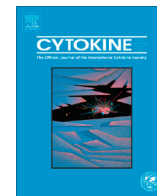




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Therapeutic effect of Active Hexose-Related Compound (AHCC) combined with CpG-ODN (oligodeoxynucleotide) in B16 melanoma murine model

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ABSTRACT

While Active Hexose Related Compound (AHCC) and CpG oligodeoxynucleotide (ODN) are separately known to modulate oxidative stress and immune responses in cancer patients, the combined effect of these two compounds is unknown. To clarify this, we investigated whether AHCC plus KSK-CpG ODN would be therapeutic in B16 melanoma mouse model, if so, and how in reduction-oxidation (redox) balance and cytokines network. We found that treatment groups (AHCC only, KSK-CpG ODN only and AHCC/KSK-CpG ODN) markedly reduced ($p < 0.001$) tumor size when compared to the positive control (PC) group. The total white blood cell (WBC) of AHCC only and KSK-CpG ODN only-treated groups showed significant lower counts than that of PC group. Next, the production of nitric oxide (NO) was significantly increased ($p < 0.01$) in AHCC/KSK-CpG ODN group compared to the PC group. Further, the redox balance was improved in AHCC/KSK-CpG ODN group through significantly low ($p < 0.001$) reactive oxygen species (ROS) production and significantly high ($p < 0.05$) glutathione peroxidase (GPx) activity compared to the PC group. Finally, AHCC/KSK-CpG ODN ($p < 0.01$) and KSK-CpG ODN ($p < 0.001$)-treated groups augmented tumor immune surveillance as shown by significantly increased level of anti-inflammatory cytokine (IL-10) and significantly decreased ($p < 0.05$) level of pro-tumorigenic IL-6 of AHCC/KSK-CpG ODN treated group as compared to the PC group. Collectively, our study indicates therapeutic effect of Active Hexose-Related Compound (AHCC) combined with KSK-CpG ODN in B16 melanoma murine model via balancing redox and cytokines network.

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1. Introduction

The annual report on the status of cancer in the United States shows that death rates continued to increase from 2000 through 2009 for melanoma of the skin and for the other types of cancers such as liver, pancreas and uterus [1]. Report showed that from 2001 to present, cancer is the leading cause of mortality in South Korea [2]. Continual rise on the number of cancer mortality underscore the need for additional prevention efforts and demands for the need to develop a more effective chemotherapeutic drugs.

While a wide array of anticancer drugs has been used, currently, these have shown to exhibit side effect such as normal tissue damage owing to the lack of specificity against cancer cells [3]. Thus, the medical research is in need of immunotherapies, which are capable of protecting and boosting the activity of normal cells such as the immune cells to fight against the cancer cells.

Of such potential candidates, as most well-known of the edible fungi family, *Lentinus edodes* has been reported to be effective towards hyperlipidemia [4], obesity [5] and cancer [6]. Active hexose correlated-compound (AHCC) is an extract obtained from *L. edodes* of Basidiomycetes family that is commercially available in Japan as a supplement for cancer patients who are undergoing chemotherapy. AHCC has been proven to stimulate cytokines (IL-2, IL-6, IL-12 and TNF- α) [7] and boost the immune system as an immunomodulator [8–10]. In addition, supplementation of AHCC showed various positive effects against cancer, such as

