cells and macrophage proliferation were assayed using a CCK-8 kit (Dojindo Molecular Technologies). The cultures were incubated with 10 μL of CCK-8 solution for 3 h. Absorbance of each well was monitored at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.8. Cytokine assay

Peritoneal macrophages of BALB/c mice were harvested from 5% thioglycollate (TG) treated mice as described previously (Salki et al., 1988). The macrophage cells (2.5×10^5 cells/mL) were suspended in complete RPMI medium containing 10% FBS were plated into 96-well culture plates. After incubation for 2 h, non-adherent cells were removed by washing 3 times with PBS, and the adherent macrophages were co-incubated with the indicated doses of HBE-III for 24 h. The concentrations of various cytokines (IL-6 and lL-12) in the culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (Pharmingen Co., San Diego, CA, USA) according to the manufacturer's protocol.

2.9. Assay of NK cell-mediated tumor cytotoxicity

Three BALB/c mice per group were injected *i.v.* with HBE-III purified from *Citrus* Hallabong peel, and their splenocytes were harvested at 3 days following HBE-III injection. Single-cell suspensions of the splenocytes were added to Yac-1 cells (1×10^5 cells/mL) to obtain effector-to-target cell ratios (E:T) of 100:1, 50:1, or 25:1 in a U-bottomed 96-well plate, and the cultures were incubated for 6 h, in 5% CO_2 incubator. After centrifugation for 5 min 900 rpm, the culture supernatants (50 μL) of each well were mixed with LDH solution (Promega, San Luis Obispo, CA, USA). After 20 min, the absorbance value of each well was measured at 490 nm. The percentage of NK cell cytotoxicity was calculated from the following formula: Cytotoxicity (%) = [(OD value of experimental group − OD value of spontaneous group)/(OD value of untreated group − OD value of spontaneous group)] × 100.

2.10. Depletion of NK cells in mice

Depletion of NK cells *in vivo* was performed according to the method described previously (Whitmore et al., 2004). The mice were injected with 50-fold diluted rabbit anti-asialo GM1 serum (500 μL/mouse, *i.p.*; Wako Pure Chemicals Industries, Ltd., Japan) at 1 and 3 days prior to tumor inoculation. Anti-asialo GM1-treated mice were injected *i.v.* with the purified *Citrus* Hallabong peel 2 days prior to tumor cell inoculation. The mice were killed 14 days after tumor inoculation, and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted microscopically.

2.11. Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA). Differences among groups were evaluated by a one-way Analysis of variance (ANOVA) and Duncan's multiple range test. All data are presented as the means ± standard deviation (SD).

3. Results and discussion

3.1. Isolation and purification of polysaccharides from Hallabong peel

Among the polysaccharides in plant leaves, pectic substance is a main component polysaccharide which can be obtained as a soluble form by water extraction. Pectic substances comprise a family of homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and substituted galacturonans (rhamnogalacturonan II, RG-II) (Albersheim et al., 1994). HG is a linear chain of 1,4-linked α-D-galacturonic acid residues in which some of the carboxyl groups are methylesterified (Ishii, 1997). RG-I is composed of a rhamnogalacturonan core with neutral carbohydrate side chains such as arabinan, galactan and arabinogalactans (Engelsen, Cros, Mackie, & Perez, 1996). RG-II is a low molecular mass (5–10 kDa) polysaccharide that contains 12 different sugar residues linked together by more than 20 different glycosyl linkages (Pérez, Rodriguez-Carvajal, & Doco, 2003). Recently various pharmacological activities have been observed in pectic substances isolated from plants (Wagner, 1990; Yamada, 1992). Especially, pharmacological activities observed in pectic substances, seemed to be dependent on not HG region but RG-I and RG-II regions (Srivastava & Kulshreshtha, 1989; Yamada, 1992). Therefore, the hydrolysis of HG region using pectinase will be easy to release the RG-I and RG-II regions which have been expected as a promising biologically active moiety.

