

Active Hemicellulose Compound (AHCC)

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Combination therapy of Active Hexose Correlated Compound Plus UFT Significantly Reduces the Metastasis of Rat Mammary Adenocarcinoma

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Abstract

Synergistic effects of active hexose correlated compound (AHCC) extracted from mushroom on the treatment with UFT against mammary adenocarcinoma, SST-2 cells, In congenitally T cell-depressed spontaneously hypertensive rats (SHR) were observed. AHCC plus UFT had slighted but significant effects on the growth of primary tumors. Pulmonary metastases were not inhibited by the treatment with AHCC plus UFT, whereas metastases to axillary lymph nodes (LN) were obviously inhibited. Combination of AHCC plus UFT showed similar synergistic anti-metastatic effects in SHR rats with accelerated pulmonary metastases following the surgical removal of the primary tumors, in vitro studies demonstrated that AHCC plus UFT enhanced the NK cell activity In tumor-bearing rats, whereas UFT alone depressed the NK cell activity. AHCC plus UFT also enhanced the NO production and cytotoxicity of peritoneal macrophages. In addition, AHCC re-stored the suppressed mRNA expression of interleukin-1 α and tumor necrosis factor- α induced by the chemotherapy. Taken together, the combination of AHCC plus UFT brought about good therapeutic effects not only on primary tumor growth but also on reducing metastasis and these effects were mediated by host immunity which was restored or activated by AHCC. AHCC may be a good candidate for a biological response modifier. (© 1998 Lippincott-Raven Publishers.)

Key words: Active Hexose Correlated Compound, biological response modifier, mammary adenocarcinoma, and metastasis.

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Introduction

Tumor cells invade from the primary lesion into the blood and/or lymphatic circulation resulting in the

formation of metastases in distant organs as well as in the regional lymph nodes (LN).^{1,2} This property of the tumor cells is mainly responsible for death in cancer, despite advances in cancer treatment. Therefore, treatment of metastases is one of the major targets in cancer treatment. Previous studies demonstrated that chemotherapy with UFT (tegafur and uracil in a 4:1 molar concentration) had significant therapeutic effects on advanced cancers with distant metastases.³⁻⁵ However, therapeutic doses of anti-cancer drugs have been reported to reduce the anti-tumor immune response^{6,7} which inhibits the micro-metastases.⁸⁻¹⁰ Therefore, various biological response modifiers (BRMs) have been used with anti-cancer drugs to restore the anti-tumor immune response reduced by chemotherapy.¹¹⁻¹⁵ Using an *in vivo* model system, we have reported that UFT plus lentinan decreased the number of pulmonary metastasis of rat mammary adenocarcinoma (SST-2) cells in congenitally T cell-depressed spontaneously hypertensive rats (SHR).^{16,17} Active hexose correlated compound (AHCC) is an extract obtained from several kinds of mushrooms which are cultured with liquid medium. The active component is an oligosaccharide and its molecular weight is about 5000 although it contains various kinds of components. Interestingly, as opposed to conventional active components which consist of a β -1,3-glucan structure as in PSK and lentinan, the oligomer of glucose in AHCC has α -1,4-linkage structures and some esterized hydroxy groups. We hypothesized that AHCC may function as a BRM in the same way that PSK and lentinan do in the chemotherapy of cancer.

In this study, we examined the effects of AHCC on the growth and metastasis of SST-2 cells in SHR rats in combination with UFT. We also examined the effects of AHCC plus UFT on the metastasis of SST-2 cells after excision of primary tumors. SST-2 is a highly metastatic cell line, which produces 100% pulmonary metastasis in syngeneic rats after *s.c.* transplantation. As SHR rats have a dysfunction of T cells but rather enhanced macrophage and NK cell activities compared with other strains of rats,¹⁸⁻²⁰ SST-2-bearing SHR rats were useful for examining the efficacy of anti-metastatic activities in various agents which function through the activation of macrophages and NK cells.

Materials and methods

Rats

Congenitally T cell-depressed SHR rats were purchased from Nippon Rat (Urawa, Japan). Throughout the experiments, 10 to 12-week-old female rats were used.

Tumors

We used a transplantable adenocarcinoma SST-2 that had originated from a spontaneous mammary adenocarcinoma in a SHR rat. The cells were maintained as a monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Gaithersburg, MD). The mean survival time of SST-2 tumor-bearing rats was about 50 days after tumor inoculation and the main cause of death was extensive metastasis to lung (our unpublished data). YAC-1 and SST-2 cells were used as target cells for NK cells and macrophages, respectively. The cells were also maintained in RPMI 1640 medium supplemented with 10% FBS.

Treatment of rats

SHR rats were inoculated s.c. with cultured SST-2 cells (1×10^6 /rat) on day 0. In this experiment, we did not set up the AHCC alone treated group, because our previous data (data not shown) indicated that AHCC alone had little effect on tumor growth and tumor treatment. Administration per os (p.o.) of UFT (15 mg/kg) (Taiho Pharmaceutical, Tokyo, Japan) was carried out every day from day 3 to 38. AHCC, an extract of several kinds of mushrooms, was supplied by Amino Up Chemical (Sapporo, Japan). According to our preliminary experiments, a rat drinks about 24 ml of water per day; we dissolved AHCC in water at the appropriate concentration of 100 mg/kg/day in 24 ml and let them drink freely from day 3 to 38. Water was administered p.o. to control rats. On day 38, rats were sacrificed, and the tumor weight and metastasis to axillary LN and lung were examined. The tumor size was measured every 3-4 days and the average diameter was estimated by the formula: $(\text{length} + \text{width})/2$. To examine the death as a result of metastasis to lung, tumor excision was performed on day 21 and all the rats were kept under observation until they died. UFT and AHCC were administered p.o. every day from day 3 to 38.

NK cell activity of spleen cells

Spleens were aseptically removed from rats on day 21, finely minced with scissors and gently teased in a loose-fitting glass homogenizer in RPMI 1640 medium. These cell suspensions were passed through four layers of gauze and washed twice in RPMI 1640 medium by centrifugation at 500 g for 5 min. The NK cell activity of the spleen cells was determined by a 4 h ^{51}Cr -release assay. Target cells of YAC-1 were pre-labeled for 1 h at 37°C with 100 mCi $\text{Na}^{51}\text{CrO}_4$, washed three times with RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 10% FBS. ^{51}Cr -labeled target cells (1×10^4) were incubated with various concentrations of effector cells in triplicate (tested at E: T ratios of 200:1-50:1) in 96-well-round bottomed microtiter plates (Coming Glass Works, Corning, NY) for 4 h at 37°C in a 5% CO_2 incubator. The radioactivity of the supernatants was counted in a Aloka g-counter (ARC-380). The percentage cytotoxicity was calculated by the following formula: $\% \text{ specific release} = [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum re-lease} - \text{spontaneous release})] \times 100$. Spontaneous release was less than 20%. All assays were performed in triplicate. The SD of triplicate wells was consistently less than 10% of the mean.

Activation of peritoneal macrophages

Rats were i.p. administered with 1 Klinische Einheit (KE) of OK-432 (Chugai Pharmaceutical, Tokyo, Japan) in 1 ml phosphate-buffered saline (PBS) for 48 h before sacrifice. The OK-432-activated peritoneal macrophages were harvested with 20 ml PBS from each group of tumor-bearing rats and prepared for further use. Flow cytometric analysis of cytotoxicity of peritoneal macrophages cytotoxicity of peritoneal macrophages on day 21 was examined against syngeneic rat adenocarcinoma SST-2 target-cells by the method as described by Slezak²¹ with some modifications. Briefly, 1×10^6 SST-2 cells were stained with 4×10^{-5} M PKH-26 (Zynaxis, Malvern, PA) for 2 min. The PKH-26-stained-target cells mixed

with freshly prepared effector cells at E: T ratios of 100:1 in 12-well culture dishes (Coming Glass Works) were cultured for 16h at 37°C, and then the cells were detached and harvested. After washing with cold PBS twice, the cells were suspended in 1 ml cold PBS and stained with 5 ml propidium iodide (PI, 50 mg/ml) (Sigma, St Louis, MO) for 30 min on ice. The PKH-26 positive and PI negative cells (PKH⁺ PI⁻ cells) were identified as living target cells and the PKH-26 positive and PI positive cells (PKH⁺ PI⁺ cells) were identified as dead target cells; only PKH-26 positive cells including PKH⁺ PI⁻ cells and PKH⁺ PI⁺ cells were gated; the PKH-26 negative cells were identified as effector cells which were gated out. All the cells were analyzed by flow cytometric analysis (FACScan, Lysys system; Becton Dickinson, Mountain View, CA). Cytotoxicity were calculated by the following formula: Cytotoxicity (%) = $\frac{\text{PKH}^+ \text{PI}^- \text{ cells}}{\text{PKH}^+ \text{PI}^- \text{ cells} + \text{PKH}^+ \text{PI}^+ \text{ cells}} \times 100$.

Quantification of nitrite

OK-432-activated macrophages (1×10^7) were mixed with SST-2 tumor cells at E: T ratios of 100:1, 50:1 and 25:1 in 12-well culture dishes for 16 h at 37°C. Since secreted NO quickly reacts with oxygen yielding nitrite, nitrite levels in culture supernatants were measured by using the Griess reagent. Briefly, 100 μ l sample aliquots were mixed with an equal volume of Griess reagent (1% sulfanilamine/0.1% naphthylethy-lene diamine dihydrochloride/2% H₃PO₄; Sigma) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate reader (Corona Electric, Katsuta, Japan). Nitrite concentration was determined using sodium nitrite as a standard and RPMI 1640 medium supplemented with 10% FBS as a blank. Reverse transcription-polymerase chain reaction (RT-PCR) total RNAs were extracted from peritoneal macro-phages on day 21 by the guanidine thiocyanate:phenol:chloroform method. Three micrograms of each RNA sample underwent cDNA synthesis in 50 μ l of reaction mixture containing 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM per each dNTP, 2 μ g/ml random primer and 1000 U M-MLV reverse transcriptase (Gibco/BRL) by incubation at 37°C for 1 h. PCR amplification of cDNA (5 μ l) was performed in 50 μ l of a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 2.5 mM MgCl₂, 0.1% Triton X-100, 200 mM each dNTP, 10 mM per each specific primer and 1 U Taq-polymerase (Promega, Madison, WI). The following primers were used: GAPDH forward primer, 5'-AC-CACCATGGAGAAGGCTGC-3'; reverse primer, 5'-CT-CAGTG TAGCCCAGGATGC-3'; iNOs forward primer, 5'-AGAGTCCTTCATGAAGCACA-3'; reverse primer, 5'-CCAAATACCGCATACTGAA-3'; tumor necrosis factor (TNF- α) forward primer, 5'-CAAGGAGGAGAAGTTCC. CAA-3'; reverse primer, 5'-CGGACTCCGTGATGTC-TAAG-3'; interleukin (IL)-1 α forward primer, 5'-GTGGTGGTGTGTCAGCAACTTC-3'; reverse primer, 5'-CCTTCAGCAACACAGGCTTG-3'.

In principle, the primer sequences were chosen from separate DNA exons of the gene. Expected sizes of amplified DNA fragments were 500, 577, 500 and 412 bp for GAPDH, iNOs, TNF- α and IL-1 α , respectively. The reactions were run for 35 cycles using a thermal cycler as follows: 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. The 9 μ l of each PCR sample was mixed with 1 ml of the sample buffer, electrophoresed through 2% agarose gel and stained with ethidium bromide.

Statistical analysis

Arithmetic means were calculated for each experiment group and tested for statistically significant differences by Student's t-test. Survival curves were analyzed by the generalized Wilcoxon test. Survival ratios were analyzed by contingency table analysis.

Results

Effects of AHCC plus LIFT on the s.c. growth and metastases of SST-2. The difference of tumor sizes between three groups was gradually increased, and, finally, on day 38, there was a significant difference of tumor sizes between the control group and AHCC plus UFT treated group ($p < 0.01$). The average tumor sizes of the control group and AHCC plus UFT treated group were 52.5 ± 4.23 and 43.9 ± 2.95 mm, respectively. Tumor weights of the control group and AHCC plus UFT treated group were 57.6 ± 4.53 and 35.1 ± 8.95 g, respectively. Metastases to axillary LN were inhibited by the treatment with AHCC plus UFT, although the inhibitory effects of AHCC plus UFT on pulmonary metastases were not clear. UFT alone had no effect on lung and axillary LN metastasis or, rather, it enhanced the metastasis

Effects on micro-metastasis to lung after excision of primary tumors

As described above, LIFT plus AHCC decreases the number of metastases. Because the metastases to lung days after tumor implantation would be more accelerated after tumor excision in SST-2 in SHR rats model.^{6, 7} We next investigated whether UFT plus AHCC had inhibitory effects on micro-metastasis after excision of primary tumors. There was no difference in tumor weights and sizes at the period of tumor excision on day 21, but mean survival time of the AHCC plus UFT treated group was significantly prolonged compared with that of the control group and UFT treated group. Three out of five rats survived, suggesting that pulmonary metastases were completely inhibited in these rats.

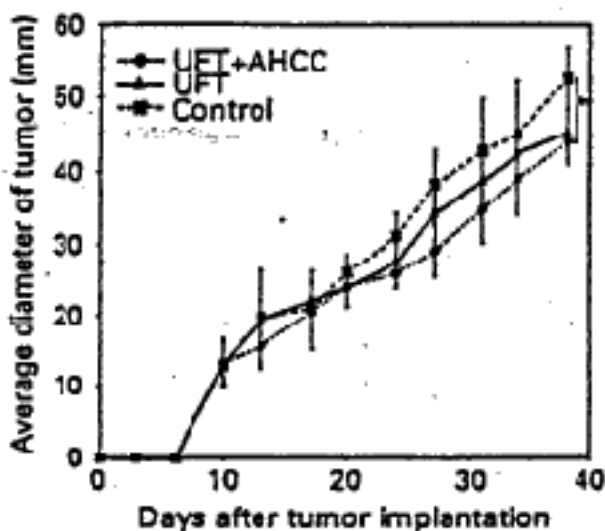


Figure 3. Growth curves of SST-2 tumors in SHR rats. SST-2 tumors were implanted s.c. on day 0. UFT (15 mg/kg) was administered p.o. every day from day 3 to 38. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 38. Each group consisted of five rats. Each point presents mean \pm SD of

average tumor diameter. Statistically significant ($r < 0.05$).

Restoration of NK cell activity by AHCC

UFT alone showed a slight but not significant effect on the survival of tumor-bearing rats and metastasis of SST-2 cells. These findings suggest that UFT may have negative effects such as inhibition of host immune response other than direct cytotoxic effects on SST-2 cells. Therefore, we investigated the NK cell activity and cytotoxicity and NO production of peritoneal macrophages in these tumor-bearing rats with or without treatment on day 21. NK cell activity was certainly depressed when UFT alone was administered. However, administration of AHCC in combination with UFT restored the NK cell activity to the comparable level of the control group.

Enhancement of NO production and cytotoxicity of peritoneal macrophages by AHCC

NO production during the effector phase of peritoneal macrophages obtained from AHCC plus UFT treated rats on day 21 was drastically enhanced at every E: T ratio. Cytotoxic activity of peritoneal macrophages was also enhanced by AHCC plus UFT, but only slightly compared with the enhancement of NO production.

Restoration of mRNA expression by AHCC

We next investigated mRNA expression in peritoneal macrophages on day 21. mRNA expression of iNOs was drastically increased by AHCC administration. mRNA expression of TNF- α , which was depressed by UFT, was restored by AHCC in combination with UFT to the level observed in the control group. Almost the same results were observed for mRNA expression of IL-1 α . GAPDH mRNA expression was the same in all experimental groups.

Discussion

Synergistic effects of AHCC on the treatment with UFT against mammary adenocarcinoma, SST-2 cells, in congenitally T cell-depressed SHR rats were observed. As far as the tumor growth on day 38 after tumor implantation was concerned, there was a significant difference in tumor sizes between the AHCC plus UFT treated and control groups, although not significant between the UFT alone treated and control groups. Although lung metastases were not inhibited by the treatment with AHCC plus UFT, metastases to axillary LN were obviously inhibited. Similar inhibitory effects of the combination of AHCC and UFT were observed in SHR rats with accelerated pulmonary metastases following the surgical removal of the primary tumor and three out of five rats survived. These results suggest that AHCC has obvious anti-metastatic activities and that it can be used for the treatment of patients with advanced cancer.

In vitro experiments showed that administration of AHCC in combination with UFT restored NK activities depressed by UFT and stimulated peritoneal macrophage cytotoxicities, NO production and cytokine production. It has been reported that both NK cells and macrophages were involved in the

inhibition of tumor metastasis, when they were activated by BRMS.22-28 Gallo-Hendrikx et al. have reported that the metastasis of human pancreatic tumor cells implanted in SCID mice was minimal, whereas the metastasis was found in more than 90% of mice carrying the beige mutation.²⁹ This result suggests that NK cells and macrophages may be more important than T and B cells in the anti-metastatic host immune system, because the beige mutation resulted in the deficient function of macrophages and NK cells. In our models, UFT alone had no effect on the growth of primary tumors but showed slight prolongation of survival of mice after removal of primary tumors. Because UFT reduced NK activities but stimulated peritoneal macrophage function, UFT may affect the growth of tumor cells indirectly at the metastatic sites by activating macrophages. The combination of UFT and AHCC significantly improved the prognosis of mice after excision of primary tumors. In vitro experiments showed that AHCC activated the NK cell activity in addition to the activation of macrophages. All together, AHCC brought about therapeutic effects in combination with UFT on primary tumor growth and metastasis, followed by the activation of NK cells and macrophages.

Conclusion

AHCC significantly reduced the metastasis of rot mammary adenocarcinoma in combination with UFT. This effect was mediated by natural host immunity, which was restored or activated by AHCC. AHCC could be used as a biological response modifier like PSK and lentinan for the treatment of advanced cancer.

Acknowledgments

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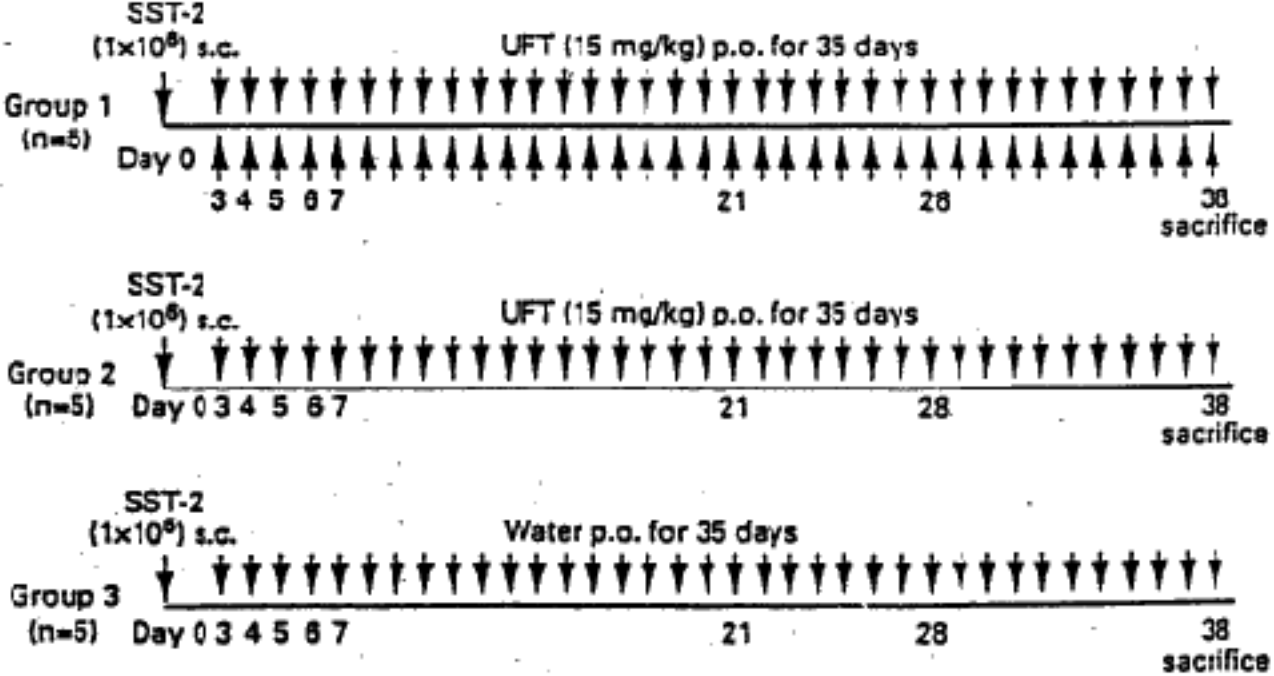


Figure 1: Protocol for observing the effect on growth of the primary tumor and metastasis. Rats were divided into three groups. Group 1: rats were administered UFT (15 mg/kg) plus AHCC (100mg/kg). Group 2: rats were administered UFT (15 mg/kg). Group 3: rats were administered water control.