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decreased tumor formation [11] and increased immune response [8,10]. Furthermore, AHCC-treated primary cultured rat hepatocytes have shown an increased value of nitric oxide (NO) production, an indicator for macrophage activity [12], while treatment of AHCC in C57BL/6 murine melanoma models have shown an increase in Ag-specific activation, such as CD4+/CD8+ and IFN- γ values [11]. Aside from immune boosting activity, reactive oxygen species (ROS) scavenging effect *in vitro* was reported in breast cancer cell line [13]. However, ROS scavenging effect *in vivo* is unknown. CpG oligodeoxynucleotide (ODN) has been well known to induce tumor regression by activating innate immunity and serving as immunologic adjuvant for established cancer immunotherapy [14]. Our novel designed KSK-CpG ODN is an active cancer immunotherapeutic agent against highly malignant tumor challenged model [15,16]. While AHCC and CpG ODN are separately known to modulate oxidative stress and immune responses in cancer patients, the combined effect of these two compounds is unknown. To clarify this, we investigated whether AHCC plus KSK-CpG ODN would be effective in B16 melanoma mouse model, if so, how in redox balance and cytokines network.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Hyclone Laboratories Incorporated (South Logan, UT). The antibiotic–antimycotic was obtained from Gibco Invitrogen Corporation (Auckland, NZ). The B16-F10 melanoma cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). AHCC (Lot No. E26-0312-2) was a donation from KCF Korea (Korea) after purity checked and was dissolved in deionized distilled water to a final concentration of 100 mg/ml. The component of the freeze-dried AHCC extract was already checked and comprised mostly of oligosaccharides, comprising around 70% of carbohydrates, of which 28% is alpha-1,4-glucan type [17]. Thus, the purity of AHCC is at least more than 99%. The LPS contamination of AHCC was negligible as previously reported [17]. KSK-CpG ODN was provided by MWG-Biotech AG (Ebersberg, Germany) and contained <0.1 EU/ml of endotoxin as determined by the Limulus assay by Bio-Whittaker (Walkersville, MD, USA) and the fluorescent probe, 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA) was acquired from Sigma Chemical Corporation (St. Louis, MO, USA). Glutathione peroxidase (GPx) activity assay kit was obtained from Biovision Incorporated (Milpitas, CA, USA). Griess reagent was purchased from Promega Corporation (Fitchburg, WI, USA). IL-1 β , IL-6, IL-10, IL-12 (p70), IFN- γ , and TNF- α multiplex kit was purchased from Bio-Rad (San Diego, CA, USA).

2.2. Cell culture

B16-F10 melanoma cell was maintained in the RPMI 1640 medium supplemented with 10% FBS and 1% Antibiotic–Antimycotic in a humidified incubator with 5% CO₂ and 37 °C incubator.

2.3. Maintenance of animals and inoculation of B16-F10 melanoma cell

Seven-week-old female C57BL/6 mice, weighing 18–20 g, obtained from Orient Bio Inc. (Seongnam, South Korea) were maintained in a pathogen-free condition at 22 \pm 2 °C and 40–60% humidity under a 12 h:12 h light dark cycle and fed with standard commercial diet. Animals were inoculated subcutaneously with 1 \times 10⁵ viable B16-F10 melanoma cells. Sizes of the tumor were

measured from 7 days after the inoculation. The animal use and care protocol for this animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC), Yonsei University Wonju Campus.

2.4. Growth and supplementation of AHCC/KSK-CpG ODN

One week after B16-F10 melanoma cell inoculation (Day 0), mice were observed to develop tumors. Mice were randomly divided into four groups: AHCC/KSK-CpG ODN group was supplemented with AHCC and KSK-CpG ODN; AHCC group was given AHCC alone; KSK-CpG ODN group was given KSK-CpG ODN alone, while the positive control group (PC) did not receive the AHCC or KSK-CpG ODN. In this study, we also added a normal group (NC) which was not inoculated with B16-F10 melanoma cell and did not receive the AHCC or KSK-CpG ODN treatment. Thereafter, the treatment with AHCC was initiated followed by intraperitoneal (i.p) injection of KSK-CpG ODN (Day 1). Briefly, AHCC was dissolved in deionized distilled water with a final concentration of 100 mg/ml. The dosage used in this study was optimized according to the individual weight of the mouse. AHCC was given daily by oral administration with a final volume of 100 μ L per mouse. While the KSK-CpG ODN group was injected i.p at a final concentration of 200 μ g/ml with a final volume of 100 μ L per mouse twice a week. Used KSK-CpG ODN has this following sequence (5'-TCGTCGTTTCGTCGTCGTTT-3'). The treatment with AHCC and KSK-CpG ODN, alone or in combination was carried out for 2 weeks. Tumor measurements were taken 7 days and 14 days after the treatment using a digital caliper. The longest longitudinal dimension (length) and the greatest traverse dimension (width) were determined. The final tumor volume in mm³ is calculated by the formula: $\frac{4}{3} \times \pi r^3$ where $r = (\text{length} + \text{width})/4$ as described elsewhere [18]. Mice were sacrificed 2 weeks (Day 14) after the treatment of AHCC and KSK-CpG and blood samples were collected for further analysis.