A crude soluble polysaccharide mixture (HBE-0) was isolated from *Citrus* Hallabong peel hydrolysates treated with pectinase from *Aspergillus niger*. HBE-0 showed a relatively high yield of crude polysaccharide (12.58 g/kg) and contained a considerable amount (1.6%) of TBA-positive materials. When HBE-0 was applied to a column of Sephadex G-75, four major carbohydrate peaks (HBE-I, HBE-II, HBE-III, and HBE-IV) were detected (Fig. 1A). The yield of HBE-III (40.4%) was higher than that of HBE-I (3.6%), HBE-II (9.6%), and HBE-IV (9.4%) (data not shown). HBE-III fraction was eluted mainly as a single peak with small retarded peak on HPSEC equipped with Supardex 75 GL column and its molecular weight (MW) was estimated to be 9 kDa (Fig. 1B). In addition, only the HBE-III fraction contained TBA-positive material, and all carbohydrates and uronic acids in HBE-III appeared to co-elute with the TBA-positive peak. From the results of chemical composition, HBE-III was composed of 52.8% neutral sugars, 43.0% uronic acid, and 4.2% Kdo-like material. It had a negative response to the Bradford assay and exhibited no absorption at 280 nm in the UV spectrum, revealing an absence of protein. Interestingly, HBE-III contained unusual sugars such as 2-methylfucose (2-Me-Fuc), 2-methylxylose (2-Me-Xyl), apiose (Api), aceric acid (AceA), Kdo, and Dha (Table 1). These sugars have been identified in RG-II as its characteristic component sugars (Hilz, Williams, Doco, Schols, & Voragen, 2006). Because Kdo and Dha are unique components of RG-II among several plant polysaccharides, the TBA-positive reaction in HBE-III indicates the possibility that HBE-III contains an RG-II polysaccharide.

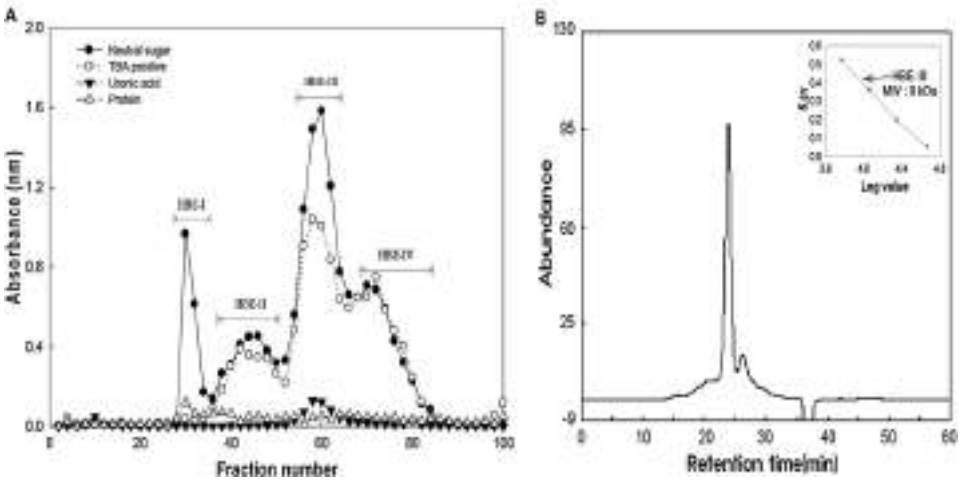

Fig. 1. Eelution profiles of crud polysaccharides (HBE-0) isolated from the pectinase digests of *Citrus* Hallabong peel on the column of Sephadex G-75 by GPC (gel permeation chromatography). ●, Neutral sugar (490 nm); ○, TBA positive material (548 nm); ▼, Uronic acid (520 nm); ▽, Protein (600 nm).

Methylation analysis indicated that HBE-III contained at least 20 different glycosidic linkages (Table 1). Methylation analysis of HBE-III showed the occurrence of terminal 2-Me-Xyl, terminal 2-Me-Fuc, 2,3,4-linked Rha, 3′-linked Api, and 2,3,3′-linked Api, being characteristic glycosidic linkages in RG-II (O'Neill, Ishii, Albersheim, & Darvill, 2004). Some results such as recovery of HBE-III from the pectinase digests of Hallabong peels, relatively low MW nature, and unusual sugar and linkage compositions being characteristic of RG-II, let us conclude that HBE-III mainly comprises RG-II structures. Recently, the health-promoting activities of RG-II (*e.g.*, inhibition of lead absorption, macrophage, Fc receptor expression-enhancing activity, and intestinal immune system modulating activity) have been reported (Park & Shin, 2007). Therefore, we finally selected the HBE-III fraction for the evaluation of biological activity.