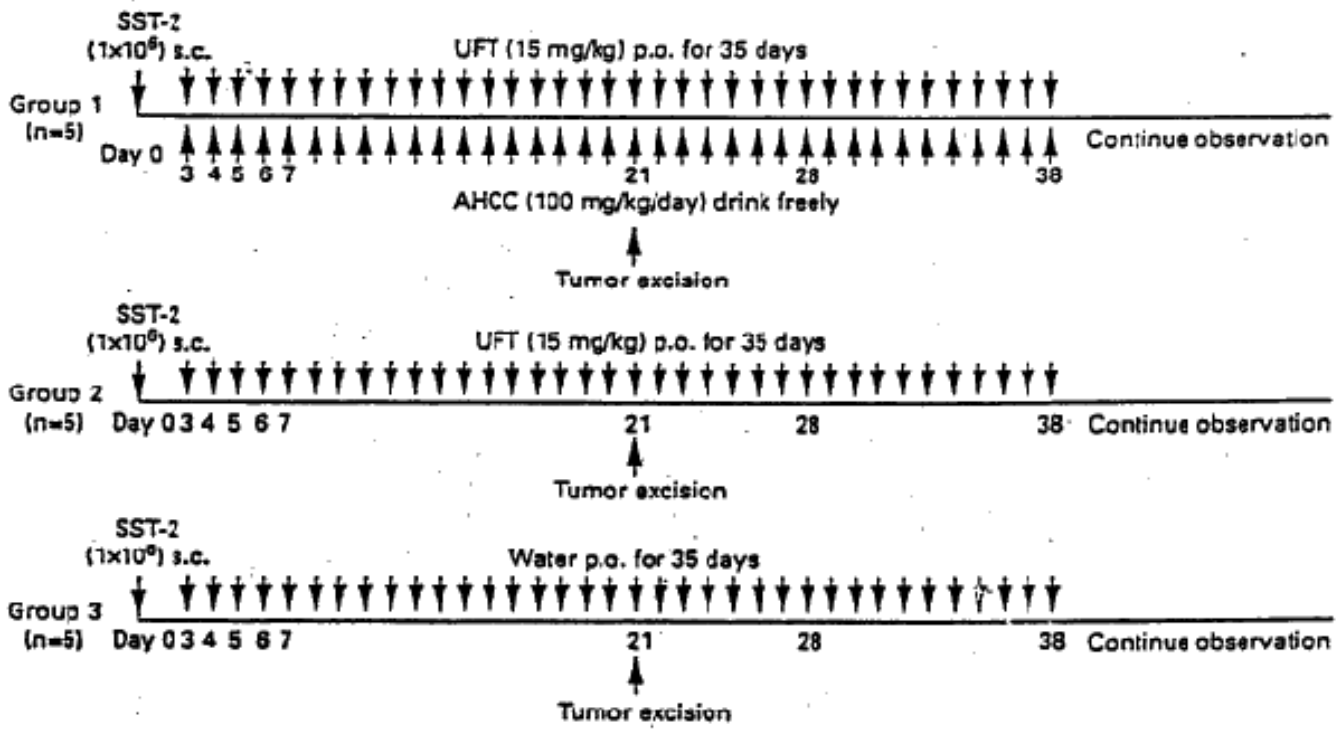


Figure 2: Protocol for observing the death by metastasis. Rats were divided into three groups. Rats were administered UFT or UFT plus AHCC described as in Figure 1. Surgical tumor excision was performed on day 21. All the rats were kept under observation until they died.

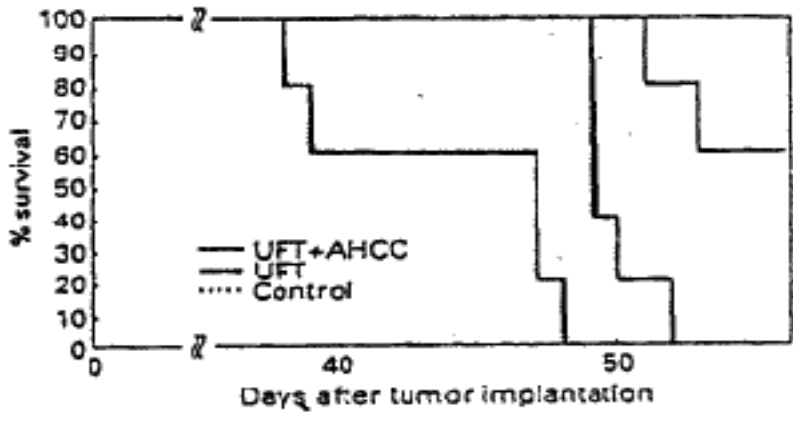


Figure 3: Survival curves of rats after surgical tumor excision on day 21. SST-2 tumors were implanted s.c. on day 0. UFT (15mg/kg) was administered p.o. every day from day 3 to 38. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 38. Surgical tumor excision was performed on day 21. Each group consists of live rats. *Statically significant (p,0.05) between the UFT plus AHCC treated group and the other two groups by the generalized Wiicoxon test.

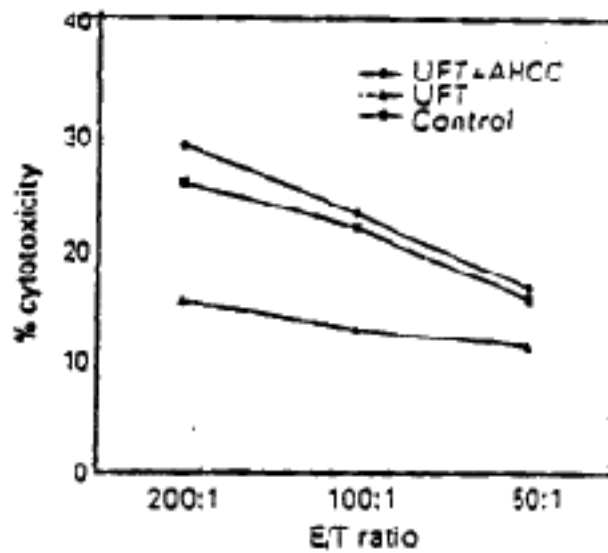


Figure 4: NK cell activity of spleen cells in SST-2-bearing rats. SST-2 tumors were implanted s.c. on day 0. UFT (15mg/kg) was administered p.o. every day from day 3 to 21. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 21. on day 21, rats were scarified and spleens were aseptically removed from three rats in each group and mixed for cytotoxicity assay.

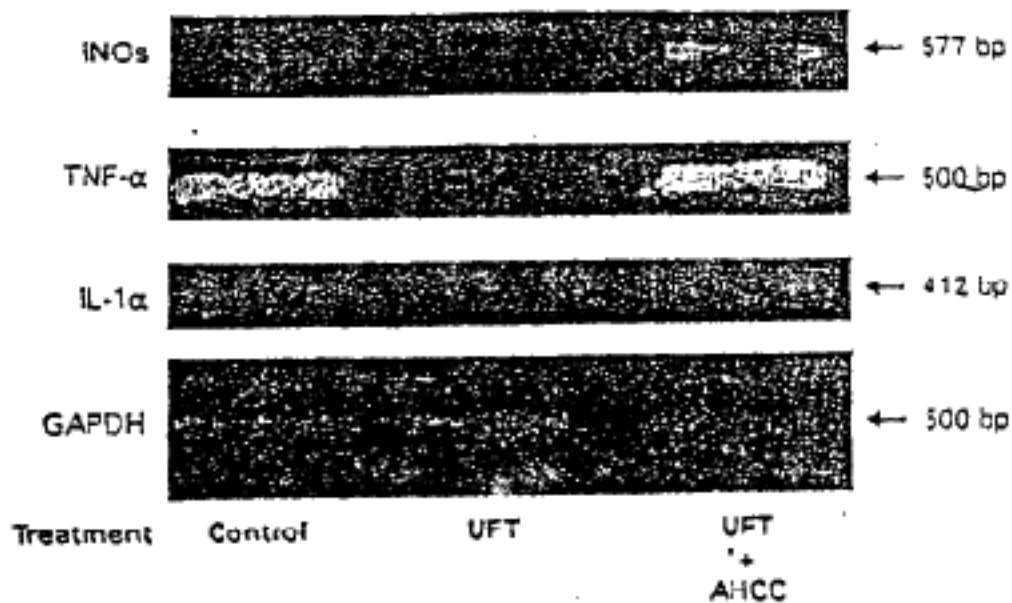


Figure 5: Expression of various cytokines and iNOs mRNA in peritoneal macrophages. SST-2 tumors were implanted s.c. on day 0. UFT (15mg/kg) was administered p.o. every day from day 3 to 21. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 21. On day 21, rats were i.p. administered 1 KE of OK-432 in 1 ml PBS. On day 23, OK-432 activated peritoneal macrophages were harvested with 20 ml PBS and total RNAs were extracted by the guandine thiocyanate:phenol:chloroform method. After reverse transcription, cDNA was amplified by PCR for 35 cycle using specific primers to detect the indicated cytokines and iNOs. PCR products were visualized under UV light.

Table 1. The effects of AHCC in combination with UFT on the growth of primary tumor and on the metastasis of SST-2 tumors in SHR rats

Treatment	Primary tumor (day 38)		Metastatic tumor			
	Diameter (mm)	Weight (g)	Lung		Axillary LN	
			Positive	Weight (g)	Positive	Weight (g)
UFT+AHCC	43.9±2.95	35.1±8.95	4/5	2.049±0.473	0/5	-
UFT	45.4±2.77	42.8±3.33	5/5	2.827±0.827	5/5	3.772±1.888
Control	52.5±4.23	57.6±4.53	5/5	1.853±0.147	3/5	8.997±11.48

All rats were sacrificed on day 38. Values are expressed as mean±SD. **p*<0.01.

Table 2. The effects of AHCC in combination with UFT on the survival of SST-2-bearing rats

Treatment	Primary tumor (day 21)		Survived/treated	Mean survival time (days)
	Diameter (mm)	Weight (g)		
UFT+AHCC	23.6±4.59	2.965±1.300	3/5	> 52.01±1.414
UFT	25.4±3.07	3.328±0.081	0/5	49.8±1.304
Control	22.3±3.48	2.828±0.538	0/5	43.8±4.818

Tumor excision was performed on day 21. All rats were kept under observation until they died. Values are expressed as mean±SD. **p*<0.01.

Table 3. NO production and cytotoxicity of peritoneal macrophages

Treatment	E:T ratio			
	NO production (μM)			Percent cytotoxicity against SST-2 cells
	100:1	50:1	25:1	
UFT+AHCC	5.44	2.74	1.53	15.32
UFT	3.08	2.00	0.78	13.27
Control	1.62	0.49	0.00	10.82

OK-432-activated peritoneal macrophages (1×10^7) were mixed with SST-2 tumor cells at E:T ratios of 100:1, 50:1 and 25:1 in 12-well culture dishes for 16 h at 37°C. Nitrite levels in culture supernatants were measured by using the Griess reagent. Cytotoxicity was assayed by flow cytometry (see Materials and methods).

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Improved Prognosis of Postoperative Hepatocellular Carcinoma Patients treated with Active Hexose Correlated Compound (AHCC)

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Abstract

Context

Active Hexose Correlated Compound (AHCC) is a newly developed functional food. In vitro experiments showed that AHCC enhanced the natural killer cell activity and could be considered as a potent biological response modifier for treating cancer patients. However, there is no report for the effects of AHCC in the clinical field.

Objective

To evaluate the efficacy of AHCC as a biological response modifier, and to determine whether AHCC improves the prognosis of hepatocellular carcinoma patients following surgical treatment.

Design

Analysis of data collected retrospectively between February 1992 and September 1999.

Setting

First Department of Surgery, Kansai Medical University in Osaka, Japan.

Patients

A total of 175 consecutive patients with histologically confirmed hepatocellular carcinoma were studied. All of the patients underwent macroscopically curative resection of a liver tumor.

Main Outcome Measure

Time to treatment failure (disease recurrence or death) after surgery.

Results

Of the 175 patients, 70 received AHCC (3g/day) orally after undergoing surgery (AHCC group). Survival and disease-free survival of patients in the AHCC group were compared with those of patients who did not receive AHCC after surgery (control group). Serum aspartate transaminase activity, γ -glutamyltransferase activity and total bilirubin were significantly

lower in the AHCC group in the 4-year period after surgery. Overall survival in the AHCC group was significantly higher than that in controls.

Conclusion

This retrospective study suggests that AHCC intake significantly improves the prognosis of postoperative hepatocellular carcinoma patients. Additional more detailed studies are needed to elucidate the mechanism of the effect of AHCC.

Introduction

Hepatocellular carcinoma (HCC) is widely distributed in different geographical areas. There is a high prevalence in Asia, and its incidence now ranks next to that of stomach cancer in Japan. Moreover, the number of patients with HCC is showing a gradual but definite increase¹. Prevention and treatment of HCC recurrence following hepatic resection has been extensively studied. Treatment have included repeated hepatic resection², interventional radiology (chemoembolization)³, percutaneous ethanol injection⁴, percutaneous microwave coagulation⁵, and administration of some hormonal agents^{6,7}. The prognosis for HCC however, remains unsatisfactory. The overall survival rate after primary surgical treatment has been about 40% at 5-years in Japan¹.

Many attempts have also been made to treat cancer by stimulating the immune system. Although several biological response modifiers (BRMs) have been developed such as BCG, Picibanil, PSK, lentinan, interferon and interleukin-12^{8,9}, the clinical efficacy of these substances has not been clearly confirmed. Active Hexose Correlated Compound (AHCC) is a functional food that was developed by Amino Up Chemical Co., Ltd. (Sapporo, Japan) in 1989. It is an extract of *Basidiomycotina* obtained by hybridization of several types of mushrooms¹⁰. Gohnam *et al.* reported that AHCC enhanced the natural killer cell activity of cancer patients and could be considered as a potent BRM for treating cancer patients¹¹. Furthermore, AHCC has been recently reported to reduce metastasis of rat mammary adenocarcinoma¹⁰, to induce detoxification enzymes in the liver, to protect the liver from CCl₄-induced liver injury¹², and to improve diabetes induced by streptozotocin¹³ in animal models. However, there is no report for the effects of AHCC in the clinical field.

This study was initiated to evaluate the effects of AHCC as orally administered BRM on the prognosis of patients with HCC following surgical treatment.

Patients:

A total of 175 patients with histologically proven hepatocellular carcinoma were studied retrospectively. All of the patients underwent macroscopically curative resection of a liver tumor at our institution between February 1992 and September 1999. Of these 175 patients, 23 cases were excluded, including 2 cases of operative death, 7 cases of hospital death, 10 cases with previous or concurrent malignancy and 4 cases who withdrew from follow-up just after discharge. Of the other 152 patients, 70 were administered AHCC (3g/day) orally after undergoing surgery until death or until the last follow-up date for living patients (AHCC group). These 70 patients were administered AHCC in accordance with the preferences of the patients and the treating physicians. The remaining 82 patients underwent hepatectomy without AHCC administration (control group). Disease-free survival and overall survival of patients in the AHCC group were compared with those in controls.

Follow-up:

All patients had a monthly follow up with routine liver biochemical tests. Every three months, all biochemical tests were performed at the central hospital laboratory. Liver ultrasound was also performed every three months. In addition, radiologists performed computed tomography and/or magnetic resonance imaging every six months. Finally, an angiographic examination was made after admission when recurrence was suspected. Once an intrahepatic recurrence had been confirmed, the patients in both groups generally received transarterial chemo-embolization.

Methods:

The perioperative clinical parameters such as patient characteristics, preoperative liver function data, operative factors and tumor characteristics were compared between the AHCC and the control groups. Overall survival, defined as the interval between the date of surgery and the date of death or last follow-up information for living patients, was evaluated. The most common cause of death was cancer, but liver failure or variceal bleeding was included among the causes of death. Disease-free survival was also evaluated, and was defined as the interval between the date of surgery and the date that diagnosis of recurrence was confirmed by a positive sonogram, computed-tomography scan, magnetic resonance imaging or hepatic angiography. AHCC was generously provided by Amino Up Chemical Co.,Ltd. It was developed by extraction from a cultured broth of *Basidiomycotina*.

Statistical Analysis:

Chi-square analysis was used to compare two or three proportions and the Student's (test was used to compare differences between two series, in order to evaluate the homogeneity of the treatment and the control group with respect to perioperative clinical factors. The data were expressed as means \pm standard deviation. Kaplan-Meier survival curves were plotted and Logrank tests were performed. Survival times were recorded in months. Two-way analysis of variance was used to compare the postoperative course of laboratory data between the two groups.

Results:

AHCC had no undesirable side effects. Only two patients in the AHCC group refused to continue the use of AHCC during the study due to slight nausea. Two patients in the control group began to take AHCC during the observation period. These four cases were censored at that time. The incidence of cirrhosis and the levels of serum albumin were significantly different preoperatively. However, these differences were disadvantageous to the AHCC group, and most patients were men with underlying viral hepatitis or cirrhosis, and most of them had well-compensated liver function.

By September 1999,34 (49%) patients had recurrences in the AHCC group and 55 (67%) had recurrences in the control group. AHCC had no significant effect ($p=0.081$) on disease-free survival. Fifteen (21%) patients had died in the AHCC group whereas 40 (49%) had died in the control group at the end of the follow-up period. Patient survival was significantly higher ($p=0.048$) in the AHCC group. The survival rate in the control group was similar to that observed among 12,595 HCC patients with hepatic resection based on data collected by the Liver Cancer Study Group of Japan from 1988 to 1995. The follow-up period ranged from 2 to 73.months in the AHCC group and from 2 to 92 months in the control group. The median follow-up period was 30 months in the AHCC group and 31 months in the control group. The 50% survival rate was 68 months in the AHCC group and at 45 months in controls.

Nine biochemical parameters were investigated for a period of 4 years after surgery in the two groups. Of these nine parameters, the serum levels of aspartate transaminase activity (AST), ?? glutamyltransferase activity (GGT) and total bilirubin were significantly lower in the AHCC group than in controls in the four-year period after surgery (Figure 2). There were no significant differences in the other 6 parameters that included the serum levels of alanine transaminase activity, alkaline phosphatase activity, albumin, platelet count, ?-fetoprotein and protein induced by vitamin K absence.

Comment:

Hepatocellular carcinoma (HCC) is a major health concern worldwide, with an incidence of approximately 1 million cases a year¹⁴. Early detection of HCC has recently become possible because of progress in diagnostic imaging and the incidence of resection for HCC has increased greatly during the last decade. As a result, short-term outcome has improved greatly and operative mortality rates have been reduced to a few per cent¹⁵. However, the long-term results are not yet satisfactory. Although, hepatic resection is the most effective form of treatment for patients with HCC, the incidence of postoperative recurrence which is the main cause of poor long-term results, remains extremely high even after hepatic resection¹⁶. Moreover, the cumulative intrahepatic recurrence rate has been reported to reach 100% at 5 years after resection of a single HCC in cirrhotic patients with viral hepatitis¹⁷.

To prevent recurrence or to prolong survival, the most widely applied option is adjuvant chemotherapy through a catheter inserted into the hepatic artery³. However, the efficacy of these agents is very low, the incidence of side effects is high, and there is no evidence suggesting that administration results in improved survival¹⁴. Furthermore, therapeutic doses of anti-cancer drugs have been reported to reduce the anti-tumor immune response and postoperative use of immunosuppressants accelerates the recurrence of malignancy¹⁸. Thus, the search for other potentially useful therapeutic approaches is necessary. However, other options, such as immunotherapy or radiation, have no definite place in daily clinical practice and should be applied only within research trials¹⁴. The disappointing status of medical treatment of HCC justifies the interest in administration of the functional food AHCC as a BRM, although its antitumor effects remain uncertain in the clinical field.

AHCC is an extract obtained from several kinds of mushrooms. The active component is an oligosaccharide and its molecular weight is about 5000 daltons¹⁰, but AHCC contains various components.

Interestingly, as opposed to conventional active components which include a β -1,3-glucan structural component which is found in PSK and lentinan, the oligomer of glucose in AHCC has β -1,4-linkage structure and some esterified hydroxy groups¹⁰. Therefore, it has been suggested that AHCC may function as a BRM in the same way that PSK and lentinan do in patients with cancer.

In vitro experiments^{??} showed that administration of AHCC restored NK cell activity depressed by UFT and stimulated peritoneal macrophage cytotoxicity, NO production and cytokine production. The combination of UFT and AHCC significantly improved prognosis in mice after excision of primary tumors. It has been reported that both NK cells and macrophages are involved in the inhibition of tumor metastasis, following activation by BRMs¹⁰. Therefore, this AHCC effect could be mediated by natural host immunity which was restored or activated by AHCC. Thus AHCC may bring about therapeutic effects on survival of HCC patients as a result of NK cell and macrophage activation. AHCC should be considered as a potent BRM and its anti-cancer activity may be through host immunomodulation.

AHCC was recently reported to protect the liver from CCl₄-induced liver injury in animal models². Therefore, the higher survival rate in the AHCC group suggests that AHCC had beneficial effects on the clinical course of patients with hepatitis or cirrhosis, in addition to anti-cancer effects. Indeed, AHCC intake seemed to inhibit the hepatitis of patients, as suggested by the reduction in postoperative levels of AST, GOT and bilirubin in our study. The effects of AHCC administration in HCC patients and their time-course are further supported by the reduction in AST, GGT and bilirubin levels. The reduction in these biochemical parameters appears to reflect a better prognosis, although we need to study a larger number of patients for a longer time period in order to confirm this preliminary observation. The mechanism of anti-cancer activity and/or the hepatitis-preventing effect of AHCC has not been explored in this study. At present, it is difficult to explain the effects of AHCC as the effect of a single ingredient, and it is similarly difficult to reach any conclusions regarding the complex effects of AHCC on patient survival.

The results of this retrospective study show that adjuvant AHCC administration improves survival of HCC patients with viral hepatitis after surgery without side effects. Moreover, improvements in the quality of life were noteworthy in the present study. Although quality of life is very difficult or even impossible to assess objectively, the general sense of well being and appetite certainly improved more in the AHCC group than in the control group. However, the validity of this observation is limited by the fact that the study was not a prospective randomized trial.