2.5. Analysis of blood samples

Mice were sacrificed through inhalation of diethyl ether for 10 s. Thereafter, blood was collected from the retro-orbital plexus and immediately put in tubes coated with an anticoagulant. The blood was mixed with an automatic mixer for 5 min. Then, the total white blood cell (WBC) and their differential counts (neutrophil, lymphocytes, monocytes, eosinophil and basophil) were determined by an automatic blood analyzer (HEMAVET HV950 FS, Drew Scientific Inc., Dallas, TX, USA). While other portions of the blood were collected and put in a commercially available red topped tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were allowed to clot for 30 min at room temperature. The clot was removed by centrifugation at 14,000 rpm for 5 min in a refrigerated centrifuge. The resulting supernatant (serum) was used for further analysis such as ROS, NO, GPx, and cytokines.

2.6. Detection of reactive oxygen species generation

The generation of intracellular ROS was detected using fluorescent probe H₂DCF-DA. Briefly, 50 μ L serum was loaded with 10 μ M of H₂DCF-DA from a stock solution of DMSO. After 30 min of loading at 37 °C incubator protected from light, serum was centrifuged and wash with 1X PBS to remove extra H₂DCF-DA. Finally, the intracellular ROS was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a DTX-880 multimode microplate reader (Beckman Coulter Inc., Fullerton, CA, USA).

2.7. Glutathione peroxidase activity assay

GPx activity in serum was measured for H₂O₂ scavenging capacity by GPx Activity Assay Kit according to the manufacturer's instruction. Absorbance was determined at 340 nm by a DTX-880 multimode microplate reader (Beckman Coulter Inc., Fullerton, CA, USA).

2.8. Production of nitric oxide assay

The NO levels in serum were spectrophotometrically assessed using Griess reagent. The absorbance at 540 nm was measured and the NO concentration was determined using a calibration curve with sodium nitrite as a standard chemical.

2.9. Cytokine assay

Serum concentrations of selected cytokines, including IL-1 β , IL-6, IL-10, IL-12p70, IFN- γ , and TNF- α were analyzed. Briefly, serum samples were assessed using a Luminex Bead-Based Suspension Array System (Bio-Rad, San Diego, CA, USA) according to the manufacturer's instructions. The plate was run on a Bio-Plex bead suspension array system by Luminex 200 Bio-Plex Instrument (Bio-Rad Hercules, CA, USA) and the raw fluorescence data were analyzed by the Bio-Plex Manager software using 5-parameter logistic method and xMAP technology.

2.10. Statistical analysis

Data values were expressed as mean \pm SEM. The mean values among groups were analyzed and compared by one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey), while for those experiments where the effects of more than one factor are considered, two-way ANOVA followed by Bonferroni posttest after confirming normal distribution was used with GraphPad Prism version 5.0 software packages (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3. Results

3.1. Body weight and tumor size

After 5 days of tumor inoculation, there was a loss of body weight among the groups, interpreted as immuno suppressed condition because of cancer progression. However, 14 days after the tumor inoculation, all groups showed regained body weight loss. Changes in body weight in each group are arranged in ascending order: NC < AHCC < AHCC/KSK-CpG ODN < KSK-CpG ODN < PC (Table 1). All groups showed similar tumor size after the first seven days of treatment. However, after 14 days of treatment, treated groups (AHCC, KSK-CpG ODN and AHCC/KSK-CpG ODN) showed significant reduced ($p < 0.001$) size of tumor when compared to the profoundly increased PC (Table 2). The percentage of change

Table 2
Tumor size and percent (%) size change.

Groups	7 days (mm ³)	14 days (mm ³)	Size change (%)	Size change in respect to PC (%)
PC	70.68 \pm 12.33	445.5 \pm 250.5	630.3 (↑)	100.0
AHCC	70.74 \pm 16.07	48.54 \pm 25.99***	31.40 (↓)	10.9
KSK-CpG ODN	61.32 \pm 13.27	57.15 \pm 23.19***	6.800 (↓)	12.8
AHCC/KSK-CpG ODN	64.58 \pm 17.05	56.10 \pm 27.98***	13.13 (↓)	12.6

Abbreviations: NC, normal control; PC, positive control; AHCC, active hexose correlated compound; KSK-CpG ODN, oligodeoxynucleotide. Data are mean \pm SEM, $n = 8$. Legend: (↑), increase; (↓), decrease.