3.2. Inhibitory effects of HBE-III on lung metastasis

The metastasizing ability of malignant tumors accounts for the poor prognosis and high mortality rate in cancer patients (Zong et al., 2012). Hence, the development of therapeutic agents that can inhibit metastasis is crucial for improving the management of cancer. The antimetastasis effect of HBE-III was detected in the mice implanted with metastatic Colon 26-M3.1 carcinoma cells. After administration for 14 days, lung metastatic colonies in HBE-III treated mice were much fewer than those in the control mice, showing that lung metastasis formation was inhibited significantly by HBE-III (Fig. 2). The antimetastatic activity of HBE-III was dose-dependent. The inhibition rates of 10, 100, and 1000 μg/mL of HBE-III were 68.7%, 76.1%, and 89.8%, respectively (data not shown). It was reported that polysaccharides

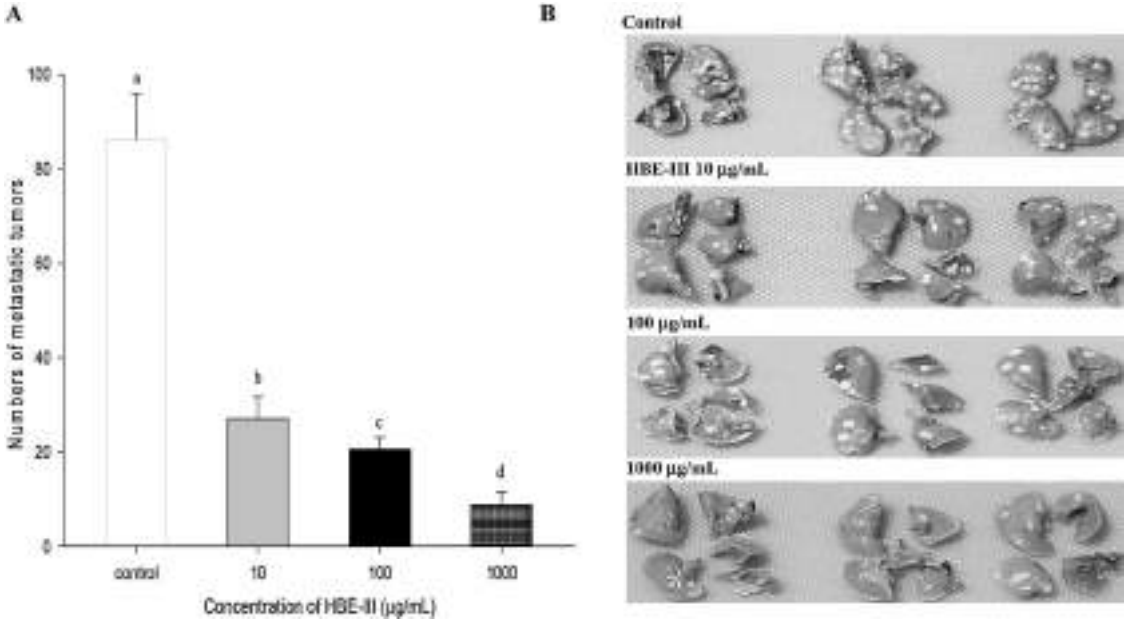

Fig. 2. Inhibitory effect of HBE-III on lung metastasis produced by *i.v.* inoculation of Colon 26-M3.1 carcinoma cells. (A) BALB/c mice ($n = 5$) per group were inoculated *i.v.* with 3×10^4 Colon 26-M3.1 colon carcinoma cells and administered intravenously with the indicated doses of HBE-III on 2 days before tumor inoculation. (B) Scanned images of mouse lungs produced by *i.v.* inoculation of Colon 26-M3.1 carcinoma cells. Mice were killed 14 days after tumor inoculation for evaluation of tumors. Bars with lower case letters (a–d) indicate significant differences ($p < 0.05$) as shown by Duncan's multiple range tests.

containing arabinogalactan residues inhibits metastasis (Uhlenbruck et al., 1986). A modified citrus pectin (MCP) containing galactose residue (Nangia-Makker et al., 2002) has also been mentioned as a possible anti metastatic agent. Inhibition of galectin-3 by pectin polysaccharide or MCP not only possesses antimetastatic properties, but also inhibits cancer cell invasion in MDA-MB-231 human metastatic breast cancer cells and human buccal metastatic cells (Sathisha, Jayaram, Nayaka, & Dharmesh, 2007). Our results also indicate that the polysaccharide from Hallabong peel is able to induce a prophylactic effect against lung metastasis and seems to induce similar antimetastatic activity with MCP. Nevertheless, our results suggest that antimetastatic principles of HBE-III may be quite different with those of MCP due to different chemical and structural characteristics between HBE-III and MCP.