AHCC intake proved beneficial for the survival of postoperative HCC patients without adverse effects. AHCC treatment is therefore a valuable adjuvant therapy for these patients. These preliminary observations need to be confirmed in larger controlled double-blind trials. A randomized prospective study of postoperative adjuvant therapy with AHCC in patients with HCC was initiated in August. AHCC, active hexose correlated compound; TACE, transarterial chemoembolization; ICG, indocyanine green; GSA, galactosyl human serum albumin; PIVKA, protein induced by vitamin K absence; NS, not significant. Data are expressed as means \pm SD.

Table 1. Clinical Background of Patients treated with AHCC and Controls

AHCC group Control group p-value

Patient Characteristics

age (years) 61.7 \pm 8.4 60.6 \pm 10.5 NS

gender (male/female) 54 / 16 68 / 14 NS

cirrhosis (yes/no) 66 / 3 62 / 19 0.001

Child score (A/B) 51 / 19 65/17 NS

alcohol intake (ml/day) 36,1 \pm 61.6 32.0 \pm 44.5 NS

hepatitis viral infection

type B/type C/ none 34/36 11 / 64 / 7 NS

preoperative TACE (yes/no) 18 / 44 / 8 36 /46 NS

TACE for hepatic recurrence (yes/no) 28/6 41 / 14 NS

Preoperative liver function data

albumin (g/L) 37.4 \pm 4.1 39.0 \pm 4.3 0.028

total bilirubin (\bullet mol/L) 15.7 \pm 5.5 15.7 \pm 5.8 NS

cholinesterase (U/L) 3765 ± 1123 3670 ± 1253 NS

aspartate transaminase (U/L) 50.7 ± 38.6 50.4 ± 30.5 NS

alkaline transaminase (U/L) 55.6 ± 45.6 49.5 ± 27.7 NS

alkaline phosphatase (U/L) 233 ± 89 2142 ± 81 NS

γ-glutamyltransferase (U/L) 71.1 ± 60.0 66.4 ± 60.3 NS

AHCC group Control group p-value

platelet count (x 10⁹/L) 135 ± 64 149 ± 75 NS

prothrombin time (%) 88.2 ± 13.1 91.7 ± 12.9 NS

antithrombin III (%) 81.0 ± 16.9 82.0 ± 17.7 NS

ICG R 15 mm (%) 18.6 ± 10.2 17.0 ± 8.5 NS

ICG K value 0.122 ± 0.036 0.124 ± 0.030 NS

redox tolerance index 0.588 ± 0.599 0.573 ± 0.441 NS

99mTc-GSA liver scintigraphy Rmax 0.432 ± 0.152 0.420 ± 0.172 NS

Operative Data

number of resected subsegments 1.87 ± 1.10 2.00 ± 1.27 NS

total blood loss (ml) 1808 ± 2309 1800 ± 2411 NS

blood transfusion (units) 3.94 ± 7.99 4.15 ± 7.27 NS

microscopic curative resection (yes/no) 55 / 15 66/16 NS

operation length (mm) 310 ± 102 294 ± 121 NS

postoperative complications (yes/no) 19 / 51 18 / 64 NS

postoperative hospital stay (days) 39.0 ± 43.0 35.3 ± 39.0 NS

Tumor Characteristics

size in diameter (cm) 4.41 ± 3.41 4.25 ± 3.28 NS

number of nodules 1.93 ± 1.94 1.66 ± 1.27 NS

differentiation (well/moderate/poor) 17/48/2 15/50 110 NS

capsule (yes/no) 60 / 9 68 / 12 NS

TNM stage 2.39 ± 0.89 2.40 ± 1.00 NS

basal α -fetoprotein (\bullet g/L) 7022 ± 38489 6602 ± 39687 NS

basal PIVKA II (AU/L) 2216 ± 6710 2891 ± 9716 NS

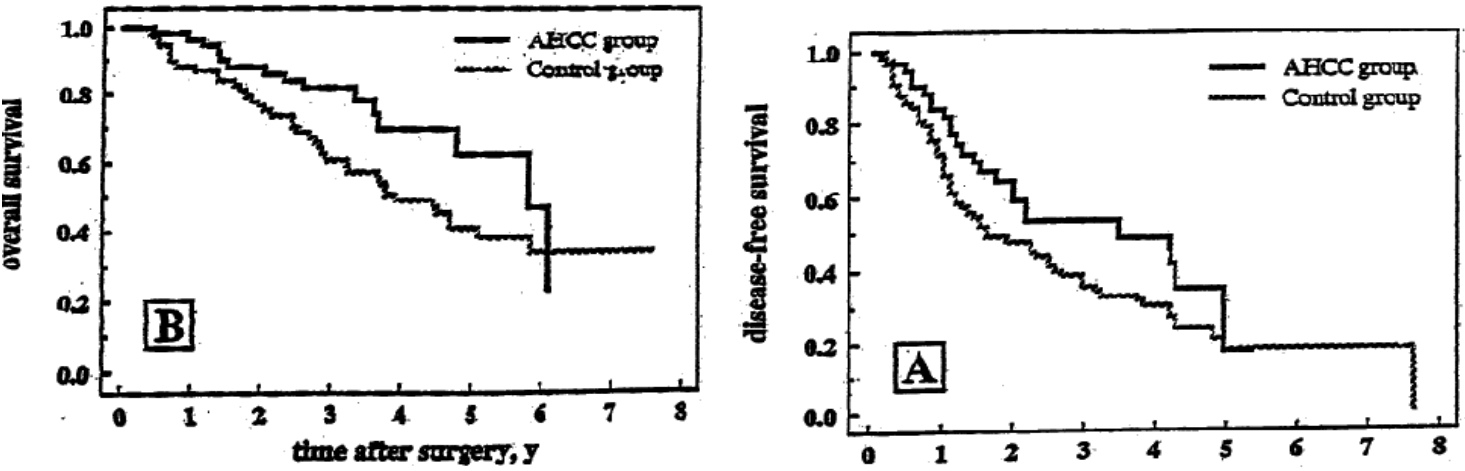


Figure 1: Kaplan-Meier estimate of disease-free survival and overall survival of patients with hepatocellular carcinoma after hepatic resection. The solid line indicates survival in the AHCC group and the dotted line represents the control group. **A**, disease-free survival. There was no significant difference between the two groups by Logrank test ($p=0.081$). **B**, overall survival. There was a significant difference between the two groups ($p=0.048$)

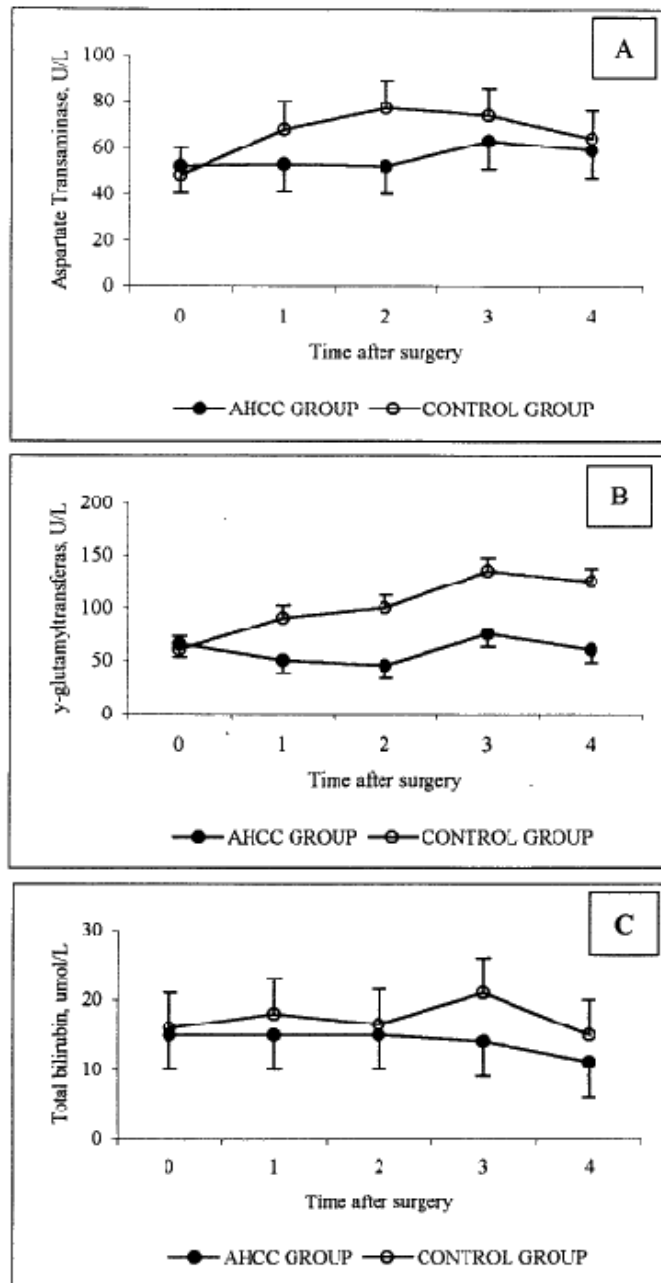


Fig. 2

Figure 2: Biochemical parameters in the patients with hepatocellular carcinoma after hepatic resection. Closed circles indicate the levels of parameters in the AHCC group and open circles indicate levels in the controls. There were significant differences in the two groups by two-way analysis of variance. **A**, aspartate transaminase activity, $p=0.030$. **B**, γ -glutamyltransferase activity, $p=0.009$. **C**, total bilirubin, $p=0.012$.

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Preventive Effects of Active Hexose Correlated Compound (AHCC) on Oxidative Stress Induced by Ferric Nitrilotriacetate in the Rat

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SUMMARY

Ferric nitrilotriacetate (Fe - NTA) is a strong oxidant, which generates highly reactive hydroxyl radical and causes injuries of various organs including the kidney and liver. The formation of 8-hydroxy - 2' -

deoxyguanosine (8- OHdG) adducts in the renal DNA is one of the earliest events after treatment with Fe - NTA. Since Active Hexose Correlated Compound (AHCC), an extract of fungi, has been shown to act as an

antioxidant, its protective effect on the oxidative stress induced by Fe - NTA was examined in the present study.

AHCC at 3% in drinking water was given to male Wistar rats for 1 week, then Fe - NTA was injected

intraperitoneally. At 3 h after the treatment with Fe - NTA, levels of 8- OHdG in the bladder urine, creatinine in the serum, thymic apoptosis, serum levels of aspartate and alanine aminotransferases were significantly increased.

All of these increases were restored to normal by the AHCC pretreatment. These results suggest that AHCC is potent in restoring the disorders of various organs induced by oxidative stressors.

Key Words: ferric nitrilotriacetate, 8- hydroxy - 2' - deoxyguanosine, creatinine, thymic apoptosis, aminotransferases, AHCC

INTRODUCTION

Reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) are constantly generated in aerobic respiration in response to both external and internal stimuli (1). Low levels of ROS play an important role in signal transduction, cell proliferation, apoptosis, immunity and defense mechanism (2-4). High levels of ROS, however, cause severe metabolic malfunctions and damage to biological macromolecules. For example, excess ROS induce oxidative damages to nucleic acids and proteins, decrease the efficiency of DNA polymerase repair and activity of signal transduction and cause cell death by apoptosis or necrosis (2,5,6). ROS induce chemical changes in bases and change in DNA conformation, causing mutation (4, 7). Damage to DNA by ROS is critical for carcinogenesis. Moreover, the accumulation of free radical - mediated damage in DNA is the major cause of the physiological changes associated with aging (8).

Animals including human possess numerous machineries of antioxidative defense by themselves. Such defense mechanisms within the organism have evolved to limit the levels of reactive oxidants and the damage that they inflict (6). Enzymes involved in such mechanisms include superoxide dismutase (SOD), glutathione peroxidase and catalase. Moreover, there exist many structural defenses, for example, sequestration of H_2O_2 -generating enzymes in peroxisomes and chelation of any free iron or copper salts in transferrin and ferritin or

ceruloplasmin to avoid the product of Fenton reaction, O₂ -9 .

Epidemiological studies have suggested that antioxidative supplements, such as dietary antioxidants of small molecules, including vitamin C (ascorbate), vitamin E (tocopherol) and carotenoids are potent in preventing carcinogenesis 10, 11. Thus, natural plant components have been expected to provide oxidative defenses against cancer and degenerative diseases caused by oxidative stress. Active Hexose Correlated Compound (AHCC™ Amino UP Chemical Co. Ltd. Sapporo) is a mixture of polysaccharides, amino acids and minerals derived from fungi. It is obtained by hot water extraction after culturing mycelia of several basidiomycetes in a liquid culture tank and treating them with several enzymes 12. The main active component of AHCC is a mixture of oligosaccharides whose average molecular weight is approximately 5,000 12, 13. The chemical analysis has revealed that polysaccharides are the major components of AHCC, consisting approximately 74%. Nearly 20% of the polysaccharides are α -1,4- glucan and its acetylated forms, which are thought to be active components 12. AHCC also contains some other polysaccharides including β - glucans. Both α - and β - glucans are shown to be responsible for the antitumor effects of Basidiomycetes 14. It has been reported that this extract shows various beneficial effects in humans and in experimental animals acting either as a biological response modifier (BRM) or an antioxidant. It was shown that AHCC was effective in suppressing growth of experiment cancers in mice and rats such as SST- 2 tumor and breast cancer 13. AHCC enhances a helper T cell 1 (Th1) response in susceptible BALB/c mice, specially increasing interleukin (IL) - 12 levels 15). It was also found out that Th1 cytokines levels such as tumor necrosis factor (TNF) - α and interferon (IFN) - γ were increased in response to AHCC treatment. AHCC is used clinically as a successful complementary therapy for cancer patients 16. AHCC treatment after surgical operation could improve the quality of life (QOL) of patients with malignant tumors and increase the survival rate by inhibiting tumor metastasis 16. AHCC can also prevent the onset of the diabetes induced by streptozotocin 17. Recently, we have found that AHCC suppresses thymic apoptosis induced by dexamethasone 18 , suggesting that AHCC acts as an antioxidant.

Ferric nitrilotriacetate (Fe - NTA) is a chemical that induces severe oxidative damage by Fenton reaction, in which the very reactive hydroxyl radical is generated¹⁹⁻²³. It acts as a potent nephrotoxin, causing high incidence of renal cell carcinoma in rats and mice^{8, 20, 24} . Fe - NTA treatment results in renal DNA damage, including formation of 8 - hydroxy - 2' - deoxyguanosine (8-OHdG) in the renal DNA through the generation of active oxygen radicals. The increase in urinary excretion of 8- OHdG reflects the oxidative DNA damage in vivo ^{19, 25}. Creatinine levels in the serum are significantly increased following Fe - NTA treatment ^{19, 23i}. Fe- NTA also can induce hepatic injuries acting through the generation of ROS²⁶. These damages done to several organs are ameliorated by antioxidants^{27, 28}.

The objective of our present study is to examine whether or not AHCC has the preventive effects on the damage induced by oxidative stress in rats treated with Fe- NTA. Our present study deals with the beneficial effects of AHCC on the damage of various organs induced by Fe - NTA.

MATERIALS AND METHODS

1. Materials. Lyophilized AHCC was prepared by Amino UP Chemical Co. Ltd. (Sapporo, Japan). Fe -NTA solution was prepared immediately before use as previously described²²). Nuclease P1 was obtained from Sigma (St. Louis, MO, USA).

2. Animals. Studies with the rats were approved by Animal Care and Use Committee, Dokkyo University School of Medicine. The animals were treated according to guidelines for the Care and Use of Laboratory Animals of the Committee. Male Wistar rats of 8- weeks old were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were kept in a room at 23 \pm 2°C with 12 h light and dark cycle and kept free access to food and water.

Twenty- one rats were divided into four groups. Two groups received 3% AHCC as a drinking water for a week. The concentration of AHCC and the duration of the treatment were chosen according to our previous results

18. Control groups received only tap water for a week. Fe - NTA (15 mg/kg body weight as Fe³⁺ + was injected intraperitoneally to two groups; one of them was AHCC - pretreatment group, and the other was the control group. Fe - NTA effects at this dose were maximal at 3h of the treatment as shown by others 27.28.

3. Blood sampling and organ preparation. Rats were sacrificed by decapitation at 3h after Fe - NTA-treatment. Blood samples were collected into sampling tubes. Serum samples were obtained after centrifuging at 4°C for 15 min at 3,000 rpm. The sera thus obtained were used for the assay of serum aminotransferases and creatinine.

Thymus glands were removed and washed in cold phosphate - buffer solution (PBS), then put into the tubes which contained collagenase- EDTA for apoptosis assay. Urine samples were taken from urinary bladder of rats for 8- OHdG assay.

4. 8- OHdG assay in the urine. Urine samples were centrifuged at 10,000 rpm, and the supernatant was used for ELISA analysis. 8- OHdG was assayed by ELISA kits, which were prepared by Japan Institute for the Control of Aging (Shizuoka, Japan) .

5. Detection of apoptosis by flow cytometry. Cell suspensions were obtained after collagenase - EDTA (0.25% in PBS) treatment and filtration through nylon mesh. The cell suspensions were washed with PBS (pH 7.4), adjusted to a concentration of 1.5×10^6 cell/mL. Then the cells were centrifuged at 200 x g for 5 min. The 200 x g centrifuged cell pellet was gently resuspended in 1.5 mL hypotonic fluorochrome solution containing propidium iodide (PI, 50 µg/mL in 0.1% sodium citrate plus 0.1% Triton x 100) , in polypropylene tubes. The tubes were placed at 4°C in the dark overnight before the flow cytometric analysis. Flow cytometry was carried out by analyzing 10,000 cells per test using a FACSCalibur (Becton Dickinson, Mountain View, CA, USA).

6. Creatinine and aminotransferase assays. Creatinine in the serum was assayed by creatinine kit. The activity of alanine aminotransferase (ALT) , aspartate aminotransferase (AST) in the serum were routinely assayed by commercial kits.

7. Statistical analysis. All the data were expressed as mean \pm SEM. Analysis of variance (ANOVA) was performed and Scheffe's multiple comparison test was applied to test for the differences between individual groups. A, p value less than 0.05 was considered statistically significant.

RESULTS

1. 8- OHdG in the bladder urine First we observed the noxious effects of Fe - NTA in the kidney and the effects of simultaneous administration of Fe- NTA and AHCC on the renal function. 8- OHdG in the bladder urine increased significantly at 3 h after treatment with Fe - NTA. A significantly lower level of 8 - OHdG in the urine of AHCC pretreated rats was observed, when compared with that of the rats treated with Fe - NTA alone (Fig. 1).

2. Creatinine in the serum The enhanced serum creatinine levels, which depend on glomerular filtration rate, are indicative of renal injury^{22, 23}. In our experiment, the creatinine levels in the serum increased significantly in the rats treated with Fe- NTA treatment alone, while AHCC - pretreatment restored the increased levels to normal (Fig. 2).

3. Thymic apoptosis Thymic apoptosis in the Fe- NTA treated rats increased significantly compared with that in the control and AHCC groups. Thymic apoptosis in the Fe- NTA -AHCC treated rats was much lower than in the rats treated with Fe - NTA alone (Fig. 5).

4. Serum AST and ALT Hepatic injuries induced by Fe - NTA were assessed by measuring the activity of serum aminotransferases. The serum levels of AST and ALT were elevated in the Fe - NTA- treated group than those

in the control and AHCC groups (Figs. 3, 4). AHCC - pretreatment decreased AST and ALT levels in the serum significantly to the normal levels.

DISCUSSION

The results of the present study have shown that Fe -NTA increases the urinary levels of 8- OHdG, and serum levels of creatinine, AST and ALT. All these changes induced by Fe - NTA have already been observed by others, showing that this toxin damages both the kidney and the liver 19, 22. 23. In the present study it was first shown that thymic apoptosis was induced by this chemical. These changes may be closely associated with oxidative stress, because Fe - NTA produces ROS in vivo. All the Fe - NTA - induced changes observed here are restored to normal by the pretreatment with AHCC for 1 week. The results seem to suggest that AHCC can protect

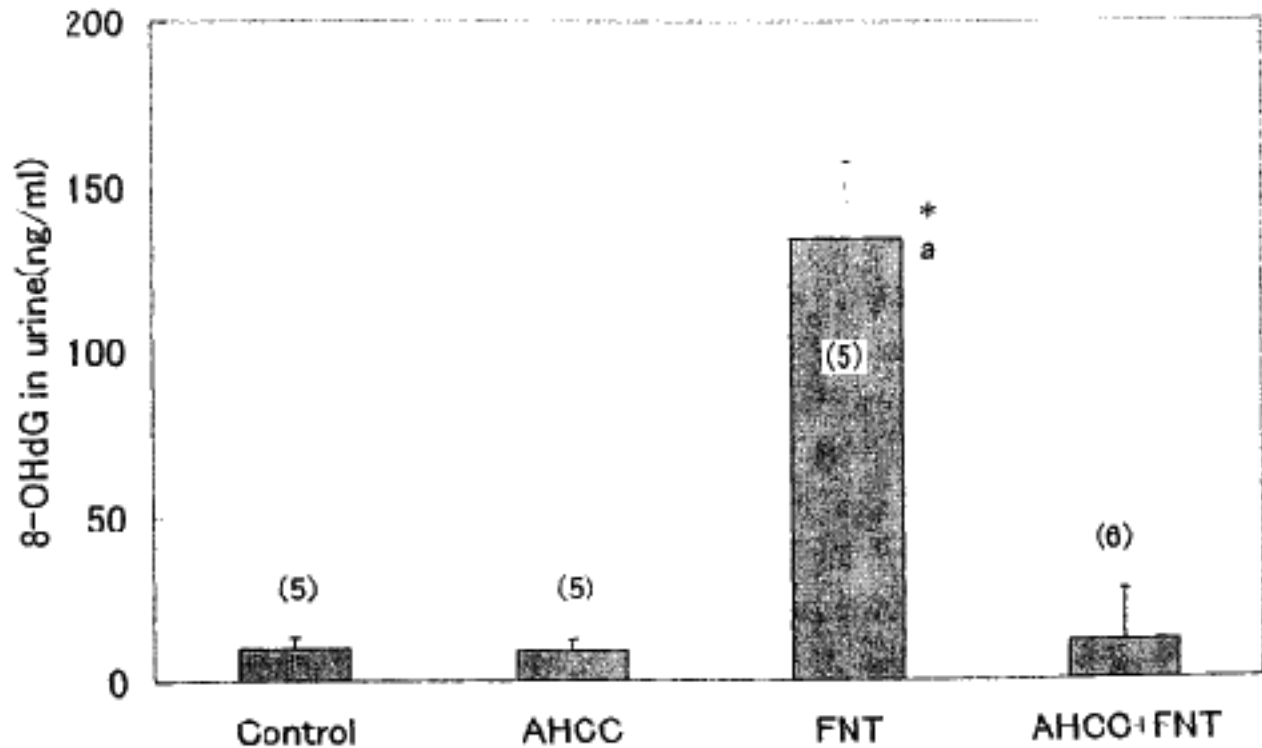


Fig. 1: Effects of Fe - NTA and AHCC on 8 - OhdG levels in urinary bladder urine. Urine samples in the urinary bladder were withdrawn immediately after sacrifice. Fe- NTA (15 mg Fe 3+ /Kg) was injected i.p.