*** Bonferroni posttest $p < 0.001$ PC vs positive control (PC) after 14 days.

in tumor size after 14 days of treatment are arranged in descending order: AHCC > AHCC/KSK-CpG ODN > KSK-CpG ODN > PC. Moreover, the change in size with respect to PC was also calculated, as shown in Table 2.

3.2. Hematological analysis

The total WBC of AHCC and KSK-CpG ODN treated groups showed significantly lower count than that of PC (Table 3). This may suggest that the WBC of AHCC and KSK-CpG ODN treated groups had already sensed the intruder, cancer cells, and rushed to the site of the tumor colonization.

3.3. Nitric oxide production

NO production was determined via measuring the concentration of nitrite in the serum. The AHCC/KSK-CpG ODN group showed a significant increase ($p < 0.01$) in the production of nitric oxide compared to the PC group (Fig. 1).

3.4. ROS production and endogenous enzyme activity

H₂DCFH-DA was used as a reporter of intracellular ROS. The total ROS production in AHCC/KSK-CpG ODN-treated group was significantly lower ($p < 0.001$) than that of the PC group. Furthermore, KSK-CpG ODN treated group alone showed a significant decrease in ROS ($p < 0.01$) compared to the PC group (Fig. 2a). GPx provides protection against the damaging effect of ROS such as hydro peroxides. The activity of the endogenous enzyme GPx was significantly increased ($p < 0.05$) in AHCC/KSK-CpG ODN group compared to the PC group (Fig. 2b).

3.5. Levels of pro-inflammatory and anti-inflammatory cytokines

The protein concentration of anti-inflammatory cytokine (IL-10) was significantly increased in KSK-CpG ODN ($p < 0.001$) and

Table 1
Effect of AHCC/KSK-CpG ODN treatment on body weight.

Group	0 day	5 days	10 days	14 days
NC	20.00 \pm 0.00	19.575 \pm 0.206	19.975 \pm 0.263	20.025 \pm 0.435
PC	20.563 \pm 0.904	20.125 \pm 0.891	20.263 \pm 1.088	20.675 \pm 0.865
AHCC	20.250 \pm 0.802	20.013 \pm 0.546	19.950 \pm 0.444	20.025 \pm 0.552
KSK-CpG ODN	20.313 \pm 0.753	19.638 \pm 0.883	19.838 \pm 0.958	20.050 \pm 0.754
AHCC/KSK-CpG ODN	19.938 \pm 0.623	19.450 \pm 0.431	19.613 \pm 0.601	19.688 \pm 0.800*

Abbreviations: NC, normal control; PC, positive control; AHCC, active hexose correlated compound; KSK-CpG ODN, oligodeoxynucleotide. Data are mean \pm SEM, $n = 8$.

* Bonferroni posttest $p < 0.05$ PC vs AHCC/KSK-CpG ODN after 14 days.

Table 3
Total white blood cells (WBC) and their differential count.

WBC and members	NC	PC	AHCC	KSK-CpG ODN	AHCC/KSK-CpG ODN
Total WBC, $\times 10^9/L$	5.045 \pm 1.436	4.338 \pm 1.523	3.395 \pm 1.737**	3.592 \pm 0.761*	3.474 \pm 0.789*
Neutrophil, $\times 10^9/L$	1.053 \pm 0.597	1.125 \pm 0.425	0.707 \pm 0.295	0.887 \pm 0.207	0.697 \pm 0.139*
Lymphocyte, $\times 10^9/L$	3.577 \pm 0.821	2.945 \pm 0.946	2.404 \pm 1.061	2.560 \pm 0.583	2.641 \pm 0.684
Monocyte, $\times 10^9/L$	0.195 \pm 0.120	0.164 \pm 0.082	0.104 \pm 0.021	0.127 \pm 0.045	0.121 \pm 0.054
Eosinophil, $\times 10^9/L$	0.165 \pm 0.186	0.081 \pm 0.102	0.021 \pm 0.021	0.014 \pm 0.009	0.010 \pm 0.010
Basophil, $\times 10^9/L$	0.058 \pm 0.082	0.034 \pm 0.041	0.011 \pm 0.012	0.004 \pm 0.005	0.019 \pm 0.003

Abbreviations: NC, normal control; PC, positive control; AHCC, active hexose correlated compound; KSK-CpG ODN, oligodeoxynucleotide. Data are mean \pm SEM., $n = 8$.