3.3. Cytotoxicity assay

We next examined the cytotoxic effects of HBE-III on tumor cells and a murine macrophage cells. The cytotoxicity assay is crucial to the investigation of antitumor strategies for eliminating cancer cells with minimal toxicity to normal cells (Kunou & Hatanaka, 1997). Colon 26-M3.1 carcinoma cells were treated with HBE-III concentrations up to 1000 μg/mL. HBE-III treatment did not affect cell viability compared to the effect of the control treatment on tumor cells at doses up to 1000 μg/mL (Fig. 3A). These results indicate that HBE-III does not directly affect tumor cell viability, is not cytotoxic, and induces proliferation of immune-related cells. Consequently, the inhibitory activity of HBE-III on tumor metastasis is not attributable to cytotoxic effects on tumor cells, and HBE-III may up-regulate the function of immune-related cells.

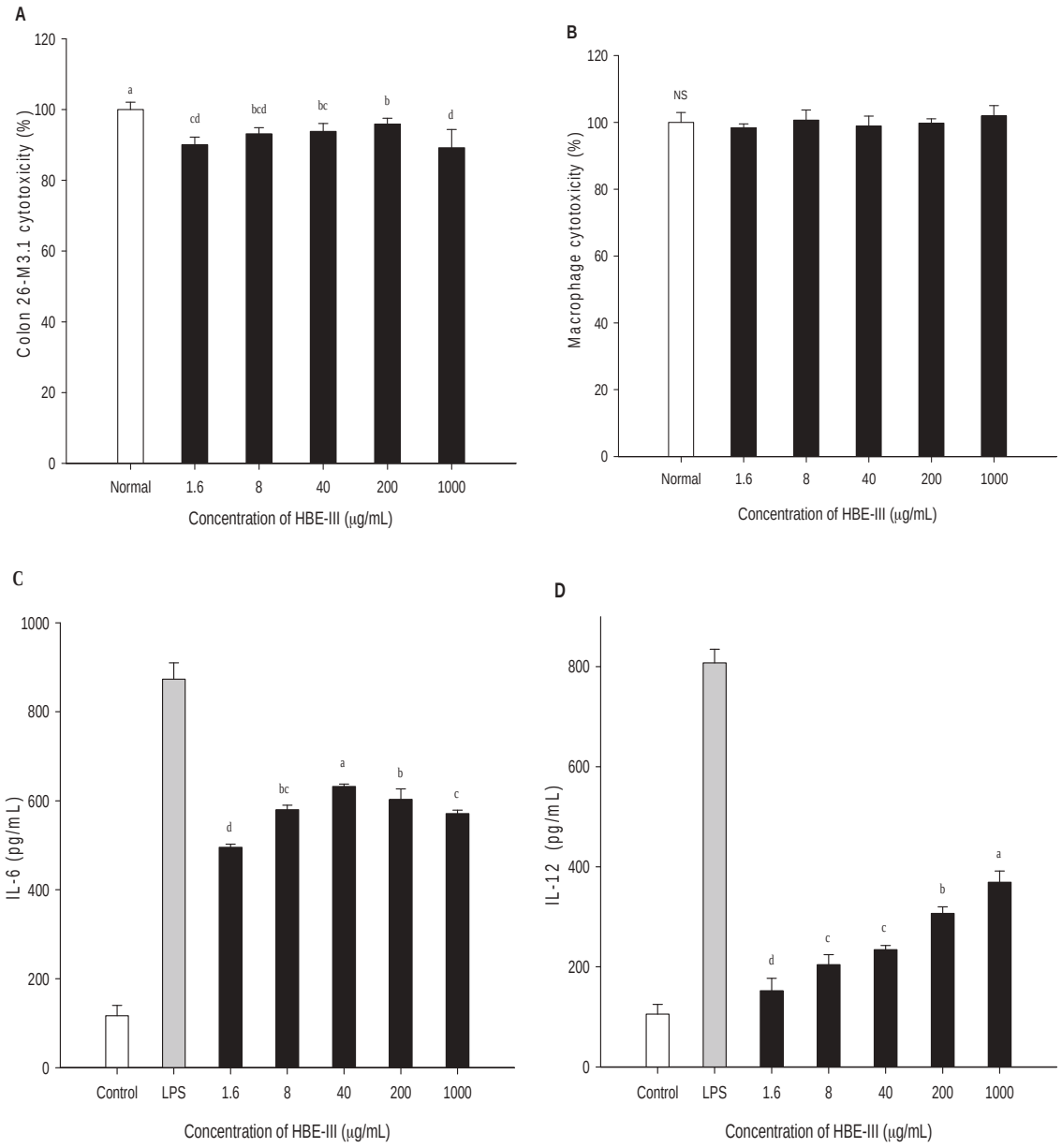

Fig. 3. Cytotoxic effect of (A) macrophages and (B) tumor cells and production of cytokines including (C) IL-6 and (D) IL-12 by HBE-III in murine peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells/well) were treated with samples in 96-well plates for 24 h. Cytotoxity was determined using the CCK-8 based colormetric assay and the concentrations of various cytokines (IL-6 and lL-12) in the culture supernatants were determined using ELISA kits. Closed symbols (●) with lower case letters (a–d) indicate significant differences between groups ($p < 0.05$) as shown by Duncan's multiple range tests.