3h before experiment. AHCC at 3% in drinking water was given for a week. Data are shown as means \pm SEM with the numbers of determination in parentheses.

*, $p < 0.001$ vs. control group.

a, $p < 0.001$ vs. AHCC + Fe-NTA group.

Abbreviations are as follows: FNT, Fe-NTA, ferric nitrilotriacetate; AHCC, Active Hexose Correlated Compound and 8 - OhdG, 8 - hydroxyl - 2' - deoxyguanosine

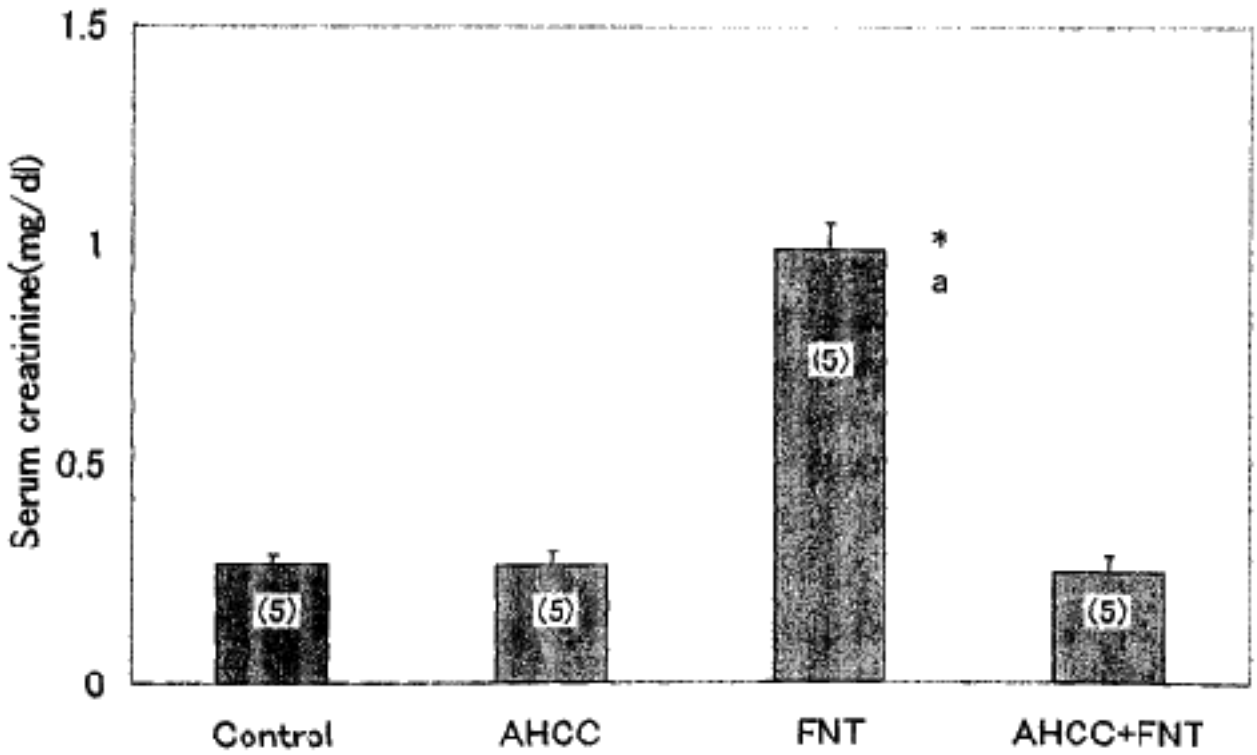


Fig. 2: Effects of Fe - NTA and AHCC on serum creatinine levels. Creatinine in the serum were assayed by creatinine kit. Data are shown as means \pm SEM with the numbers of determination in parentheses.

*, $p < 0.001$ vs. control group.

a, $p < 0.001$ vs. AHCC + Fe-NTA group.

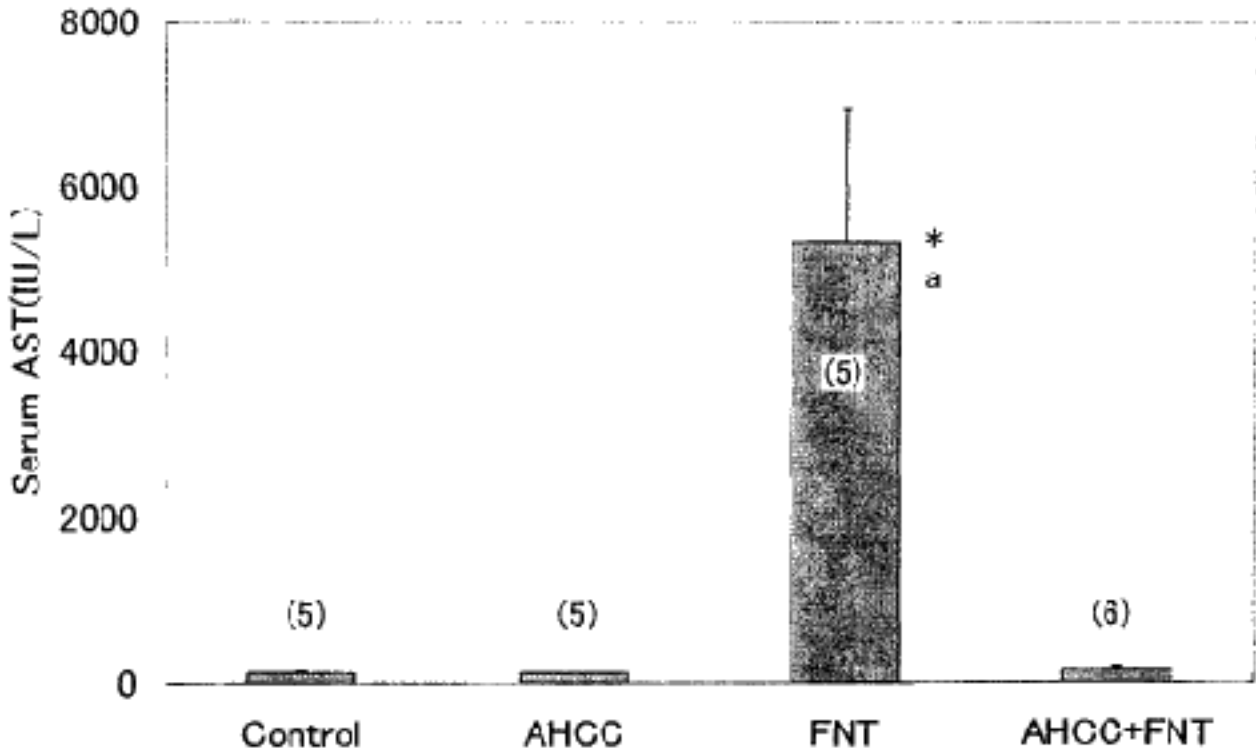


Fig. 3: Effects of Fe - NTA and AHCC on serum alanine aminotransferase (ALT) levels. ALT was assayed by commercial kit. Data are shown as means \pm SEM with the numbers of determination in parentheses.

*, $p < 0.001$ vs. control group.

a, $p < 0.001$ vs. AHCC + Fe-NTA group.

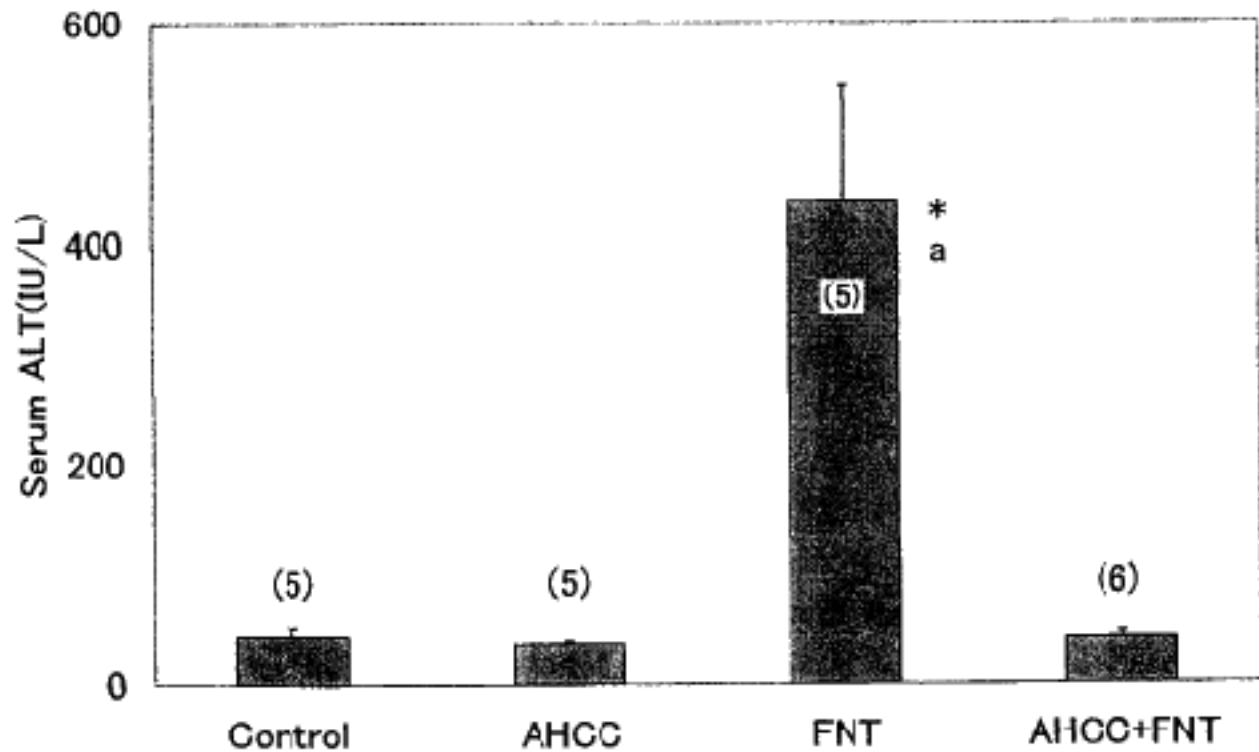


Fig. 4: Effects of Fe - NTA and AHCC on serum aspartate aminotransferase (AST) levels. AST was assayed by commercial kit. Data are shown as means \pm SEM with the numbers of determination in parentheses.

*, $p < 0.001$ vs. control group.

a, $p < 0.001$ vs. AHCC + Fe-NTA group.

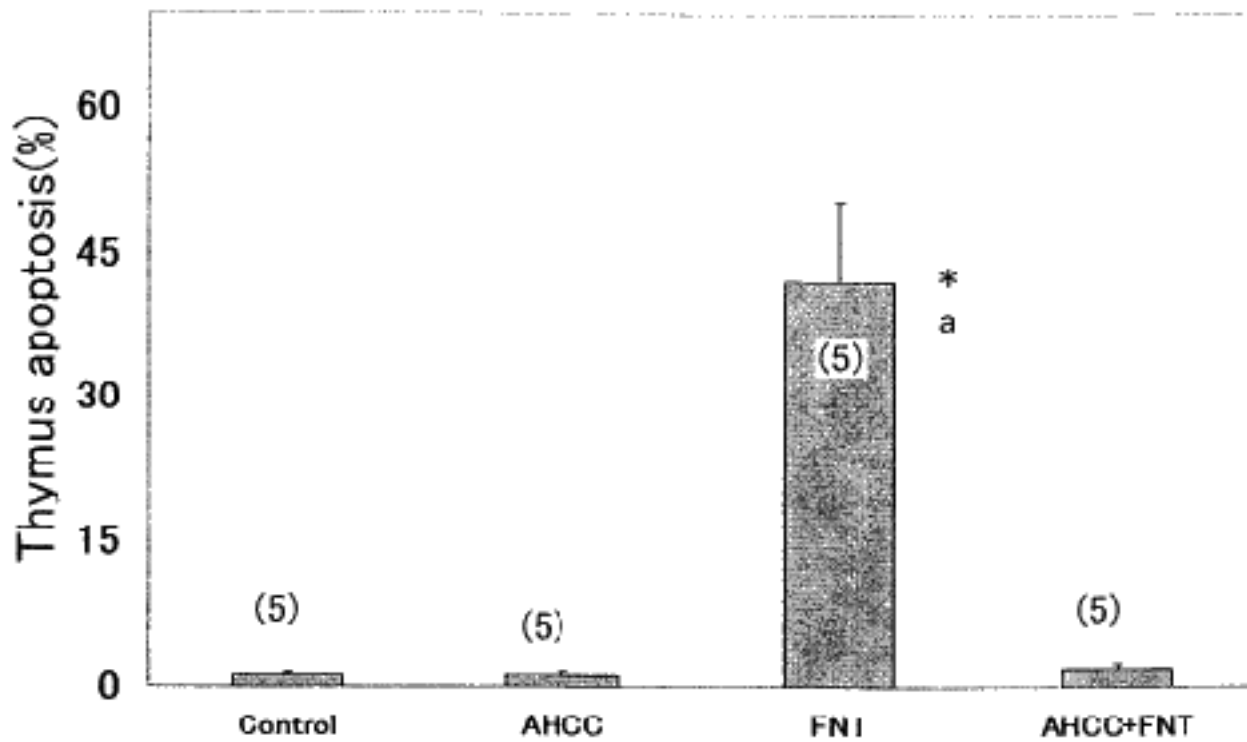


Fig. 5: Effects of Fe - NTA and AHCC on the thymic apoptosis. Apoptosis was estimated by flow cytometry using FACSCalibur. Data are shown as means \pm SEM with the numbers of determination in parentheses. *, $p < 0.001$ vs. control group. a, $p < 0.001$ vs. AHCC + Fe-NTA group.

damages of various organs caused by ROS.

Fe - NTA is a renal toxicant and carcinogen in rats and mice 20, 24. This chemical increases markedly the urinary excretion of 8- OHdG which is a useful marker for measuring the level of oxidative DNA damage 19, 25. The damage in DNA is closely associated with aging and carcinogenesis 8, 19, 20, 23, 24. A common form of DNA damage is the formation of hydroxylated bases, which is considered to be an important event in carcinogenesis induced by oxidative stress. The formation of 8- OHdG has been shown to be suppressed by antioxidants such as vitamin E, vitamin C and methionine 10, 11. Many other antioxidants have been shown to protect the injuries induced by Fe - NTA. For example, N - acetylcysteine (NAC), a precursor of intracellular cysteine and glutathione, prevents renal damage induced by Fe - NTA in the rat 28. Alpha- tocopherol, a lipid-soluble antioxidant, ameliorates renal proliferative response and toxicity induced by Fe - NTA 27. AHCC decreased the Fe - NTA - increased 8- OHdG levels in the urine, suggesting that this compound prevents mutation by scavenging ROS.

It is reported that Fe - NTA increases the hepatic ornithine decarboxylase (ODC) and that the pretreatment of rats with butylhydroxytoluene (BHT), an antioxidant, suppresses the increase in the enzyme activity 26. Fe - NTA injected intraperitoneally into rats increases plasma levels of the two aminotransferases, ALT and AST and AHCC pretreatment normalized the elevated enzyme activities. These findings suggest that AHCC protect the hepatic damage induced by oxidative stress of Fe - NTA.

Thymic apoptosis is induced by glucocorticoids and oxidative stress and suppressed by antioxidants such as melatonin 30. Recently we have found that AHCC suppresses thymic apoptosis induced by dexamethasone 18. All these findings suggest that AHCC acts as an antioxidant. It is not known yet if AHCC acts directly as an antioxidant to scavenge ROS or induces some enzymes, which cleavage ROS. It still remains unclarified which components of AHCC are responsible for its protective effect against oxidative stress. Our preliminary experiments have revealed that AHCC itself has no antioxidant effect on thymocytes in vitro (data not shown). The study is currently under way to test the effects of individual α - and β glucans purified from AHCC.

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AHCC on Immobilization Stress in the Rat: Beneficial Effects of Active Hexose Correlated Compound

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Dokkyo Journal of Medical Sciences p. 559 28(1): 559 ~ 565, 2001

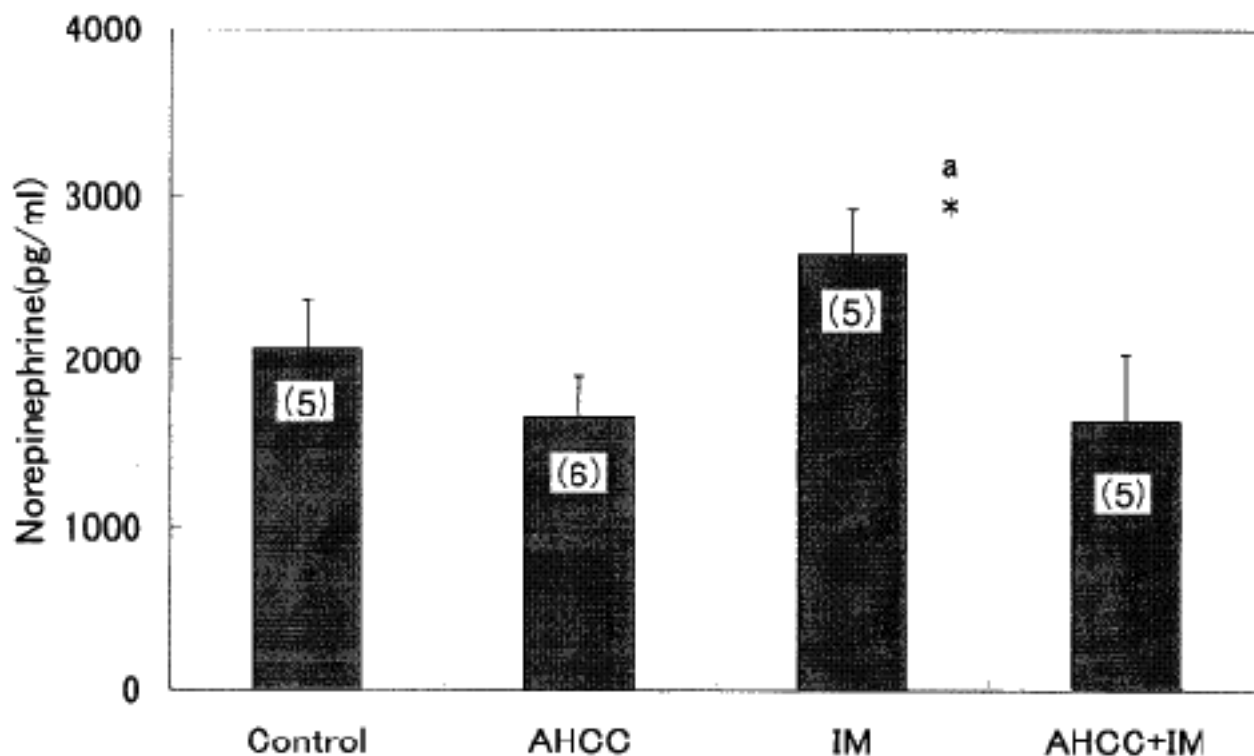


Fig. 1: Effect of immobilization and AHCC on norepinephrine (NE) levels in the plasma. NE was assayed by high-performance liquid chromatography (HPLC) with electrochemical detection. Data are shown as means \pm SEM with the number of determinations in parentheses. *, $p < 0.05$ vs. control a, $p < 0.05$ vs. AHCC + IM Abbreviations used are as follows: IM, immobilization; AHCC, Active Hexose Correlated Compound.