* Nonparametric Tukey's multiple comparison test $p < 0.05$ versus PC.

** Nonparametric Tukey's multiple comparison test $p < 0.01$ versus PC.

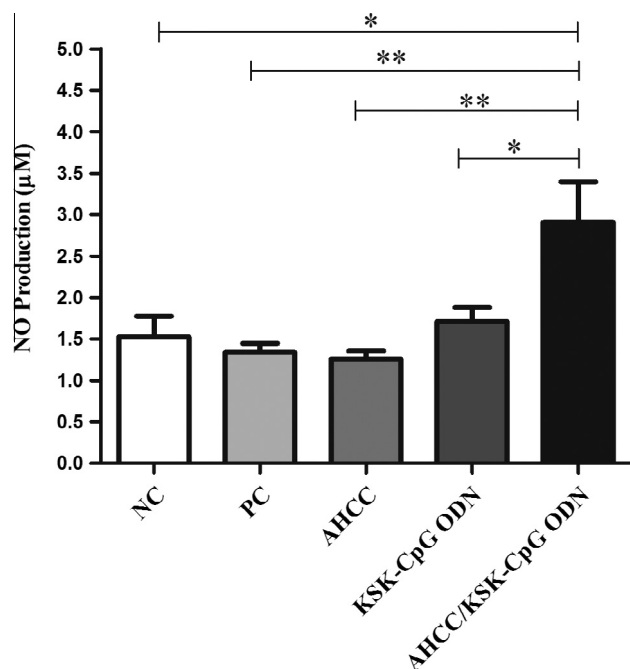


Fig. 1. The level of nitric oxide (NO) in melanoma mice orally administered with active hexose correlated compound AHCC/CpG for 14 days. The nitrite present in the mice serum was used as an indicator of NO production using the Griess reagents at indicated time point (10 min). The absorbance was determined at 540 nm using microplate reader. Positive control (PC) group showed lower NO production compared to the significantly higher NO production of AHCC/KSK-CpG ODN group. Values are mean \pm SEM, $n = 8$. ** $p < 0.01$ vs. positive control.

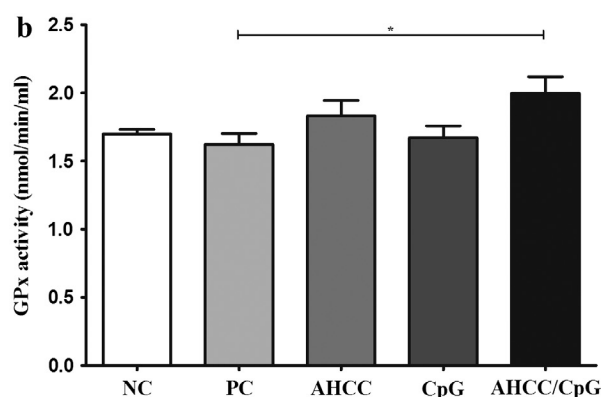
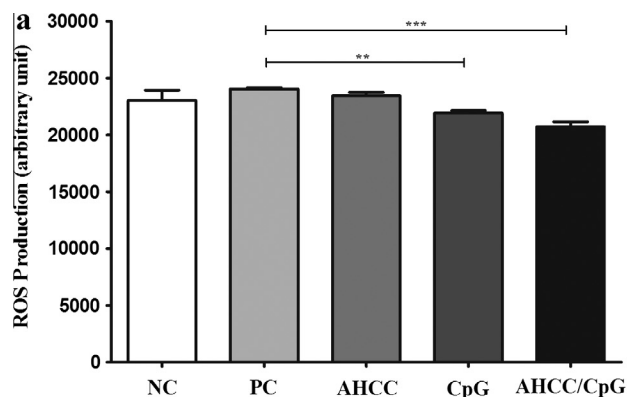


Fig. 2. Effect of active hexose correlated compound AHCC/KSK-CpG ODN on ROS (a) and endogenous enzyme glutathione peroxidase activity (b). Mice sera were stained with $H_2DCF-DA$ to detect intracellular reactive oxygen species (ROS) production using a fluorometric microplate reader. Positive control (PC) had higher ROS production compared to the significantly lower ROS production of AHCC/KSK-CpG ODN. KSK-CpG ODN treated group also showed significantly decreased ROS generation. Glutathione peroxidase (GPx) activity of mice serum was directly assayed and PC group showed lower activity, while the group treated with AHCC/KSK-CpG ODN showed increased activity. Values are mean \pm SEM, $n = 8$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. positive control.