Table 1
Chemical properties of the purified compound (HBE-III) from the pectinase digests of *Citrus* Hallabong peel.

Chemical composition[a]	HBE-III (%)			
Molecular weight on HPSEC	9.0 kDa			
Neutral sugar	52.8			
Uronic acid	43.0			
Kdo-like	4.2			
Protein	0.0			
Sugar component[b] (Mole%)[c]		Methylation analysis		
		Position of methyl group	Doduced glycosidic linkage	HBE-III (Mole%)
2-Me-Fuc	3.8	3,4	Terminal (*p*)[d]	5.5
Rha	10.2	2,3,4	Terminal (*p*)	3.5
		3,4	2 (*p*)	2.0
		2,4	3 (*p*)	3.7
		4	2,3 (*p*)	0.4
		~	2,3,4 (*p*)	2.4
Fuc	3.2	2	3,4 (*p*)	4.1
2-Me-Xyl	4.6	3,4	Terminal (*f*)[d]	3.5
Ara	12.4	2,3,5	Terminal (*f*)	5.8
		4	2,3 (*p*)	1.7
Xyl	0.0			
Api	2.2	2,3-	3′ (*f*)	8.3
		~	2,3′ (*f*)	2.4
AceA	2.5	3	2 (*f*)	0.3
Man	0.8			
Gal	9.8	2,3,4,6	Terminal (*p*)	4.2
		2,3,4	6 (*p*)	1.3
		3,6-	2,4 (*p*)	3.7
Glc	3.3			
GalA	37.6	2,3,4	Terminal (*p*)	10.8
		2,3	4 (*p*)	14.3
		3	2,4 (*p*)	7.2
		2	3,4 (*p*)	7.2
GlcA	5.4	3,4	2 (*p*)	3.6
Dha	1.8	2,4,6,7	5 (*p*)[e]	2.0
Kdo	2.4	2,4,6,7,8	5 (*p*)[e]	2.2

[a] Based on dry material.
[b] Sugar components were analyzed using alditol acetates by GC.
[c] Mole% calculated from the detected total carbohydrate.
[d] (*p*) and (*f*) mean pyranoside and furanoside, respectively.
[e] The linkages of Kdo and Dha residues were determined in a separate experiment.

3.4. *Effect of HBE-III on the production of IL-6 and IL-12 by peritoneal macrophages*