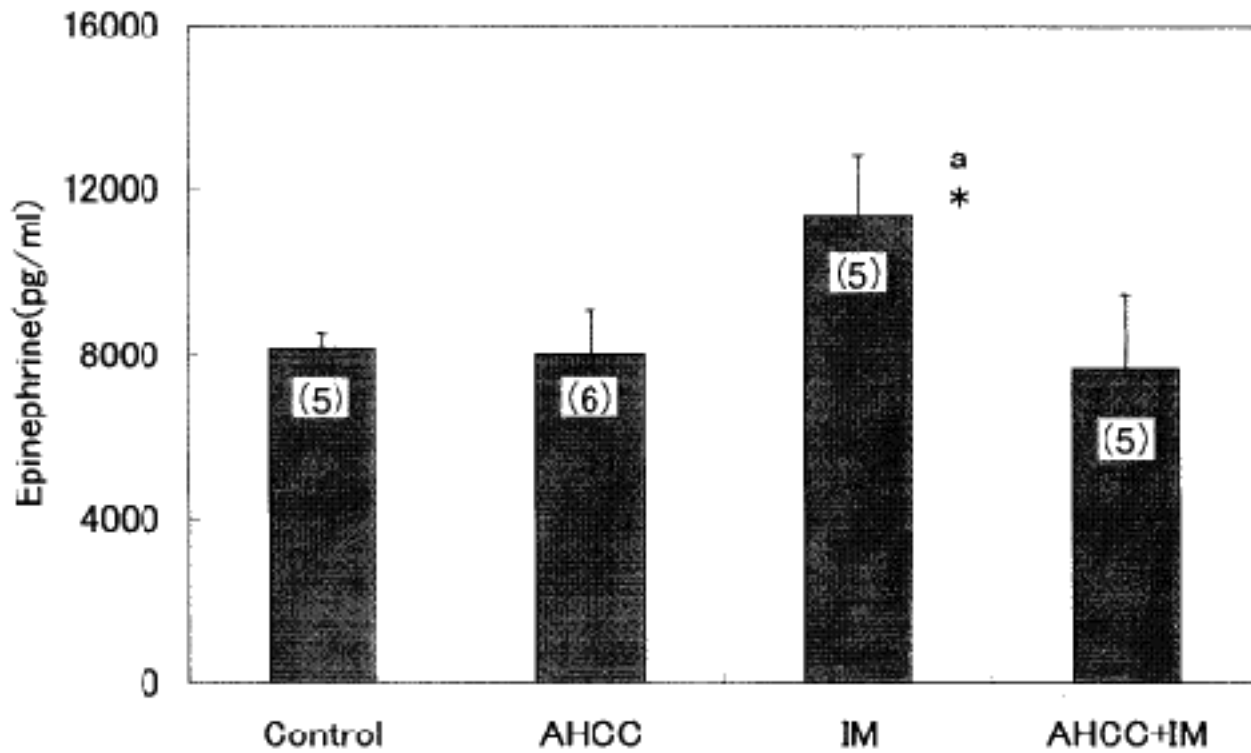


Fig. 2: Effect of immobilization and AHCC on epinephrine (EP) levels in the plasma. EP was assayed by high-performance liquid chromatography (HPLC). Data are shown as means \pm SEM with the number of determinations in parentheses. *, $p < 0.05$ vs. control a, $p < 0.05$ vs. AHCC + IM.

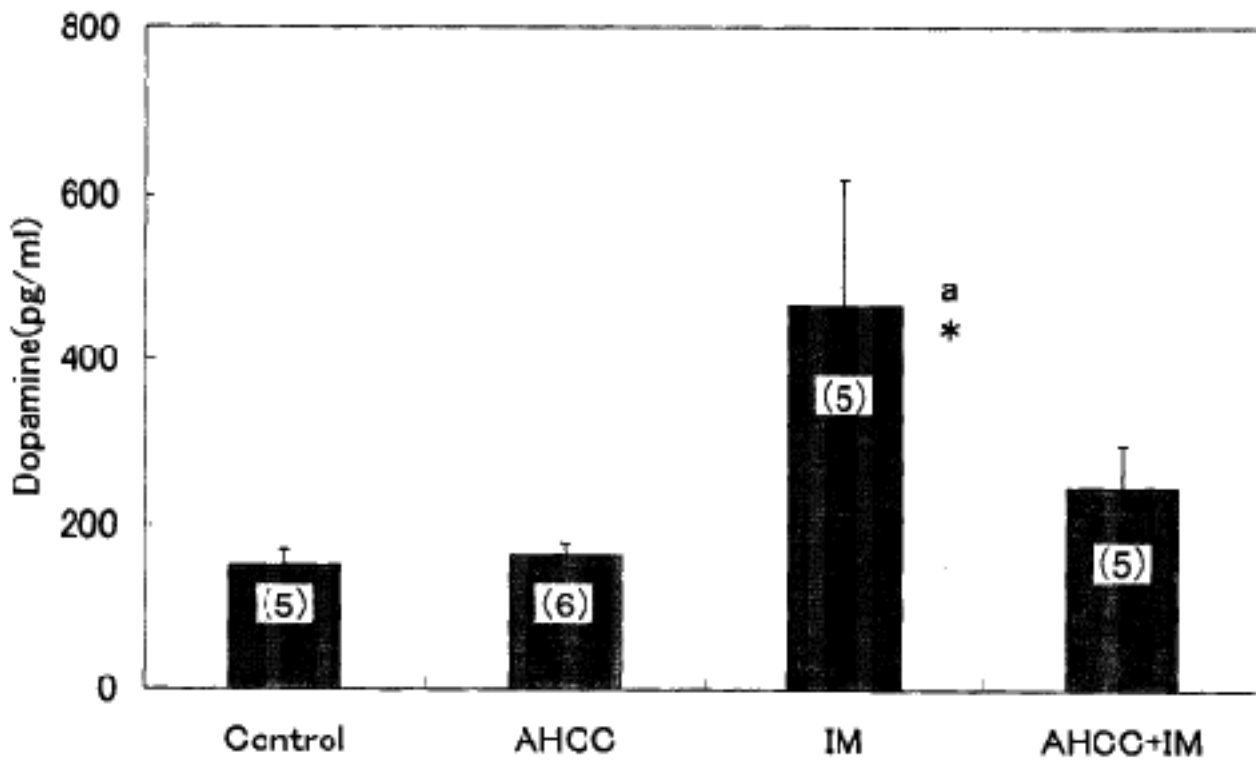


Fig. 3: Effect of immobilization and AHCC on dopamine (DA) levels in the plasma. DA was assayed by high-performance liquid chromatography (HPLC). Data are shown as means \pm SEM with the number of determinations in

parentheses. *, $p < 0.05$ vs. control a, $p < 0.05$ vs. AHCC + IM

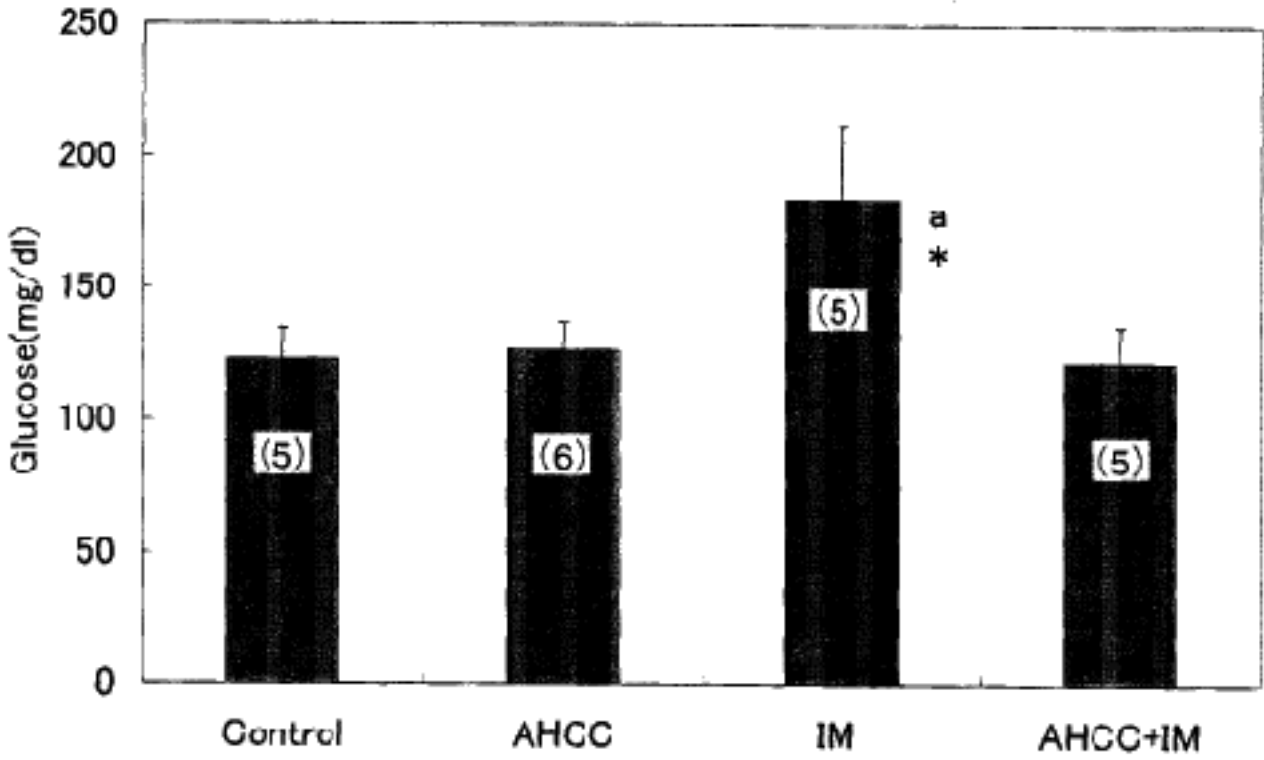


Fig. 4: Effect of immobilization and AHCC on glucose levels in the serum. Glucose in the serum was assayed by glucose assay kit. Data are shown as means \pm SEM with the number of determinations in parentheses. *, $p < 0.05$ vs. control a, $p < 0.05$ vs. AHCC + IM

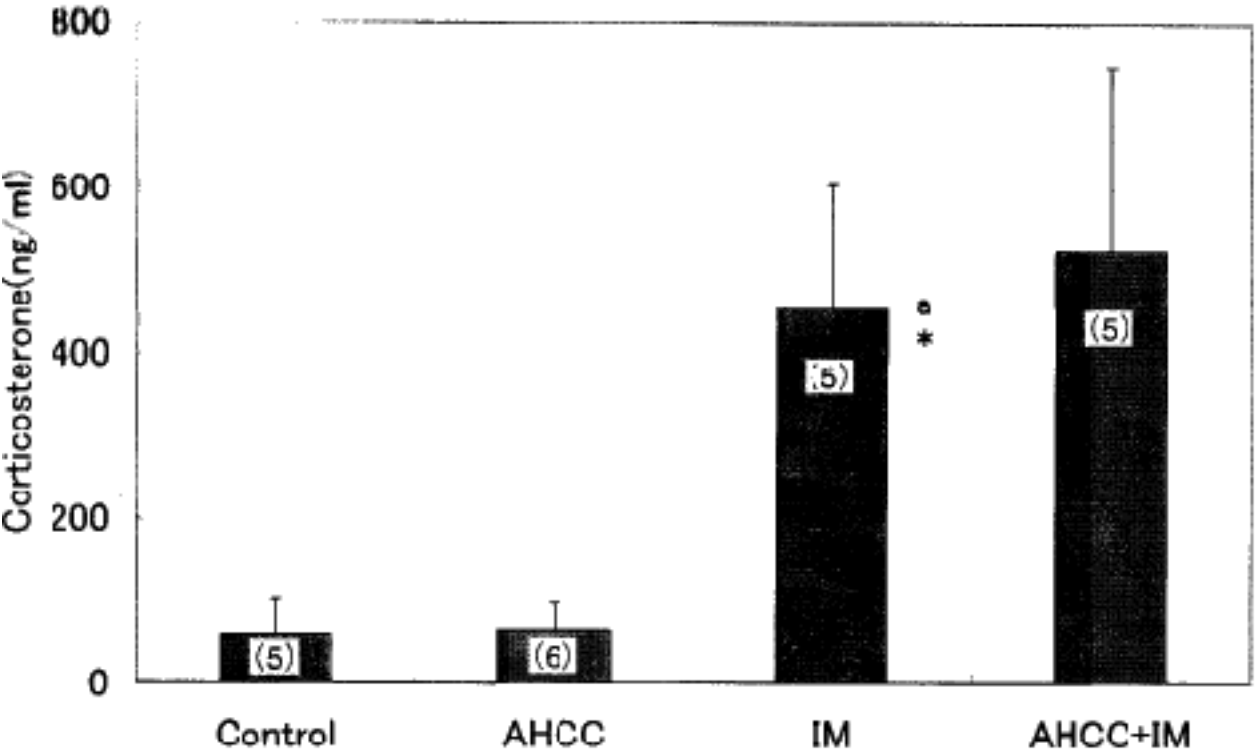


Fig. 5: Effect of immobilization and AHCC on corticosterone levels in the plasma. Corticosterone was assayed by high-performance commercial radioimmunoassay. Data are shown as means \pm SEM with the number of determinations in parentheses. *, $p < 0.05$ vs. control a, $p < 0.05$ vs. AHCC + IM

Protective Effects of Active Hexose Correlated Compound (AHCC) on the Onset of Diabetes Induced by Streptozotocin in the Rat

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Biomedical Research 20 (3) 145-152, 1999

Abstract

Effects of Active Hexose Correlated Compound (AHCC) on the onset of diabetes were studied in rats treated with Streptozotocin (STZ). AHCC was given to male rats at 4% in drinking water. A single i.v. injection of STZ (40mg/kg body weight) to rats resulted in an increase in blood glucose levels, a decrease in serum insulin levels, suppression of body weight gain, and an increase in serum GOT and GPT activities and serum levels of lipid peroxides. Treatment of AHCC restored these parameters to normal. Insulin immunoreactive B-cells in Langerhans islets reduced in number after treatment with STZ, while insulin immunoreactivity in the islets was normalized when AHCC was administered to STZ-treated rats. These results show that AHCC treatment is effective on the prevention of diabetes onset induced by STZ.

Crude extracts derived from fungi of Basidiomycetes family such as lingzhi {Ganoder-malucidum, reishi) and zhuling (Polyporus umbellatus, chorei) are still used as components of Chinese traditional medicine. These fungi contain polysaccharides as well as other bioactive substances whose, actions include regulation of the immune system, antitumor action, hypo-glycemic effect, improvement of lipid metabolism and diuretic effect (26).

Recent advances in culture techniques have enabled us to culture various species of Basidiomycetes. Active Hexose Correlated Compound (AHCC™, Amino UP Chemical Co; Ltd., Sappro) is a mixture of polysaccharides, amino acids and minerals derived from fungi. It is obtained by hot water extraction after culturing mycelia of several basidiomycetes in a liquid culture tank and then treating them with some enzymes.

AHCC has been successfully used as a biological response modifier (BRM) in various disorders but only a little is known about its mechanism of actions. Recently we have reported that AHCC has hepatoprotective and detoxicating effects due to induction of hepatic enzymes and antioxidant action (23). It was also found that AHCC prolonged the life span and stimulated cytokine secretion from macrophages in mice bearing SST-2 tumor when treated together with 5-fluorouracil (5-FU)(14). Furthermore, it was shown that AHCC was effective in preventing lung metastasis in breast cancer-bearing mice when treated simultaneously with 5-FU (14). Sun et al. (13, 22) described that AHCC diminished the side effects caused by antitumor agents such as cyclophosphamide, 5-FU and cytosine arabinoside. In other words, the disorders in hematopoietic function, depilation and he-patotoxicity induced by these pharmaceuticals were all ameliorated by AHCC. These results suggest that AHCC restores the depression of the immune system and reduces side effects caused by antitumor agents, resulting in the prolongation of life span in experimental animals.

It is reported that AHCC treatment for 2 years of patients with hepatoma who had undergone hepatectomy suppressed the remission and prolonged their life span significantly when compared with control patients (10). Yagita et al. (28) found that oral administration of AHCC to patients with malignant tumors resulted in an increase in serum levels of tumor necrosis factor (TNF)- α , interferon- γ , and interleukin-12 and a decrease in serum immunosuppressive acidic protein (IAP) and tumor growth factor (TGF)- β levels. AHCC has clinically shown to be beneficial for the treatment of other diseases. In addition to patients with malignant tumors (28), diabetic patients have been shown to respond to AHCC (11). Polysaccharides which lower elevated blood glucose levels were found in several fungi. For example, ganoderan A, B and C, polysaccharides derived from Ganoderma lucidum have hypo-glycemic activity in alloxan-treated mice (24). It is not known whether or not AHCC contain such anti-diabetogenic polysaccharides.

In diabetes, the metabolism of glucose, proteins and lipids is abnormal due to the deficit in glucagon and insulin secretion, leading to various metabolic disorders (4) and onset of complications (8, 21). It is also reported that diabetics are highly sensitive to oxidative

stress (1, 17, 25).

The objective of the present study is to examine the preventive effects of AHCC on the onset of diabetes in rats treated with streptozotocin (STZ). This chemical is thought to induce type I diabetes possibly by stimulating xanthine oxidase (XOD) reaction in β -cells of Langerhans islets to increase O₂ radicals, which in turn destroy the cells (9). Thus, the STZ-induced diabetes can be regarded as an experimental model of type I diabetes. The present study deals with the preventive effects of AHCC on the impairment of islet cells.

Materials and Methods

Materials: STZ was obtained from Sigma Chemical Co. (St. Louis, MO, USA). It was dissolved in 0.05 M citrate buffer, pH 4.5 at a final concentration of 40 mg/mL. The solution was stored in darkness at 4°C until use. Lyophilized AHCC was obtained from Amino UP Chemical Co. Ltd. (Sapporo, Japan).

Animals: Male Wistar rats of 4 weeks old were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were housed in a room kept at 22±2°C with 12h light and dark cycle (light on 8 : 00-20 : 00 h) and kept free access to food and water. The general conditions of the rats were observed every day. The amount of water intake was measured by subtracting the amount of residual of water from that measured the previous day.

A group of rats were given only AHCC at 4% in their drinking water for 3 weeks (AHCC-treated group). STZ at a dose of 40 mg per kg body weight was injected through the tail vein to 2 groups of rats (STZ-treated group). One of the STZ-treated groups received 4% AHCC in drinking water for one week prior to the treatment and for 2 weeks thereafter (STZ-AHCC treated group).

Blood sampling and organ preparation. Blood was collected via the tail vein of the rats into EDTA-coated blood sampling tubes at 10:00 a.m. on the same day immediately after STZ treatment and at 7 and 14 days after STZ treatment. At 14 days after STZ treatment the rats were sacrificed by decapitation, and their livers and pancreases were removed. These organs were quickly rinsed in ice-cold saline solution. Pancreases were immediately fixed in 10% formalin.

Biochemical analyses. Blood samples were centrifuged at 4°C for 15 min at 3,000 rpm and the sera thus obtained were used for biochemical analyses. Serum GOT and GPT, blood glucose, lipid peroxides (LPO) and insulin were measured by Henry's method (5), glucose oxidase method, Yagi's method (27) and Insulin-EIA test (Wako, Osaka), respectively.

Immunocytochemistry. The pancreatic tissue was fixed in 10% buffered formalin for 2 days, and embedded in paraffin according to the conventional procedure. Paraffin sections were serially cut at 5 μ m in thickness and immunostained. Localization of insulin in pancreatic islets was visualized by the streptavidin-biotinylated horse-radish peroxidase complex immunoenzymatic technique using Histofine SAB-PO (M) kits (Nichirei, Tokyo, Japan) according to the protocol of the manufacturer. Briefly, a monoclonal antibody against rat insulin was employed as the first antibody. Reaction products were formed with 3, 3'-diaminobenzidine tetra-hydrochloride in the presence of hydrogen peroxide. The immunoreaction specificity was determined using normal serum instead of the specific antiserum.

Statistical analysis. All the data were expressed as mean±SD. Statistical analysis was performed by Dunnett's multiple comparison test to control and non parametric method by Scheffe's multiple comparison test. A P value less than 0.05 was considered statistically significant.

Results

General Conditions

Poor general conditions such as depilation and deterioration in quality of fur were noticed in the STZ-treated group. AHCC treatment resulted in an improvement of these conditions. AHCC alone showed no effect on body weight changes. A significant decrease in body weight gain was observed during 14 days after STZ treatment when compared with intact controls. The body weight gain of the STZ-AHCC treated rats was nearly the same as that of the intact control.

Water intake

An increase in the daily water consumption was noticed in the STZ-treated group (Fig. 3). AHCC given alone increased somewhat the water consumption but decreased the increased water intake when given to the STZ-treated rats.

Biochemical analyses

A significant rise in blood glucose levels was observed in the STZ-treated group up to 14th day after the drug treatment, reaching its maximum on 7 days after the treatment (Fig. 4). The blood glucose level at 14 days after STZ treatment was more than twice that in the control group. AHCC treatment nearly normalized the elevated levels of blood glucose in STZ-treated rats. The serum insulin levels decreased following STZ treatment and remained low till 14 days (Fig. 5). AHCC treatment restored the decreased insulin levels to normal.

The serum levels of GOT, GPT and LPO were greater in the STZ-treated group than in the intact control and STZ-AHCC treated group at 14 days after the treatments.

Histology of the pancreas

A prominent decrease in the number of b-cells in the Islets of Langergans was observed in the STZ-treated group when compared with control group. The immunoreactivity of insulin in b-cells of the STZ- Paraffin sections obtained from the control and treated rats were immunostained with anti-insulin antibody. A prominent decrease in the number of insulin immunoreactive cells was recognized in STZ-treated group, while appreciable insulin cells were found in the STZ-AHCC treated group.