AHCC/KSK-CpG ODN ($p < 0.01$) treated group as compared to the PC group (Fig. 3d). The level of pro-tumorigenic IL-6 in AHCC/KSK-CpG ODN treated group was significantly decreased ($p < 0.05$) as compared to the PC group (Fig. 3c). However, the levels of IL-1 β , TNF- α , IL-12p70 and IFN- γ showed no statistically significant changes compared to the control groups (NC, PC) (Fig. 3).

4. Discussion

Our study indicates positive effect of AHCC combined with KSK-CpG ODN in B16 melanoma murine model via balancing redox and cytokines network. First, to prove antitumor activity, we measured tumor size in each group. Oral administration of AHCC followed by intraperitoneal injection of KSK-CpG ODN significantly reduced tumor size (Table 2). This is consistent with the other groups receiving AHCC alone and KSK-CpG ODN alone when compared to the dramatic increase in tumor size of the PC group (Table 2). Our results are in agreement with previous report that treatment with AHCC delayed melanoma and lymphoma formation and reduced tumor size [11]. In line with this, our previous study showed that KSK-CpG ODN, new toll-like receptor (TLR) 9 agonist is able to block the lung metastasis of B16 melanoma [15]. Taken together, our results clearly indicate that combination therapy of AHCC plus immunological adjuvant, KSK-CpG ODN is effective in B16-F10 melanoma murine model. This is the first report on combination therapy of functional food plus toll-like receptor (TLR) 9 agonist in tumor model.

To delineate the moleculo-cellular mechanism such as immunity and redox balance of combined treatment with AHCC plus KSK-CpG ODN in murine melanoma cancer model, we designed