Activated macrophages release various cytokines, such as interleukin (IL)-12, IL-6, IL-1β, and tumor necrosis factor (TNF)-α; these cytokines induce immune responses to elicit potent antitumor or antimetastatic activities (Kounsar, Rather, Ganai, & Zargar, 2011). Therefore, we examined the effect of HBE-III on the secretion of IL-6 and IL-12 by incubating peritoneal macrophages with doses up to 1000 μg/mL. As shown in Fig. 3, the treatment of peritoneal macrophages with HBE-III significantly increased the production of IL-12 in a dose-dependent manner (Fig. 3D). At a concentration of 1000 μg/mL, HBE-III caused a 248% increase in IL-12 production, compared to the untreated cells. Many *in vitro* studies have shown that cytokines such as IL-2, IL-12, and IL-18 can activate resting NK cells (Shida, Suzuki, Kiyoshima-Shibata, Shimada, & Nanno, 2006). Activated NK cells can lyse or inhibit metastasis and growth of a wide variety of tumor cells (Huang et al., 2005). IL-12 is produced mainly by macrophages and is a NK cell-stimulatory factor (Munder, Mallo, Eichmann, & Modolell, 1998). IL-12, a multifunctional cytokine, is critical for eliciting tumor immunity (Munder et al., 1998). The production of IL-6 showed maximal activity at an HBE-III treatment of 40 μg/mL, after which it declined (Fig. 3C). The enhancement of IL-6 production was 324% at 1.6 μg/mL and reached 440% at 40 μg/mL compared to the control group. In addition, the levels of IL-6 were lower than those induced by 5 μg/mL

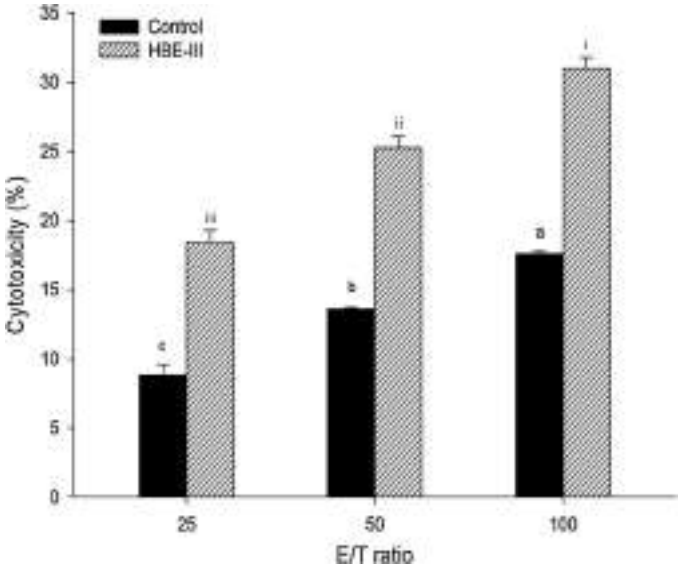

Fig. 4. Effect of HBE-III on the enhancement of NK cell activity. NK cell activity was determined by LDH assay as described in Section 2. Bars with lower case letters (a–c or i–iii) indicate significant differences ($p < 0.05$) as shown by Duncan's multiple range tests.

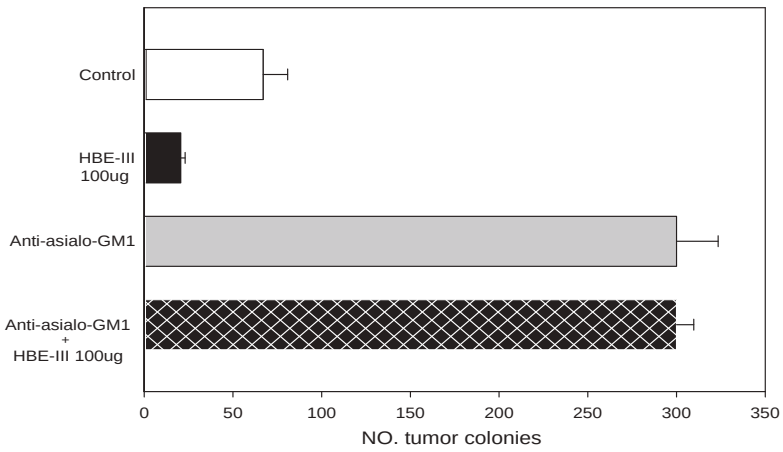

Fig. 5. Effect of NK cell depletion on HBE-III-induced inhibition of lung metastasis. To deplete NK cells *in vivo*, mouse anti-asialo GM1 serum was injected into mice 1 and 3 days before inoculation of colon 26-M3.1 carcinoma cells. Mice were treated with HBE-III (100 μg, *i.v.*) 2 days before tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.

lipopolysaccharide (LPS), which indicates that inflammation pathways were not involved. IL-6 is also considered to be a major immune and inflammatory mediator (Tanigawa, Craig, Stoolman, & Chang, 2000). As an inflammatory cytokine, IL-6 plays an important role in activating T cells and rejecting tumor cells (Cheng, Wan, Wang, Jin, & Xu, 2008). These results suggest that HBE-III can stimulate cytokine production in macrophages and enhance macrophage-mediated cytotoxicity, which may explain its antitumor activity (Park, Lee, Cho, Kim, & Shin, 2012). Thus, the antitumor effect of HBE-III may be related to its potentiation of IL-6 and IL-12 production in tumor-bearing mice.