Discussion

Streptozotocin (STZ) was originally discovered as a metabolite of *Streptomyces achromogenes* var. *streptozotocis* by a research group in Upjohn Co. Ltd. in 1959. At first, there were expectations over possible clinical applications of its antibacterial and antitumor activities but it was soon found that diabetes was induced as a side-effect of STZ treatment. This chemical has widely been used as a diabetogenic agent there-after. Okamoto suggested that STZ induces diabetes through damaging DNA in the nuclei of pancreatic b-cells by alkylation, leading to an increase in poly (ADP-ribose) synthase. The increase in the enzyme activity results in a drastic decrease in nicotinamide adenine dinu-cleotide (NAD) concentrations of the yff-cells and then a decrease in the number of b-cells and death of the cells. All these changes may induce dys-function of the pancreas.

According to Kawada, STZ transported into b-cells through glucose transporter GLUT-2 located on their cell membranes is activated inside the cells and injures their mitochondria. This inevitably leads to a reduction of ATP generation through electron transport system and an increase in ADP concentrations. Subsequent degradation of ADP provides hypoxanthine, a substrate of xanthine oxidase (XOD). When XOD reaction takes place in the b-cells where XOD activity is intrinsically very high, O₂ radi-cals are produced, resulting in cell damage and the onset of diabetes. STZ also activates XOD directly and augments O₂" generation.

The present study showed that the body weight of the STZ-treated rats decreased soon after STZ administration. This finding was in close agreement with that of other investigators (2, 3, 7, 12). In the STZ-AHCC treated group, however, no significant decrease in body weight was observed, suggesting that AHCC normalized the weight change caused by STZ.

A significant increase in water intake, one of the general signs of diabetes, was observed in the STZ-treated rats. The water consumption was a little greater in the AHCC-treated groups than in the control, presumably because the rats had preference to AHCC-containing water which is rich in polysaccharides, amino acids, lipids and minerals. The water consumption of the STZ-treated group is much greater than that of the AHCC-treated groups. AHCC nearly normalized the water consumption and body weight gain of STZ-treated rats, suggesting that AHCC may have prevented the onset of diabetes.

In the STZ-treated group, blood glucose rose immediately after the treatment and reached a quite high level at 14th day. The serum insulin levels decreased significantly in the STZ-treated group, indicating that their pancreatic b-cells were damaged. On the other

hand, the blood glucose levels in the STZ-AHCC treated group were much lower than in STZ-treated group but slightly higher than in the control group. These results showed that mild diabetes did occur in the STZ-AHCC-treated rats, even though the serum insulin levels were restored to normal in STZ-AHCC treated rats.

The serum levels of GOT, GPT and LPO increased in the STZ-treated group, while they were normalized by AHCC treatment. Rhee et al (20) reported that the increase in the serum levels of GOT, GPT and LPO was due to the oxidative damage in the pancreas, liver, kidney and other organs caused by STZ through the increase in the free radical production. They suggested that the production of free radicals was augmented by the increase in arachidonate concentrations and the activity of lipoxygenase and cyclooxygenase. Furthermore, they observed the suppressive effects of antioxidants such as vitamin E on the onset of diabetes. The results of our present study suggest that AHCC suppresses the production of free radicals induced by STZ, whereby symptoms of diabetes were diminished.

Diabetogenic substances such as STZ preferentially destroy nuclei of β -cells. Apoptosis has recently been suggested to be involved in STZ-induced degeneration of islet cells (15). Since β -cells of the islets decreased in number, one can expect that apoptosis may have occurred following STZ administration. The STZ-induced decrease in insulin immunoreactivity in islet β -cells was restored by AHCC. These findings suggest that AHCC prevents cellular damage induced by STZ and preserve the capability of insulin secretion.

In conclusion, the results of our present study show that AHCC can prevent the onset of STZ-induced diabetes by protecting β -cells from degeneration and by diminishing oxidative injuries of cells in various organs. It remains to be clarified the mechanism by which AHCC acts as an antioxidant.

The author would like to thank Professor S. Matsuzaki (Dokkyo University School of Medicine) for his critical review of the manuscript. Thanks are also due to Amino UP Chemical Co. Ltd for the supply of AHCC.

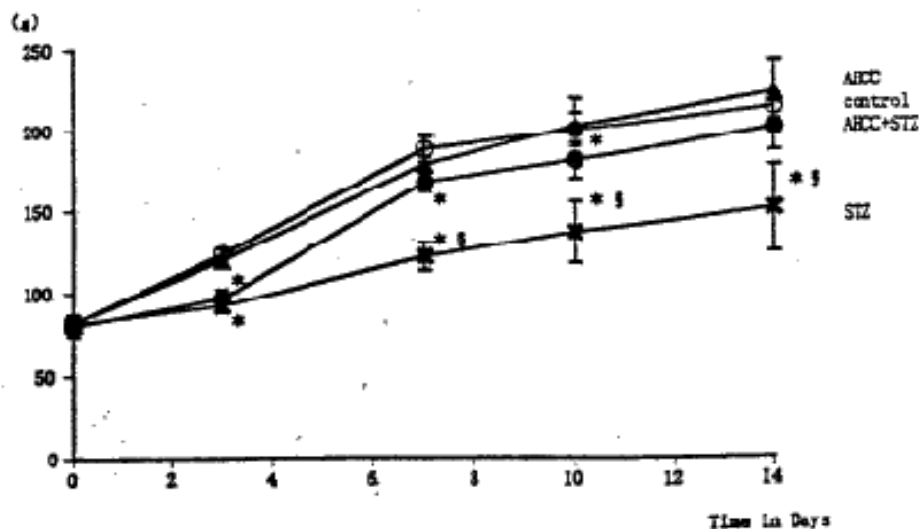


Fig. 1 Effects of AHCC and STZ on the body weight. Single intravenous injection of STZ reduced significantly the body weight gain. The body weight in the STZ-AHCC treated group was significantly larger than that in the STZ-treated group. Values are the means \pm SD of 6 determinations in each group. * $P < 0.01$ vs. control group. § $P < 0.01$ vs. STZ-AHCC group.

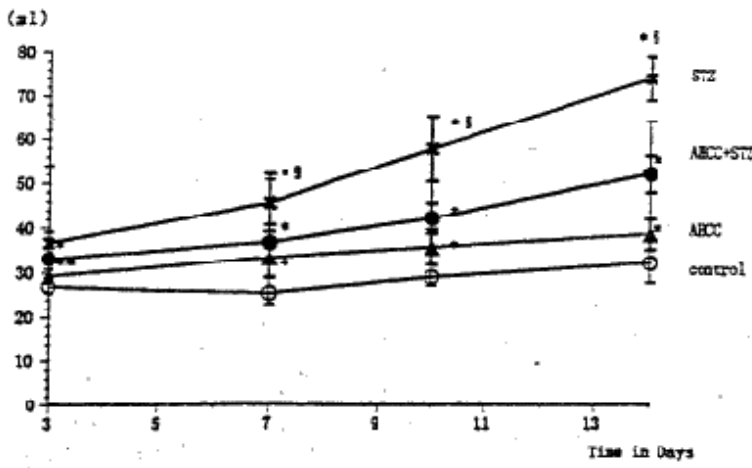


Fig. 2 Effects of AHCC and STZ on water intake. Single intravenous injection of STZ caused significant increase in the water intake. Treatment of the diabetic rats with AHCC nearly normalized the water intake. The values are the means \pm SD of 6 determinations in each group. * $P < 0.01$ vs. control group. § $P < 0.01$ vs. AHCC-STZ group.

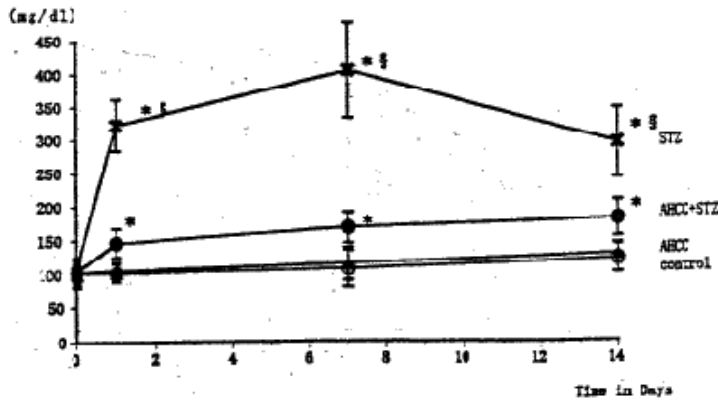


Fig. 3 Effects of STZ and AHCC on serum glucose levels..

Single intravenous injection of STZ caused marked elevation of the blood glucose, reaching its maximum at 7 days of the treatment.. Treatment of the diabetic rats with AHCC nearly normalized the elevated serum glucose level. The values are the means \pm SD of 6 determinations in each group. * $P < 0.01$ vs. control group. § $P < 0.01$ vs. AHCC-STZ group.

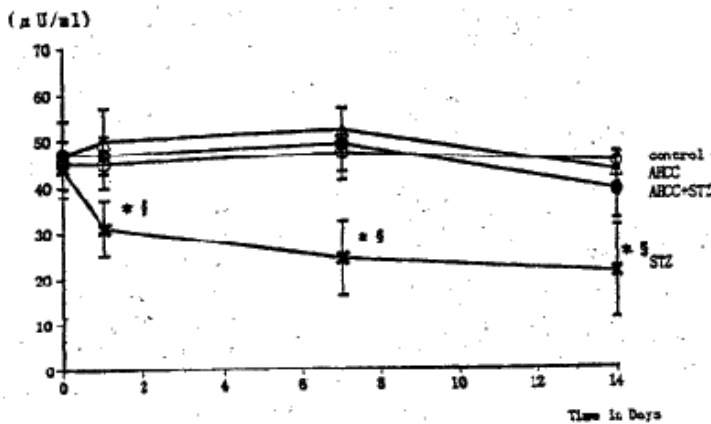


Fig. 4 Effects of STZ and AHCC on serum insulin levels..

Single intravenous injection of STZ caused a significant decrease in the immunoreactive insulin level. AHCC restored the decreased serum insulin in diabetic with rats. Values are the means \pm SD of 6 determinations in each group.

* P<0.01 vs. control group. § P<0.01 vs. AHCC-STZ group.

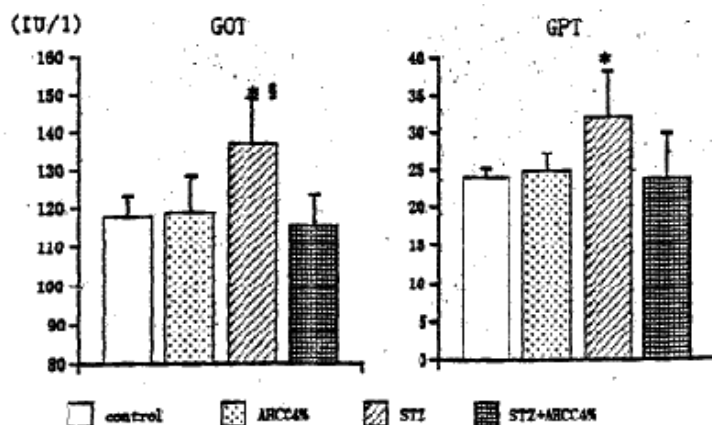


Fig. 5 Effects of STZ and AHCC on the activity of serum GOT and GPT. Rats were sacrificed at 14 days after STZ treatment and their sera were taken. The activity of the transaminases (GOT and GPT) was assayed as described in the text. Values are the means \pm SD of 6 determinations in each group. * P<0.01 vs. control group. § P<0.01 vs. AHCC-STZ group.

Table 1: Effects of STZ and AHCC on serum lipid peroxides (LPO)

After 14 days	Control	AHCC treated	STZ treated	AHCC-STZ treated
Lipid peroxides (LPO) (nmol MDA/ml)	5.29 \pm 0.72	5.22 \pm 0.62	6.26 \pm 0.81*§	5.13 \pm 0.67

* P<0.01 vs. control group. § P<0.01 vs. AHCC-STZ group.

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Preventive Effects of AHCC on Carbon Tetrachloride Induced Liver Injury in Mice

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Introduction

In the natural world, it is said that approximately 5,900 genera, 6,400 kinds of mycelia inhabit. Lingzhi {Ganoderma lucidum, reishi), zhuling (Polyporus umbellatus, chorei) are popular Chinese traditional medicine derived from fungi of basidiomycetes family. Those mycelia are identified to contain various physiologically active substances, such as polysaccharide with β -1, 3-glucan structure. It has been reported its activities; anti-tumor activity, accommodation activity on immune system, hypoglycemic activity, etc.

Recent advance of culturing techniques has enabled artificial culture of basidiomycetes. Active Hexose Correlated Compounds (AHCC; from Amino Up Chemical Co., Ltd.) is a mixture containing polysaccharide obtained by culturing in a liquid culture tank followed by enzyme reactions and hot water extraction.

AHCC has been observed and reported its bioactivity as Biological Response Modifiers (BRM) or nonspecific immunoreactive activator. Especially in clinical studies, the effects of AHCC have been reported; improvement of adult diseases such as diabetes or hepatic disease, cancer cell atrophy and inhibition of metastases in tumor patients, the survival time prolongation, reducing side effects caused by chemotherapy, etc.

Although there are many clinical studies reported, actual pharmacological mechanism is known only a little. To investigate its pharmacological mechanism as the first step, we prepared an acute carbon tetrachloride hepatitis model in mice which symptom is said to be similar to drug liver injury in human. This is the report of the investigation of the effect of AHCC administered per os (p.o.) on liver function change and drug metabolizing enzymes in liver.

Materials and Methods

Animals. Male ICR mice of 7 weeks old, body weight 34-38 gm. were purchased from Nippon Clea Co., Ltd. The animals were kept free access to food (CE-2; Nippon Clear) and water for one week as pre-breeding period.

Materials. All the material drugs were purchased from Wakopure Industry Co., Ltd. and Sigma Chemical Co. AHCC freeze-dried powder was supplied by AMINO UP CHEMICAL CO., LTD.

Methods

Preparation of food and its administration- AHCC was dissolved in water at 50% concentration. Mice were treated with the solution by oral at the dose of 1g/kg/day for three days. On the fourth day, 20% solution of CCl₄ diluted with olive oil (Japan Pharmacopoeia) was administered i.p. at the dose of 2ml/kg/day for five days. Those mice were divided into four groups with five mice each: control group with normal mice, injured group treated with CCl₄, AHCC treated group, AHCC and CCl₄ co-administrated group (CCl₄ was administered after AHCC treatment).

Blood and organ preparation- On the final day of CCl₄ administration, mice were given free access to water not giving any food. 12 hours later, blood sample was collected via carotid. The samples were centrifuged (3,000 r.p.m., 5 min) and serum was obtained. The liver removed was rinsed with in ice-cold saline solution (0.9%) and weighted. Livers were immediately stored at -80°C.

Serum analysis - Glutamate pyruvate transaminase (GPT) activity in serum was measured by Rate method (GPT-UV Test Wako), Albumin was measured by BCG method (Albumin-B Test Wako), Total protein (TP) was measured by Lowry method, Globulin (G) was calculated by TP-A, Triglyceride (TG) was measured by Acetylacetone method (Triglyceride Test Wako)

Hepatic enzyme preparation- Frozen stored livers were homogenated at ice by adding 4 times of 0.154MKCl-0.05M Tris (pH 7.4)-1mM EDTA. This liver homogenize was first centrifuged 1,000r.p.m., 5°C for 5 min, then 10,000r.p.m. for 5°C for 15min., finally the supernatant fraction was obtained. Then the supernatant fraction obtained by 10,000r.p.m. was centrifuged 38,000r.p.m., 5°C for 60min and supernatant (cytosol) fraction was obtained for GST measurement. Microsome fraction was obtained by adding twice as much as the liver weight of cold 0.154MKCl-0.05M Tris (pH 7.4)-1mM EDTA-20% glycerol solution in the precipitate gained from 38,000r.p.m. The microsome fraction was applied for the measurements of Protein, P450, LPO, UDP-GT, ERDM and AHH.

Lipid peroxidation (LPO) measurement- The measurement was based on Yagi method. 0.05ml of liver microsome fraction was centrifuged 3,000r.p.m., 10min with 0.5ml of 1/12N H₂S₀₄ 4.0ml, 10% phosphotungstic acid aqueous solution, and TBA reagent was added in the precipitate for 60min heating in boiling water. Obtained fluosubstance was extracted by n-butanol and measured its fluorescence intensity with spectrophotofluorometer.

The measurement of P450 content- The measurement was based on Omura and Sato method. Two cells of enzyme solution 3ml which is equivalent to liver weight (250mg) were prepared with hydrosulfite sodium OIM in each cell. One of them were treated with CO for 30 seconds, and another was used as the control. The content of P450 was calculated from the highest peak of 450nm.

The measurement of each enzyme activity- Glutathione S-transferase (GST) measurement was measured by Habig method. Uridine phosphate glucuronyl transferase (UDP-GT) by Mulder method, Arylhydrocarbon Hydroxylase (AHH) by Dehnen method, Benzyloxyresorufin 0-dealkylation (BROD) by Lubet method, Erythromycin N-demethylase (ERMD) by Wrighton. Histopatbological examination- The liver was fixed in formalin and the prepared slide was stained with haematoxylin-eosin (HE).

Statistical analysis- All the data were expressed as means \pm SD. Statistical analysis was performed by ANOVA method and significant difference was judged by Kruskal-wallis test. A P value less than 0.05 was considered as significant difference.

Results

1. General condition in mice

In CCl₄ administration group, some toxicity was observed: the decrease of active movement, hair xanthosis, and inhibition of body weight gain. On the other hand, in the AHCC and CCl₄ combined treatment group, those symptoms were reduced. Comparing to the CCl₄ group, which showed liver weight gain by more than 70%, a significant inhibition in liver weight gain was observed in AHCC co-administration group.

2. Biochemical parameter change in serum and microsome fraction

Comparing to the serum GPT activity increased to 369.5 (IU) in CCl₄ group, it was 164.7 (IU) in AHCC co-administration group. Not any significant change in triglyceride, albumin or globulin was observed.

LPO in liver microsome fraction in CCl₄ group was 287 nmol/ml which was more than twice as much as in control group. On the other hand, LPO in AHCC co-administration group was 152 nmol/ml, the lipid hyperoxidation was significantly inhibited. AHCC administration group also showed lower lipid hyperoxidation comparing to control group).

3. Phase I drug metabolizing enzyme activity

Cytochrome P450 content in liver microsome fraction was decreased in CCl₄ group, however, AHCC co-administration inhibit P450 reduction. P450 content in AHCC group was increased compared with control group. By the measurement of drug metabolizing enzyme activities, BROD activity was not observed. Also, ERDM activity was remarkably inhibited in CCl₄ group and induced in AHCC group, however, there was no significant difference between AHCC co-administration group and control group.

AHH activity was tending to be induced in CCl₄ group and AHCC group, the significance is not clear as in co-administration group (Fig. 2).

4. Phase II drug metabolizing enzyme activity

The measurement of drug metabolizing enzymes showed that CCl₄ suppressed GST activity and UDP-GT activity. This effect was not observed when AHCC was administrated together. Additionally, GST activity was induced in AHCC group.

5. Histopathological changes

In CCl₄ group, widespread centrilobular necrosis and inflammatory cell infiltration were observed. On the other hand, in CCl₄ and AHCC co-administration group, centrilobular necrosis was rarely observed and small part of inflammatory cell infiltration was observed.

Discussion

As a liver poisonous substance, which induces experimental liver injury, CCl₄ is generally used and known to induce acute liver injury by short-term administration.

Acute liver injury model was used in this experiment to find a protecting effect of AHCC on liver. AHCC is known to have immune stimulate effect or anti-tumor effect as bioactivity, and it is expected to have another various activities in plant polysaccharide like AHCC. It is considered that this experiment made one of its activities clear.

It was observed the degradation of appetite, weight loss, auxesis of liver, sGPT level increase, etc. in CCl₄ induced liver injury mice prepared for this experiment.

Regarding organ weight, AHCC significantly inhibit liver weight gain. Liver of mouse administered CCl₄ is known to become fatty liver because of disorder of lipid metabolism, and olive oil used as a solubilizer is also a cause of adiposity in liver. As a result of histopathological examination, adipose degeneration in liver was significantly inhibited in AHCC co-

administrated group more than in CCl₄ group. This result made us think AHCC had some effect on fatty liver.

For serum parameter, sGPT level, a general index of hepatitis, which elevate when liver cell is injured, was suppressed. This result suggests that radical derived from CCl₄ has a preventive effect for attacking liver cell.

Liver cytochrome P450 measured in this research was decreased in CCl₄ group. For the cause of decreasing P450, since P460 content usually depend on the content of protein, the possibilities are whether heme protein change of composition or metabolism damaged or the change of P450 active part, e.g. CO bind inhibition, has happened. On the other hand, P450 tend to be induced in AHCC group. In AHCC and in CCl₄ group, it was considered that P450 content went to the normal level by the counteraction of these two substances.

Furthermore, AHH as P450 metabolic enzyme belongs to CYP1A isozyme. It is a general compound, metabolic enzyme especially for aromatic compound, and has strong relation to metabolic activation of cancergenesis. ERDM belongs to CYP3A molecule and has intimate relation to metabolism of endocrine substances such as antibiotic or hormone. Liver drug metabolizing enzyme is known as the most important enzymes when endogenic substances like hormone or xenobiotics are absorbed and go thorough liver, and also they are known as the enzyme easy to change its amount or activity when liver was injured. It is also known that these enzymes are suppressed and cause endocrine disorder when hepatic failure (liver abortive) is happened, various general symptoms, feebleness or febricula, arise by adrenal hormone disorder or sex hormone metabolism disorder. On the other hand, it is considered that AHCC induce these enzymes and maintain the balance of biofunctions; detoxication of external substances, metabolism, metabolism of endogenic substances (hormone). Also, it was suggested that the dose of AHCC need to have an adjusted when it is administered with P460 drug metabolize enzyme.