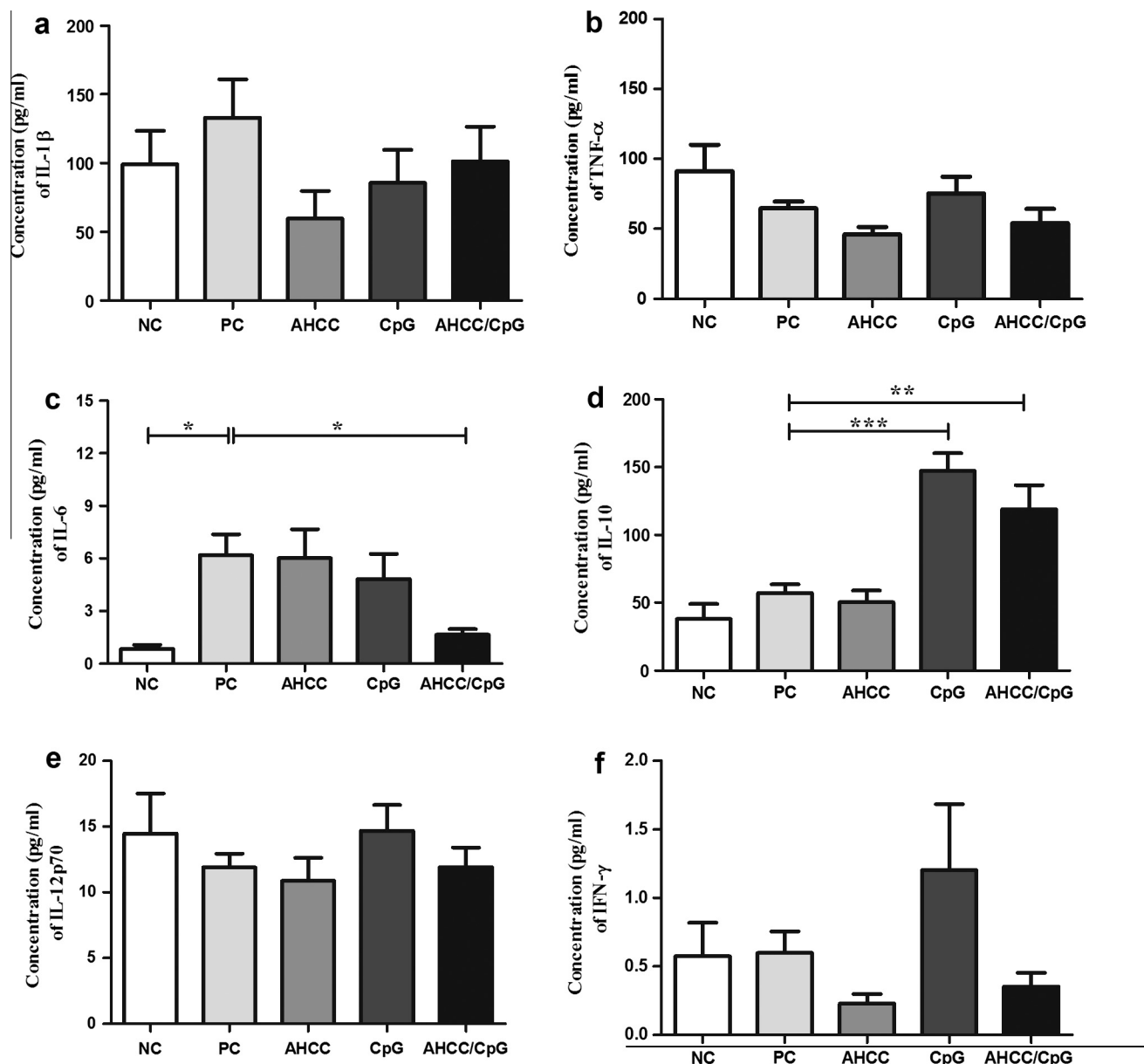


Fig. 3. Quantitative level of pro-inflammatory and anti-inflammatory cytokines treated with active hexose correlated compound AHCC/KSK-CpG ODN from melanoma mice. Selected cytokines such as IL-1 β (a), TNF- α (b), IL-6 (c), IL-10 (d), IL-12p70 (e) and interferon- γ (f) in serum level were measured using a Luminex Bead-Based Suspension Array System. Increased protein level of IL-10 (d) was detected in KSK-CpG ODN and AHCC/KSK-CpG ODN-treated mice. However, protein level of IL-6 (c) in AHCC/KSK-CpG ODN-treated mice showed significantly decreased compared to Positive Control (PC) group nonetheless comparable to the Negative Control (NC) group. Values are mean \pm SEM, $n = 8$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to corresponding controls (PC and NC).

the redox and immunologic assay. One of the hallmarks of cancer cells is highly-proliferating cell, in turn requiring high ROS concentration to meet this demand [19]. Low-level of ROS is crucial for signal transduction before their elimination. In this study, we measured ROS production and the endogenous antioxidant (GPx) activity. AHCC/KSK-CpG ODN group showed equilibrium between the ROS and glutathione peroxidase, an endogenous antioxidant. This is strongly supported by significant lower ROS production and higher activity of glutathione peroxidase. However, the positive control group showed the imbalance between pro-oxidant (ROS) and anti-oxidant, which might lead to oxidative stress. Furthermore, this imbalance between pro-oxidant/anti-oxidant favors the alteration and damage of molecules such as DNA, RNA, lipids and proteins hence leading to the mutations driving carcinogenesis [20]. Acharya and colleagues (2010) proposed that abnormal increased of ROS abolished the redox balance and promote

tumor formation by initiating an aberrant induction of signaling networks that cause tumorigenesis [21]. In relation to this, positive group has higher ROS level and was shown to have dramatic increase of tumor size. By contrast, AHCC/KSK-CpG ODN group showed significantly lowest ROS production and highest GPx activity. Considering the reduced tumor size, our results suggest that AHCC/KSK-CpG ODN treatment would provide protection against melanoma through recovering redox balance. This has an important implication that our combined therapy with medicinal food plus TLR9 agonist would be synergistic in alleviating tumor or drug-induced oxidative status, finally relieving the toxicity as well as increasing quality of life. However, this remains to be further verified.