3.5. Effect of HBE-III on NK cell activity

We then examined the effect of HBE-III on NK cell activity based on cytotoxicity against Yac-1 cells; splenocytes obtained from the mice treated (*i.v.*) with HBE-III (1000 μg/mL) 3 days before the assay showed a higher cytotoxicity than those from the untreated group, in an E:T ratio-dependent manner (Fig. 4). NK cells are known to be an important effector to suppress tumor growth and metastasis, and activated macrophages and NK cells are known to be the effectors responsible for natural immunity against tumors (Barlozzari, Leonhardt, Wiltrout, Herberman, & Reynolds, 1985). Therefore, we next addressed whether the inhibitory effect of HBE-III (100 μg) on tumor metastasis was associated with NK cell activity. Pretreatment with anti-asialo GM1 serum, which selectively eliminates NK cells, enhanced the frequency of experimental lung metastasis (Fig. 5). The removal of NK cells partially abolished the antitumor effect of HBE-III on lung metastasis of Colon 26-M3.1 carcinoma cells, indicating that the inhibitory effect of HBE-III on tumor metastasis was mediated by NK cell activation. In brief, HBE-III enhanced NK cell activity, and *in vivo* removal of NK cells abrogated the antitumor effect of HBE-III. NK cells are best known for their capacity to kill tumor cells and there is evidence for their role in controlling infection in the earliest phases of immune responses. One of the primary objectives of immunomodulation is to enhance the number and vitality of NK cells (Yi et al., 2012). Hence, the activation of NK cells is a good indicator of the antitumor properties of a compound. Thus, our results suggest that the polysaccharide fraction, HBE-III, from Hallabong peel inhibited lung metastasis produced by Colon 26-M3.1 carcinoma cells, and NK cell activation followed by the production of cytokines from macrophages by treatment with HBE-III play essential roles in the prevention of tumor cell metastasis in the lung.

4. Conclusions

Tumor metastasis is a major cause of death in cancer patients and is defined as a series of discrete biological processes that lead to the spread of tumor cells from the primary neoplasm to a distant location. However, the treatment of metastasis is still far from satisfactory, due in part to a lack of effective drugs. Thus, it is important to find new promising agents with antimetastatic activity for improving the management of cancer. Meanwhile, Korean *Citrus* Hallabong fruits are used for juice manufacture and their peels, the primary byproduct, are usually discarded. However, citrus peels are an attractive source of phytochemicals, which have health benefits. No previous study has reported the isolation of phytochemical constituents from Hallabong peels. Therefore, we sought to develop new physiologically active materials from Korean *Citrus* Hallabong peels, the main byproduct in Hallabong juice industry. Polysaccharides were isolated from pectinase digests of Hallabong peels and their chemical characteristics and antimetastatic activities were examined. The crude polysaccharide mixture from Hallabong peels was successfully purified to yield four fractions (HBE-I, HBE-II, HBE-III, and HBE-IV). Among the purified fractions, HBE-III exhibited a high yield and the polysaccharide was composed of 52.8% neutral sugars, 43.0% uronic acid, and 4.2% Kdo-like material. Interestingly, HBE-III contained unusual sugars that are characteristic of RG-II. The polysaccharide fraction (HBE-III) of Hallabong peels significantly inhibited lung metastasis and markedly increased the levels of IL-6 and IL-12 from peritoneal macrophages. Furthermore, HBE-III significantly augmented NK cytotoxicity against Yac-1 tumor cells. However, the depletion of NK cells by anti-asialo GM1 serum abolished the inhibitory effect of HBE-III on lung metastasis of Colon 26-M3.1 cells. In addition, HBE-III has low toxicity and side effects toward normal cell and may have potential use as a novel therapeutic agent. Thus, these results suggest that the polysaccharide fraction (HBE-III) of Korean *Citrus* Hallabong peels showed antitumor activity by improving immune system functions. HBE-III could act as an antitumor agent with immunomodulatory activity and could be further explored as a novel therapeutic agent.

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