Another type of drug metabolizing enzyme is phase II drug metabolizing enzyme: GST and UDP-GT. GST strongly relates to detoxification as glutathione conjugation reaction. That is GST detoxification hydrophobic compound by protein-bounding and operate bioprotective reaction against detriments absorbed or taken in the body or formed by metabolism under normal conditions. UDP-GT is also known to detoxification various kinds of external exogenous materials or endogenic substances such as O-, N-, S- and C-glucuronic acid conjugate as a reaction of glucuronic acid conjugation. AHCC showed inhibition effect on declining phase II drug metabolizing enzyme activity caused by CCl₄, also GST activity induced effect was observed in AHCC administered group. That is one of the protective mechanism for liver injury caused by CCl₄ is to accelerate detoxification of CCl₄. CCl₄ becomes trichloromethylradical. This radical is supposed to form lipid peroxidation followed by hyperoxidation reaction to damage cytoplasmic or cell membrane which cause liver injury. LPO level in AHC and CCl₄ co-administrated group suppressed compared more than in CCl₄ group, and it was reduced more in AHCC group than in control group. According to these results, it is considered that AHCC had another effect to erase radical and prevent liver injury caused by the radical.

In conclusion, AHCC showed protective effect for acute liver injury in mice regarding general condition, serum parameter, liver drug metabolize enzymes. The most significant effects of AHCC are the inhibition of liver auxesis, reducing general poisoning symptom, and inducement of detoxic enzymes. It is necessary to explicate the pharmacological functions of these protective effects.

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Abbreviations

A: albumin, AHCC: active hexose correlatedd compounds, AHH: arylhydrocarbon hydroxylase, BRM: biological response modifiers, BROD: benzyl oxyresorufm 0-dealkylation, CCl₄: carbon tetrachloride, ERMD: erythromycin N -demethylase,

G: Globulin, GPT: glutamate pyruvate transaminase, GST: glutathione S-transferase, TG; tryglyceride, TP: total protein, UDP-GT: uridine phosphate glucuronyl transferase.

Improving Effect of Active Hexose Correlated Compound (AHCC) on the Prognosis of Hepatitis C and Carcinoma Patients

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The 34th Congress of the European Society for Surgical Research (Bern, Switzerland) 1999.

Medicinal mushrooms and their extracts have been reported to have a variety of biological effects including immunomodulation (1,2), anti-tumor properties (2-5), and beneficial influences on serum lipids, blood pressure and blood sugar (6-9). Active hexose correlated compound (AHCC), a newly developed extract from a hybridization of several kinds of mushrooms, is one such product whose most active ingredient, among many, is thought to be an oligosaccharide with a molecular weight of approximately 5,000. Research suggests that the immune effects are produced through stimulation of NK cells, killer T-cells and cytokine production, specifically gamma interferon, IL-12 and TNF-alpha (2,6).

To illustrate the clinical influence of AHCC therapy on viral loads in Hepatitis C patients and on CD4 counts in patients with breast cancer, we present the following preliminary case reports:

Case 1: 35 YO Female with Hepatitis C

A.G. presented 8/93 for treatment of Hepatitis C. She was diagnosed 7/92 with elevated liver enzymes and positive Hepatitis C antibodies, and had a history of IV drug abuse. She was prescribed a dietary and nutritional supplement regimen as well as intravenous (IV) vitamin treatments, all of which were focused on immune support and anti-viral effect. Blood work was largely normal for five years except for slight, fluctuating elevations in the liver enzymes AST (GOT), ALT (GPT) and GGT. She also reported occasional pain and tingling in the area of the liver.

Blood work 11/98 showed a Hepatitis C Virus RNA level of 2,160,900 by PCR testing (nl<2000 copies/MCP). AHCC was added within a week after this test -6 gms in divided doses and no other adjustments to the patients treatment protocol were made. Follow-up testing 3/99 showed a decrease in Hepatitis C Virus RNA to 1,573,400 (a 27.2 % decrease in 4 months).

Case 2: 64 YO Female with Hepatitis C

M.F. presented 11/98 for treatment of Hepatitis C, irritable bowel syndrome (IBS) and allergies. The hepatitis diagnosis was made 2-3 years earlier when liver enzymes significantly increased, although there was some evidence of elevated liver enzymes up to 35 years earlier. At presentation, the liver enzymes Gamma GT and AST were high (100.0 and 67.0 respectively), and the WBC count was low (3.4 thous/ml). Hepatitis C Virus RNA by PCR testing, done 1/99, showed a count of 1,475,000. Nutritional supplementation and IV vitamin therapy did not produce significant results as liver enzymes were still high and the patient was still fatigued. AHCC was begun 3/99, 6 gms in divided doses. Follow-up testing showed Hepatitis C Virus RNA level of 167,000 (a dramatic 89% decrease in 4 months). Liver enzyme

levels were essentially unchanged, however, the patient reported significant improvement in energy.

Case 3: 47 YO Male with Hepatitis C and Prostate Cancer

D.F. presented 1/98 with Hepatitis C and prostate cancer (PSA 14.2, Gleason 6). He was diagnosed with moderately differentiated adenocarcinoma with high heterogeneity, when he experienced painless hematuria 11/97. Hepatitis C was first treated in 1974 with acupuncture and homeopathy with resolution of increased liver enzymes.

The patient was treated with total androgen blockage (TAB) starting in 5/98 to stabilize the prostate cancer - his PSA decreased to 4.9. The last Lupron was administered 1/99 and the last Casodex was taken 2/99. Measurement 12/98 of Hepatitis C Vims RNA by PCR testing was 2,498,200. D.F. began AHCC 1/99. 2/99 AHCC was increased to 6 gms per day in divided doses. Follow-up testing done 7/99 showed a significant decrease in the level of Hepatitis C Virus RNA to 499,600 (an 80% reduction in 6 months).

Case 4: 48 YO Female with Metastatic Breast Cancer

M.K. presented 12/96 for treatment of breast adenocarcinoma, poorly differentiated her original diagnosis was made in 1990 when she had a lumpectomy. An evaluation of hip pain in 1996 showed bilateral breast masses, positive bilateral axillary lymph nodes, and metastases to the sternum, ilia and sacrum. After a bilateral mastectomy, an aggressive alternative medical therapy was begun (all standard treatments were refused). After various alternative protocols, the patient stabilized regarding extent of metastases, pain control, and immune function, although she reported that she was not feeling well.

M.K. had AHCC added to tier treatment protocol 2/99 - 6 grams per day in divided doses. After 10 weeks on AHCC she showed a significant improvement in reported well-being as well as improved immunological parameters. These improved results (measured 4/16/99 compared with 1/28/99) included: (a) Total WBC 3.6 vs 2.8 Th/mm³; (b) Helper-Inducer T-cells (CD4) 275 vs. 232; (c) Total lymphocytes 810 vs 742; (d) Total T lymphocytes 492 vs 460; (e) B lymphocytes 124 vs 68; and (f) Helper/Suppressor ratio 1.3 vs 1.1.

These cases illustrate the clinical influences of AHCC therapy. Clearly more evaluations are necessary with larger numbers of patients over a longer study period using a double blind approach. At the same time, systematic clinical and basic research is required to elaborate on the mechanisms of benefit and the indications for AHCC's optimal use. Until then, our preliminary clinical experience suggests that AHCC has significant potential benefit in this population group worthy of further research.

Impaired Th1-Related Immune System in Cancer Patients: Comparable Down-Regulation In Early Stage And Advanced Stage

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Summary

Background

Interleukin 12 (IL-12), Interferon- γ (IFN- γ) and Tumor necrosis factor- β (TNF- β) are known to induce proliferation and differentiation of Th1 cells. These "Th1-related" cytokines are shown to negatively modulate the growth of tumors. In animal models, Th1 cells are shown to be activated shortly after the implantation of tumor cells and exert antitumor effects, but suppressed and overwhelmed by expanding Th2 cells as tumor cells grow. In this study, we ask whether Th1 system including these cytokines is actually modulated in cancer patients.

Methods

Patients diagnosed cancer of various organs, and 100 control subjects without cancer were enrolled to the study. The production of Th1-related cytokines (IL-12, INF- γ and TNF- β) by peripheral blood mononuclear cells, and the induction of Th subsets (Th1 and Th2) were measured and compared between patients and control subjects after mitogen stimulation in vitro. Natural killer (NK) cell activity was also measured. To evaluate the influence of disease progress we selected stomach cancer and colon cancer due to their common criteria for staging. Each parameter was compared between cancer patients in early stage, in advanced stage and control subjects.

Findings

Phytohemagglutinin(PHA)-induced IL-12, IFN- γ and TNF- β in cancer were significantly lower than those in control. Th1 cell induction was also lower in cancer than that in control we found no significant difference in Th2 induction between cancer and control. NK cell activity was not up- or down-regulated in cancer patients. When patients with either stomach cancer or colon cancer were grouped to two stages based on the common criteria for staging, suppressed cytokines and Th1 cells were seen in the early stage as well as the advanced stage. None of these parameters differed between the two stages of cancer. Collectively, results show that suppressed Th1-related immunological parameters already exist at early stage of cancer and persist through the disease progress.

Interpretation

Th1-related immune system appears already impaired in early stage of cancer which suggests either; (a) tumor cells even in small size have significant effects on immune system and have already down-regulated Th1-related function or; (b) defective Th1 system precedes the emergence of tumor cells. Our data may favor that the latter is the case. Our results also suggest that Th1- related immune parameters would be useful immunological markers for cancer screening.

Introduction

Although cancer therapies have progressed it is still not easy to find the disease in its early stage. Because the incidence of cancer is still increasing globally periodical health check is getting more important and preventing medicine need to be highlighted. But unfortunately, conventional methods including hematological, biochemical, urinal examinations as well as X-rays are not sensitive enough to detect cancer in the early stage. In this concern, the development of better techniques to detect cancer in the early stage.

The acquired immunity is characterized by the function that immune competent cells including T cell and B cells recognized foreign antigen with antigen specific manner. From animal experiments the antigen specific mechanism¹ was shown to be activated and functioning in the cancer immunity whereas antigen non-specific mechanism² is also functioning. The finding of Th1 and Th2 subsets based on the cytokine production pattern gave us a tremendous help in understanding immune system. Studies have revealed that IFN- γ , and TNF- β produced by Th1 cells and IL-12 by APC through the cross talk between these cells are key factors for the differentiation of Th cells to Th1 cells which are responsible for cancer immunity. Together, these cytokines seem to modulate and optimize the immune responses including antitumor responses originally initiated with antigen specific or non-specific manner.

In this study, we shed light on Th1-related cytokines and Th subsets in cancer patients and asked whether these parameters are actually modulated in cancer. To the aim mitogen-induced cytokines and Th subsets were measured in cancer patients and compared with those in control subjects. Then we selected stomach cancer and colon cancer for the evaluation of the influence of disease progress on immunological parameters. We found impaired Th1 - related immunological parameters not only in advanced stage but also in early stage of cancer, suggesting that defective anti-tumor immunity already exists in early stage of cancer. We propose that Th1-related immunological parameters may be useful markers for cancer detection at early stage.

Methods

Patients

Patients who had been diagnosed cancer, aged 16 - 87 years, at various organs were enrolled (Table 1). Patients who had experienced therapies including surgical operation, chemotherapy and radiation were not included. When we selected patients with stomach cancer or colon cancer for the evaluation of the

influence of disease progress on immunological parameters. They were grouped to the early stage, cancer cells stay within mucosa (m) or submucosa (sm), or the advanced stage; cancer cells invade beyond the submucosa (Table 2). 100 individuals without cancer, aged 27 - 72, were enrolled as controls. Consent was obtained from all the patients and control subjects.

Study Design

For cytokine assay Heparinized peripheral blood cells were diluted by phosphate buffered saline without calcium and magnesium (PBS (-)). Centrifuged on Ficoll-Conray at x400 G for 20 min, mononuclear cells were collected. After washing, mononuclear cells were resuspended in RPMI-1640 (IBL, Co., Fujioka, Gunma, Japan) with 10% FCS (Cancera, Rexdale, Ont.) with the concentration of 1×10^6 cells/ml of lymphocytes. Lymphocytes ($2 \times 10^6/200 \mu l$) and $20 \mu g/ml$ of phytohemagglutinin P (PHA-p; Difco, Laboratories, Detroit, MI) were added to 96 holes microtiter plates, with $220 \mu l$ in final volume. After incubation at $37^\circ C$ for 24 hours with 5% CO_2 supernatants were collected for cytokine assay. IL-12 was measured by enzyme linked immuno solvent assay (ELISA) kit (R&D Systems, Minneapolis, MN). IFN- γ and TNF- β were measured by ELISA kits (IFN- γ ; Biosource, Nivelles, Belgium, TNF- β ; JIMRO Gunma, Japan).

All cytokines were measured with the way as manufacturers recommended.

For identification of Th cell subsets Heparinized peripheral blood were stimulated with 25 ng/ml of PHorbol 12-Myristate 13 Acetate (PMA, Sigma Chemical Co., St. Louis, MO), $1 \mu g/ml$ of ionomycin (Sigma Chemical Co., St. Louis, MO) and $10 \mu g/ml$ of Brefeldin-A (Sigma Chemical co., St. Louis, MO) at $37^\circ C$ with 5% CO_2 for 4 hours. $300 \mu l$ of stimulated blood was added by $20 \mu l$ of peridinin chlorophyll protein (percept)-conjugated anti-CD4 antibody (Immunotech, Marseille, France) incubated at room temperature for 15 min. $4 \mu l$ of x 10 Lysing Solution (BDIS, San Jose, CA) was added and incubated for fixing cell surface epitopes, lysing red blood cells and pretreatment for permialization. Then $1.5 \mu l$ of x 10 Lysing Solution (BDIS, San Jose, CA) was added and incubated at room temperature and for 10 min. For staining of intracellular cytokins $20 \mu l$ of fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ antibody (BDIS, San Jose, CA) and phycoerythrin-conjugated anti-IL-4 antibody (BDIS, San Jose, CA) were added and incubated for 30 min. at room temperature. FITC-conjugated IgG2a/PE-conjugated IgG1 antibodies (BDIIS, San Jose, CA) were used as negative controls. The FACS analysis was done with FACScan (BDIS, San Jose, CA).

For the assay of NK cell activity target cells (K-652) 3×10^6 were incubated with $100 \mu Ci$ of ^{51}Cr -Sodium Chromate (Diichi Radiosotope, Tokyo, Japan) at $37^\circ C$ for 1 hour. After washing $0.1 \times 10^5/10 \mu l$ of target cells were mixed with $1 \times 10^6/200 \mu l$ of effector cells. After incubation at $37^\circ C$ for 3.5 hour supernatant was collected and radioactivity was measured NK cell activity was calculated with following formula. NK cell activity (% lysis) = $(\text{Test cpm} - \text{spontaneous cpm}) / (\text{Total cpm} - \text{spontaneous cpm})$

Statistical Analysis

The analyses were performed with a commercially available statistical package (SPSS Ver 9.0). Because all cytokines, Th subsets and NK activities are not normally distributed we used Mann-Whitney U test for the comparison of the two distributions and Kruskal-Wallis test with Bonferroni test for the three distributions. The results are presented using median values with 25th and 75th percentiles.

Results

Patients (206 men, 215 women; age range 16 - 87 years [median 57]) and 100 controls were enrolled (61 men, 39 women, age range 27 - 72 years [median 53]). PHA-induced median IL-12 was 7.8 pg/ml in the patients and 28.5 pg/ml in the control group ($p < 0.001$, Mann Whitney U test, Fig. 1). The median INF- γ and TNF- β were 6.8 IU/ml and 1420 pg/ml in the whole patients and lower than 29.9 IU/ml and 2450 pg/ml in the control respectively (INF- γ ; $p < 0.001$, TNF- β ; $p < 0.001$, Mann Whitney U test). The median Th1 subset was 22.1% in the patients and 27.5% in the control ($p < 0.001$, Mann Whitney U test). The median Th2 subset was 2.6% in whole patients and had no difference to 2.4% in control ($p = 0.21$ Mann Whitney U test). NK cell activity was 42.5% in cancer and higher than 34.5% in control ($p < 0.001$, Mann Whitney U test).

To elucidate the influence of disease progress to the immune parameters, we selected patients with stomach cancer or colon and categorized them to two groups; early stage and advanced stage. When cytokines were compared between the early stage and control, all of three cytokines (IL-12; 7.8 pg/ml, INF- γ ; 6.1 IU/ml, TNF- β ; 1305 pg/ml) in the early stage were lower than those (IL-12; 28.5 pg/ml, INF- γ ; 29.9 IU/ml, TNF- β ; 2450 pg/ml) in control respectively (IL-12; $p < 0.001$, INF- γ ; $p < 0.001$, TNF- β ; $p < 0.001$, Kruskal Wallis and Bonferroni test). On the other hand, there was no significant difference between cytokines (IL-12; 7.8 pg/ml, INF- γ ; 6.1 IU/ml, TNF- β ; 1340 pg/ml) in the advanced stage respectively (IL-12; $p = 1.000$, INF- γ ; $p = 1.000$, Kruskal Wallis and Bonferroni test)(Fig. 2). The median of Th1 subset in the early stage was 19.0% and lower than 27.5% in the control ($p < 0.001$, Kruskal Wallis and Bonferroni test) (Fig. 2). The median Th1 subset (19.0%) in the early stage was not different from that (23.5%) in the advanced stage ($p = 0.34$, Kruskal Wallis and Bonferroni test) (Fig. 2). Th2 subset was not significantly different between three groups (data not shown) (Fig. 2). NK cell activity was not significantly different between three groups either (data not shown) (Fig. 2).

Discussion

Our results demonstrate that: (a) Mitogen-induced Th1-related cytokines (IL-12, INF- γ ; TNF- β) were lower in patients with cancer than those in controls respectively; (b) Th1 subset induced in cancer was lower than that in control, whereas there was no difference of Th2 subset between cancer and control; (c) NK cells activity was up-regulated in cancer compared to that in control; (d) Th1-related cytokines in early stage of cancer were down-regulated and comparable to those in advanced stage; (e) Th1 Induction in early stage was down-regulated to the comparable level in advanced stage; (f) No difference was found in NK cell activity when patients were grouped to the two stages.

IL-12 is originally found as a cytokine that stimulates natural killer cells.³ Studies revealed that IL-12 induces IFN- γ production by NK cells⁴ and T cells⁵, stimulates the proliferation of T cells⁵ and NK cells³ and augments NK cell-mediated cytotoxicity^{6, 7} and cytolytic T cell responses^{8, 9, 10}. Since the finding of Th1 and Th2 subsets¹¹ the mechanism of positive interaction between IL-12 and IFN- γ for Th1 induction has been defined.^{4, 12-14} Activated antigen presenting cells (APCs) produce IL-12, which stimulates Th1 cells to proliferate and differentiate to effector cells including cytotoxic T cells and delayed type hypersensitivity (DTH)-committed T cells. In the presence of IL-12 Th1 cells produce IFN- γ , hypersensitivity (DTH)-committed T cells. In the presence of IL-12 Th1 cells produce IFN- γ , with which the Th1 cell itself proliferates and, in turn, IFN- γ augments IL-12 production by APCs. On the other hand Th2 cells, which are mainly involved in allergic reaction and Ig production, are dependent on APC-derived IL-1, instead of IL-12, and produce IL-4 with which Th2 cells proliferate. Th1 cells are negatively regulated with IL-10^{15, 16} and IL-4 produced by Th2 cells with the mechanism of blocking macrophage-derived IL-12 production.¹⁵ Thus Th1 and Th2 negatively regulates each other and are mutually inhibitory.

Which subset of Th cells to be activated is dependent on multiple factors. For example, in BALB/C mice, infected with *Leishmania*, Th2 cells are activated and lead to Ig production, especially IgE and the mouse cannot deplete the organism. In contrast, in C57/BL6 mice Th1 cells are activated with *Leishmania* and exert cell-mediated immunity¹⁶ leading to the depletion of the organism. This demonstrates that same antigens are to activate differential Th subsets if provided by particular generic background.¹ Other studies show that a peptide can activate either Th1 or Th2 cells in the same mice depending on the provided accessory signals,^{18, 19} which means antigen specific immunological response can be modulated to either Th1 dominant or Th2 dominant.^{13, 20, 21} In other words, the decision to which subset Th cells differentiate is modulated by accessory signals including cytokines.

In cancer immunity responsive subset is again Th1 cells. For example, in a mouse model, shortly after implantation of tumor cells Th1 cells were activated to show antitumor responses in the presence of Th1-related cytokines. But later as tumor increases in size, Th1 system is down regulated and overwhelmed by Th2 system, which allows tumor cells to grow.²⁵ Therefore, IFN- γ and IL-12²²⁻²⁴ also seem to participate in T cell-mediated antitumor immunity. One question here is whether the anti-tumor activity can be initiated or mediated by antigen specific manner. Ogata et al. showed that C3H/He mice initially bore a MCH-1-A1 tumor and received surgical resection of the tumor rejected a second challenge of same tumor cells³. This illustrates that once tumor cells are successfully removed even with the way other than immune mechanism the antitumor immunity can be established. In addition they showed C57BL/6 x C3H/He mice successfully rejected OV-HM tumor with i.p. injection of IL-12, otherwise the tumor will outgrow. Of importance is provided by findings that the mouse initially bearing the OV-HM tumor and treated with IL-12 only rejects a second challenge of the same OV-HM tumor but not unrelated MCH-1-A1 tumor cells. In similar experiments the anti-tumor activity of IL-12 was completely abrogated with administration of anti-IFN- γ mAb before IL-12 treatment. These results demonstrate that IL-12 and IFN- γ are actually enhancing cancer immunity which is mediated, at least in part, by "antigen specific mechanisms. On the other hand, Siders et al demonstrated that a recombinant adenovirus encoding IL-12 can inhibit hepatic metastasis of murine tumors even after the depletion of T cells in normal mice or even

after the depletion of NK cells in SCID mice², which suggests that IL-12 also evokes its antitumor activity initiated with antigen non-specific manner.