Next, we sought to unveil the potential immune mechanism of synergistic treatment with AHCC/KSK-CpG ODN by measuring cytokine release and nitric oxide production. The development of

cancer is not solely influenced by the status of oxidative stress, but also inflammation. Cytokines are implicated to control various immune functions including cancer [22]. In contrast, some cytokines serve as a niche on premalignant cells, favoring their growth and survival, and others are pro-tumorigenic, which would contribute to maintaining the tumor-promoting inflammation [23,24]. In that context, to delineate the effect of AHCC/KSK-CpG ODN on cytokine network, we measured several cytokine concentrations using a multiplex-bead array system for the highest sensitivity and precision. Of note, the pro-tumorigenic cytokine IL-6 was significantly decreased in AHCC/KSK-CpG ODN group when compared to the positive control group. Cumulative studies showed that IL-6 is one of the best featured pro-tumorigenic cytokines, involved in cancer owing to its ability to activate the oncogenic transcription factors such as NF- κ B, and STAT3 in epithelial cells [25–29]. Moreover, IL-6 is implicated in the growth of cancer cell lines or primary tumors [30]. Accordingly, we found that the AHCC/KSK-CpG ODN group showed significantly increased level of IL-10, a potent anti-inflammatory cytokine when compared to the positive control group. While in our B16 melanoma model, IL-10 level is evidently the highest of all groups, supporting known reports, AHCC/KSK-CpG ODN group showed a significant increase in the level of the potent anti-inflammatory cytokine, IL-10. Mumm JB and colleagues (2011) demonstrated that IL-10 induces essential mechanism for effective antitumor immune surveillance and is required against the incidence and progression of endogenously arising skin tumors [31]. In line with this, we found that the concentration of IL-10 is inversely correlated to the percent change in tumor size. KSK-CpG ODN treatment alone and combined treatment of AHCC/KSK-CpG ODN showed significantly increased concentration of IL-10 and showed significantly reduced tumor size, consequently indicating a significant correlation between two parameters (data not shown). This suggests that IL-10 might inhibit the tumor progression however, this remains to be further verified. Previous study demonstrated that IL-10 gene transfer in murine mammary tumor cells increased the expression of the inducible nitric oxide synthase (iNOS) which leads to the elevated levels of nitric oxide [32]. In parallel, we found a significant increase in the nitric oxide production in AHCC/CpG group compared to that in the positive control group. These might be linked to the significant increase in IL-10 level. Nitric oxide is able to exert a potent anti-tumor property through tumor cell lysis, apoptosis, and inhibition of tumor proliferation [33–36]. Several lines of evidences showed that higher concentration of NO can induce apoptosis in tumor cells [37,38]. Viewed together, our data reveal the potential therapeutic effect of AHCC as anti-cancer natural product combined with KSK-CpG ODN through preventing inflammation-promoted cancer cell growth, inhibiting the expression of pro-tumorigenic cytokine (IL-6), inducing the potent anti-inflammatory cytokine (IL-10), and boosting antitumor property (nitric oxide).

Another clinical implication of this study is that combined therapy with AHCC/KSK-CpG ODN would be effective against highly immunogenic tumor such as melanoma. Our conjecture is that the positive effect of AHCC/KSK-CpG ODN on cancer might be attributable to the oligosaccharides (comprising around 70% of carbohydrates, of which 28% is alpha-1, 4-glucan type), amino acids, lipids and minerals. In addition, previous findings explored the potential of AHCC as potent antioxidant [39,40] and suggested that AHCC does not scavenge ROS directly; instead it may stimulate certain antioxidant mechanisms *in vivo* [40]. Parallel to this, in our present study, we showed that treatment with AHCC alone did not reduce the ROS production, however has enhanced the activity of the endogenous enzyme glutathione peroxidase. From the point of immunologic biomarker (NO, cytokines), AHCC showed weaker activity. By contrast, KSK-CpG ODN, as verified in NO and cytokines

profiles, showed higher immunomodulation than AHCC, albeit with little redox activity. KSK-CpG ODN designed for human usage was previously demonstrated to have an anti-cancer activity in B16 melanoma and EL4 lymphoma murine model by way of augmenting Th1 cytokine release, the activity of NK cell and cytotoxic T lymphocyte [15]. Given these, the combined therapy would significantly complement and further enforce the positive effects of KSK-CpG ODN against melanoma in the context of immune augmentation as well as redox balance. However, despite the positive evidence of AHCC and KSK-CpG ODN on cancer, the exact mechanism of combined action is still unclear. Further combination therapy with different dose and schedule in different tumor model is imperative for preclinical and clinical application.

5. Conclusion

Collectively, our study indicates therapeutic effect of Active Hexose-Related Compound (AHCC) combined with KSK-CpG ODN in B16 melanoma murine model via balancing redox and cytokines network.

Author disclosure statement

The authors declare no competing financial interests.

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