In this study we showed that the induction of IL-12 and IFN- γ as well as TNF- β in whole patients with cancer were lower than those in controls, which appeared compatible with above findings in mice. When patients with stomach cancer or colon cancer were grouped to the early stage and advanced stage, we expected that significantly different amounts of cytokines could be observed between the two stages. But surprisingly, these cytokines are already down regulated at the early stage and we found no difference between early stage and advanced stage. This provides us two possible cases; (a) even at clinically early stage of the cancer immune system is already impaired due to the presence of cancer and it is actually advanced stage immunologically; (b) immunological impairment precedes the emergence of cancer. If the latter is the case, it should be taken into account that cytokine abnormality itself may be involved in the etiology of cancer by down-regulating the anti-cancer defense mechanism. In this study we employed in vitro mitogenic stimulation to evaluate cytokine production and Th subset induction using mitogenic stimulation. Obviously in this system we are determining the function of not only tumor specific T cell clone(s) but also other T cells. Therefore, the fact that we could detect abnormal Th1 function in this system suggests that detected abnormalities are not limited to tumor specific Th1 subset and its precursor but also other T cell repertoires that would be committed to other specificity. This idea may favor the latter interpretation mentioned above.

In the present study NK cell function was not impaired in cancer at any stage and did not appear to be related to cancer susceptibility though it was shown, in a study, that NK cells were required for IL-12 induced antitumor response in mice.²⁷

Taken together, our data strongly suggest that, even in early stages of cancer, the Th1 system is already impaired and this is quite different from the condition of the animal model, where the Th1 system is activated shortly after the implantation of the tumor. Also, we should pay more attention to the possible role of immunological conditions for the emergence of cancer. It should be interesting to measure these parameters before and after the surgical resection of early cancer. Our results also provide chances to detect cancer at an early stage or even earlier than clinical onset by means of immunological parameters.

Finally, it is well accepted that multiple factors including gene level, environmental, nutritional, hormonal, etc. are involved in oncogenesis. But these factors may be too prevalent to stay the incident of cancer to the level actually observed. We think that preventive mechanism by immune system is a key factor to determine whether cancer cells can survive or will be deleted.

Table 1:

	Cancer Patients (n=421)	Controls (n=100)

<u>Demography</u>		
Sex (male/female)	206/215	61/39
Median age (years)	57	53
Age range (years)	16-87	27-72
<u>Organ (male/female)</u>		
Thyroid gland	1/6	
Lung	51/30	
Esophagus	16/2	
Stomach	38/43	
Colon	29/27	
Rectum	23/7	
Liver	12/10	
Gall bladder	3/1	
Bile duct	3/2	
Pancreas	10/8	
Kidney	3/1	
Ureter	0/1	
Bladder	6/0	
Prostate	10/0	
Uterus	0/20	
Ovary	0/21	
Breast	0/37	

Table 2:

Demography	Early Cancer (n=24)	Advanced Cancer (n=113)
Stomach		
Sex (male/female)	5/10	33/33
Median age (years)	55	53
Age range (years)	33-74	19-82
Colon (caccum0sigmold)		
Sex (male/female)	5/4	24/23
Median age (years)	56	63
Age range (years)	37-72	32-84
Total		
Sex (male/female)	67/70	
Median age (years)	57	
Age range (years)	19-84	

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Enhancement of NK Cell Activity in Cancer Patients by Active Hemicellulose Compound (AHCC)

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In the present study, we examined the effect of Active Hemicellulose Compound (AHCC) on natural killer (NK) cell activity of three cancer patients. AHCC is hemicellulose which originated from rice and is biologically modified using carbohydrasae separated from *Lentinus Edodes* to increase its immunomodulatory function. The patients had different types of advanced cancer: rhabdomyosarcoma multiple myeloma and breast cancer. Patients were given AHCC at 3 and 6 g/d for 2 weeks, then NK cell cytotoxicity was measured by 4-hr ⁵¹Cr-release assay using K562 tumor cells as targets, and NK cell populations were determined by flow cytometry using CD56 and CD3 monoclonal antibodies. As for the patient with rhabdomyosarcoma, treatment with AHCC resulted in: 1-significant increase in NK function (91-135%) at effector: target ratios = 25 to 100:1, 2-fold increase in NK population (15.7% vs. 7.4% before treatment), and 3-three fold Increase in PEL counts (3 X 10⁶ cells/ml blood vs. 1 X 10⁶ cells/ml pretreatment). The increase in the above mentioned parameters was observed at the end of the treatment period, and continued at a high level at 1 week after cessation of treatment, than it declined at two weeks post treatment. Treatment with AHCC demonstrated a similar pattern of enhancement in NK anticancer activity for multiple myeloma (87%), and for breast cancer (93%). In addition, 2-3 fold elevation of NK populations was also observed in both patients.

We conclude that AHCC is a promising immunomodulator and its augmentatory effect on NK cytotoxicity may explain one mechanism by which AHCC exerts anticancer activity.

AHCC, commercially known as Lentin Gold, was offered by Daiwa Pharmaceutical Co., Ltd, Head Office 1-16-19 Sangenjaya Setagaya-Ku, Tokyo 151 Japan. Research was supported by NIH/MBRS (RR-08140).

TABLE I. Effects of Different Treatments on Body Weights, Liver Weights

Samples	Change of body weight (g/5 days)	% of control liver weight/body weight (%)
Control	-1.6 ± 0.9 [†]	100.0 ± 6.5
CCl ₄	-2.4 ± 1.1	173.5 ± 37.1*
AHCC	-0.6 ± 0.5	107.1 ± 3.3
CCl ₄ + AHCC	-1.25 ± 0.9	135.0 ± 9.0 [~]

[†] Values represent means ± SD of 4 or 5 mice.

* Statistical significances of differences vs. control are indicated with $p < 0.05$.

[~] Statistical significance of differences vs. CCl₄ treatment is indicated with $p < 0.05$.

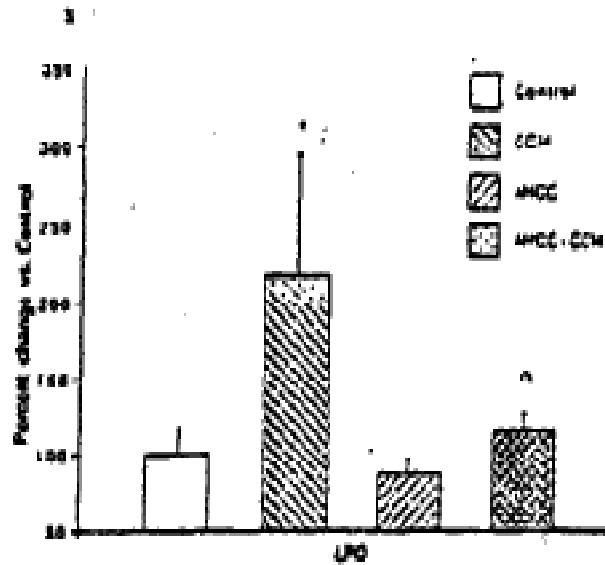


Figure 1: Changes of levels of LPO from CCl₄ or AHCC. Treated Hepatic Microsomes in Mice Values represent mean ± SD of 4-5 mice per group and are shown as percent changes compared with control. The value of the control (mean ± SD) is 131.94 ± 21.53 nmol/mg.protein.

*Significantly different from untreated group at $p < 0.05$. [~]Significantly different from CCl₄ treated group at $p < 0.05$.

TABLE II. Effects of Different Treatments on Serum Parameters

	Glutamate pyruvate transaminase (IU/l)	Triglyceride (mg/dl)	Albumin (g/dl)	Albumin/Creatinin
Control	51.4 ± 11.5 [†]	84.5 ± 39.6	4.6 ± 0.2	2.0 ± 0.3
CCl ₄	369.5 ± 152.8*	82.7 ± 24.5	5.1 ± 0.5	1.9 ± 0.4
AHCC	66.0 ± 35.9	93.3 ± 36.6	4.6 ± 0.1	1.9 ± 0.2
CCl ₄ + AHCC	164.7 ± 10.8 [~]	65.5 ± 24.1	4.8 ± 0.5	1.6 ± 0.2

[†] Values represent means ± SD of 4 or 5 mice.

* Statistical significances of differences vs. control are indicated with $p < 0.05$

[~] Statistical significance of differences vs. CCl₄ treatment is indicated with $p < 0.05$

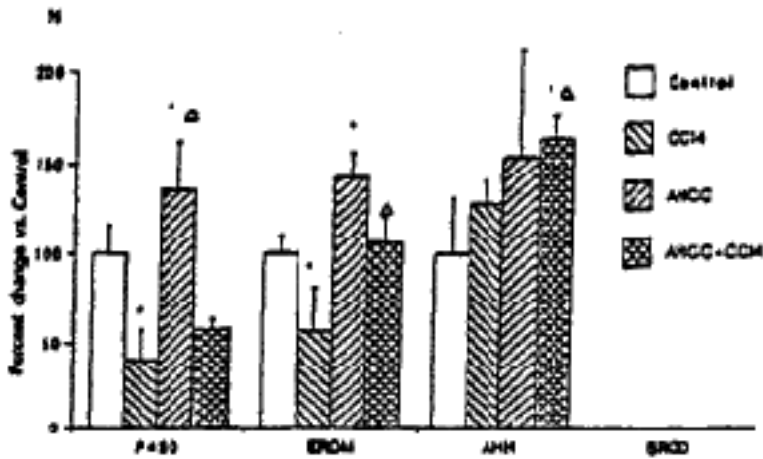


Figure 2: Changes of Contents of Cytochrome. P450 Activities of ERDM, AHH and BROD from CCI, or AHCC. Treated Hepatic Microsomes in Mice Values represent mean \pm SD of 4-5 mice per group and are shown as percent changes compared with control. The values of the control (mean \pm SD) are ERDM: 1.82 ± 0.17 . AHH: 6.4 ± 1.93 , BROD: $0 \text{ nmol/min/mg.protein}$.

*Significantly different from untreated group at $p < 0.05$. ^ΔSignificantly different from CCI treated group at $p < 0.05$.

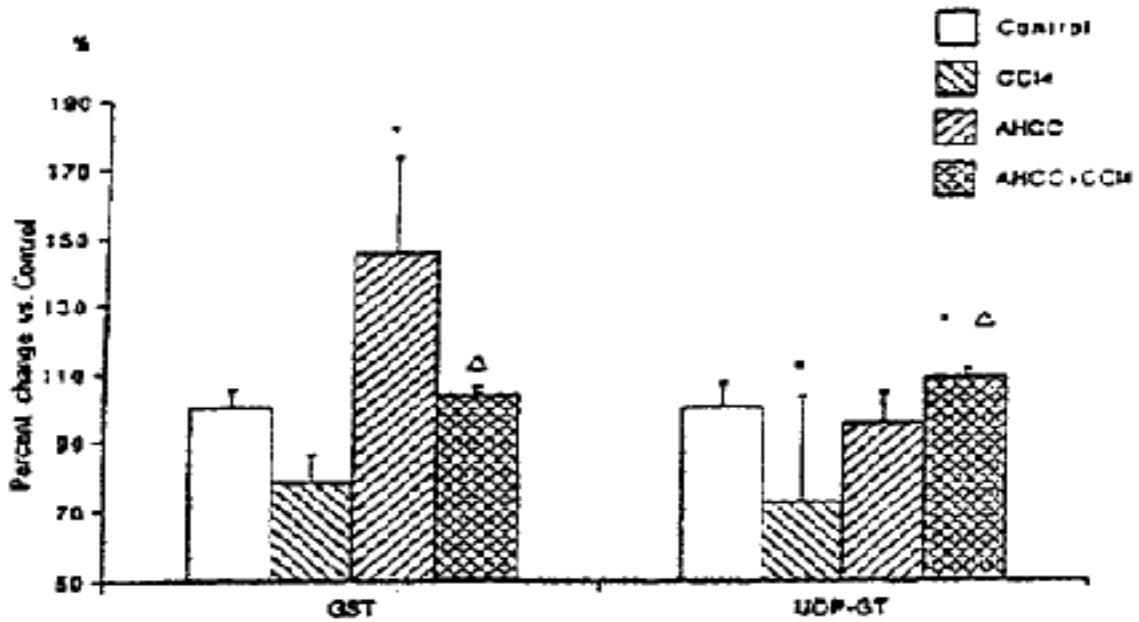


Figure 3: Changes of Activities of GST and UDP-GT from CCI or AHCC. Treated Hepatic Microsomes in Mice Values represent mean \pm SD of 4-5 mice per group and are shown as percent changes compared with control. The values of the control (mean \pm SD) are GST: 102.13 ± 5.18 nmol/min/mg UDP-GT: 18.14 ± 1.25 nmol/min/mg.protein.

*Significantly different from untreated group at $p < 0.05$. ^ΔSignificantly different from CCI treated group at $p < 0.05$.

Active Hexose Correlated Compound (AHCC) Improves Immunological Parameters And Performance Status of Patients with Solid Tumors

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Summary

Active Hexose Correlated Compound (AHCC), a member of Phyto-polysaccharide extract, is known to show Biological Response Modifiers (BRM)- like activity. Because Interleukin 12 (IL-12), interferon- γ (IFN- γ) negatively modulate tumor growth we evaluate the possible effect of AHCC on the production of IL-12 and IFN- γ as well as NK cell activity which also plays a critical role in cancer immunity. 38 patients with solid tumors were given AHCC orally for 6 months and blood was drawn every 2 months to verify the affects of AHCC on their immune function. Peripheral, blood lymphocytes (2×10^5 , /200 \bullet 1) re-suspended in RPMI1640 with 10% FCS were stimulated with 20 \bullet g/ml of Phytohemagglutinin (PHA) in microtiter plates for 24 hours at 37 \bullet C. Supernatant was collected for cytokine assay. IL-12 was measured by the enzyme linked immuno solvent assay (ELISA) kit (R&D Systems, Minneapolis, MN) and IFN- γ was measured by the ELISA kit (Biosource, Nivelles, Belgium). For the assay of NK cell activity ^{51}Cr -sodium chromate-labeled target cells (K-562; 1×10^4 /10 \bullet 1) were mixed with effector cells (1×10^6 /200 \bullet 1) and incubated for 3.5 hours at 37 \bullet C. Supernatant fluid was collected and radioactivity was measured. Performance Status (PS) as an indicator of QOL was also evaluated before and after the intake of AHCC, The basal levels of two cytokines and NIK activity in patients with tumors were lower than those in normal control. All of three immunological parameters of patents increased to the normal levels after the intake of the compound. These results demonstrate that AHCC improves both immunological abnormalities and clinical conditions.

Keywords: AHCC, Cancer, Interleukin- 12, Interferon-gamma, Natural Killer cell activity, *1:Comfort Medical Foundation, Immuni-x Xo., Ltd., *2: Amino Up Chemical Co., Ltd., *3: Comfort Hospital, *4: Shonan Kamakura General Hospital

92563 Reduction of Side Effects of Anticancer Drugs by Active Hexose Correlated Compound (AHCC)

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Summary

Active Hexose Correlated Compound (AHCC), mycelia extracts of cultured basidiomycetes, was examined in animal models to relieve side effects, such as anemia, alopecia and liver injury induced by anticancer drugs. 1. Male ddY mice were treated with fluorouracil (5-FU, 50 mg/kg) or cyclophosphamide (CY, 100 mg/kg) or both daily for 14 days by i.p. and AHCC was given in a 5% diet (or the same period. Treatment with 5-FU or/and CY resulted in body weight loss, decrease in the numbers of peripheral cells in blood and polychromatic erythro-cytes in bone marrows and were significantly restored by coadministration with AHCC. 2. Male and female SD rats received cytosine arabinoside (Ara-C, Lp., 50 mg/kg. daily) for 7 days. AHCC was given for the same period by either p.o. (500 mg/kg). Lp. (500 mg/kg) or local swabbing 0% AHCC-solution). The rats treated with Ara-C showed severe alopecia (50-100% hair loss). However, coadministration of Ara-C with AHCC protected from alopecia, especially when AHCC was given p.o. in which only slight alopecia (0-50% hair loss) was observed. 3. Male ddY mice were treated with mercaptopurine (6-MP, 30 mg/kg) and methotrexate (MTX. 2.5 mg/kg) by p.o. for 4 weeks. AHCC was given at a dose of 1 g/kg simultaneously. Treatment with 6-MP and MTX resulted in the decreases of body weight gain, serum albumin and triglyceride levels, liver drug-metabolism enzyme activities and the increases of liver weights. sGPT and sGOT levels obviously. The liver injury was significantly improved by the coadministration with AHCC. The results show that AHCC relieved the side effects induced by anticancer drugs in animals.

Preventive Effect of Active Hexose Correlated Compound (AHCC) on Hexose Correlated Compound (AHCC)

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Poster Presentation

Summary

Many attempts have been made to stimulate the immune system for cancer treatment. Although several biological response modifiers have been developed such as BGG, Picibanil, PSK, interferon and interleukin-2, the clinical efficacy of the substances has not been clearly elucidated. Active Hexose Correlated Compound (AHCC) is a newly developed biological response modifier. It is an extract of Basidiomycetes which is obtained by hybridization of several types of mushrooms. This study was initiated to evaluate the preventive effect of AHCC on the recurrence of hepatocellular carcinoma patients after surgical treatment.

In this study, 121 patients with histologically proven hepatocellular carcinoma were included. All of the patients underwent macroscopically curative resection of a liver tumor. In 38 patients, AHCC (3-6 g/day) was administered orally after surgery (group A) and 18 patients began to take AHCC after recurrences were verified (group B). The other 65 patients served as controls (group C). The longest follow up periods were 39, 65, and 56 months for groups A, B, and C respectively.

There were no significant differences between the three groups in the distribution of age, gender, clinical stage, hepatitis type B and/or C viral infection, amount of bleeding during surgery and resections volumes of the liver. The survival rate in group A was significantly higher than that in groups C. The disease-free survival rate in group A was also significantly higher than that in groups B and C. One year after surgery, the serum levels of tumor markers (α -fetoprotein and PIVKA II) in group A were significantly lower than those in groups B and C.

This retrospective study suggests that AHCC intake has a preventive effect in postoperative hepatocellular carcinoma patients. Further detailed studies are needed to elucidate the mechanism of the effect of AHCC.

Active Hemicellulose Compound (AHCC) Enhances NK Cell Activity of Aged Mice in Vivo

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Active Hemicellulose Compound (AHCC) is hemicellulose which originated from rice and is biologically modified using carbohydrase separated from *Lentinus Edodes* to increase its immunomodulatory function. In the present investigation, we evaluated the ability of AHCC to stimulate in vivo NK cytotoxic reactivity. Old C57BL/6 mice were injected i.p. daily at a concentration of 30 mg/kg/day. At 2, 5 and 14 days at post treatment, peritoneal erudite and spleen were examined for tissue cellularity and NK activity using 4-hr. Cr-release assay against YAC-1 tumor cells. The results demonstrate that; 1) AHCC generated peritoneal cytotoxic cells having the characteristics of NK cells with high levels of granularity. The induction was observed as early as 2 days, (750-900% of control) and maintained at high levels with continuous injections, 2) Peritoneal macrophage did not exhibit antitumor activity nor act as accessory cells for NK cells, 3) No significant induction of splenic NK cell activity, and 4) AHCC treated mice induced a significant increase in peritoneal (300-500%) and splenic cellularity (150-190%), suggesting that AHCC acts a mitogen factor in vivo. AHCC could be considered as a potent biological response modifier and its anti-cancer activity may be through post NK immunomodulation.

AHCC is commercially known as Lentin Gold, and offered by Daiwa Pharmaceutical Co. LTD, Head of Office 1-16-19 Sengenjaya Setagaya-ku, Tokyo 151 Japan.

NK-Immunomodulation by Active Hemicellulose Compound

(AHCC) in 17 Cancer Patients

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The present study was designed to examine the immunomodulatory function of active hemicellulose compound (AHCC). AHCC is an extract of *Mycellia basidiomycota* which was originated by hybridization of several types of mushrooms. Seventeen cancer patients with different advanced malignancies participated in the study: ovarian carcinoma (3), multiple myeloma (2), stomach (2), breast (5), lung (2), rhabdomyosarcoma (1), and prostate (2). Patients received AHCC 3 g/day orally for 2-6 months. NK cell activity was examined by 4-hour Cr release assay against sensitive K562 and resistant Raji tumor cells. Results showed significant enhancement of NK activity against K562 as early as 2 weeks (2-to 3-fold of base-line). Activity was further increased at subsequent time periods up to 6 months posttreatment with AHCC. NK activation was also detected against Raji cells) but at later stages, i.e. 1-2 months (2- to 10-fold). AHCC appears to activate NK cells by increasing their binding capacity to tumor cell targets (2-fold), and also by increasing NK cell granularity as examined microscopically, in cytospin preparation, and biochemically. On the other hand, flow cytometry analysis showed no significant change in the percentage of NK cells (CD3-, CD16+/CD56+), We conclude that AHCC is a potent immunomodulator and may be useful in immunotherapy of cancer.