Anti-Cancer Drugs 1998, 9, pp. 343–350

Research paper

Combination therapy of active hexose correlated compound plus UFT significantly reduces the metastasis of rat mammary adenocarcinoma

Kazuhiro Matsushita, Yasuhiro Kuramitsu, Youichi Ohiro, Manabu Obara, Masanobu Kobayashi, Yong-Qing Li and Masuo Hosokawa

Laboratory of Pathology, Cancer Institute, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060, Japan. Tel: (+81) 11 706 5070; Fax: (+81) 11 709 6468.

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 slight but significant effects on the growth of primary tumors. Pulmonary metastases were not inhibited by the treatment with AHCC plus UFT, whereas metastases to axillay 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 restored the suppressed mRNA expression of interleukin-1α 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, metastasis.

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

This work was supported by Grants-in Aid from the Minsitry of Education, Science, Sports, and Culture, and the Ministry of Health and Welfare, Japan. We wish to acknowledge Amino Up Chemical Co., Ltd, for kindly providing AHCC.

Correspondence to M Hosokawa

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

The experimental protocol is shown in Figure 1. 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

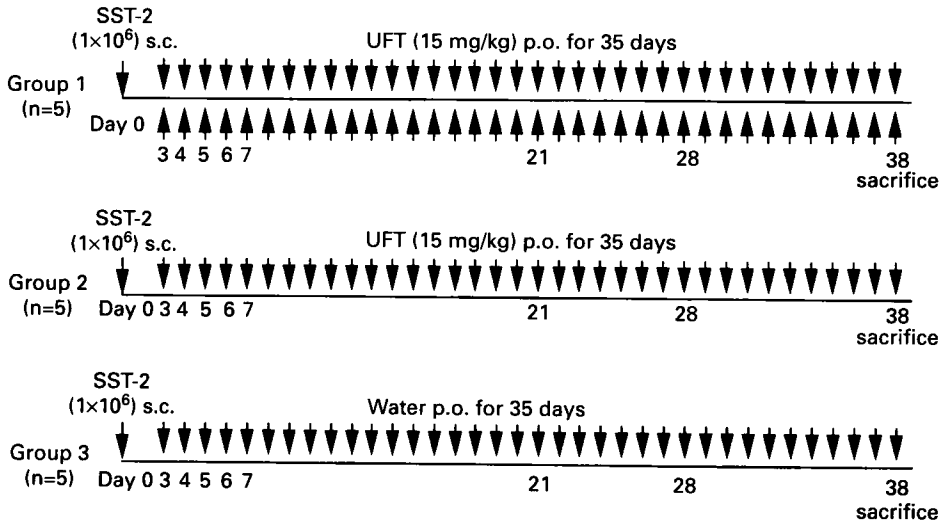

Figure 1. Protocol for observing the effect on growth of the primary tumor and metastasis. Rats were divided into three groups. Group 1: rats were administered UFT (15 mg/kg) plus AHCC (100 mg/kg). Group 2: rats were administered UFT (15 mg/kg). Group 3: rats were administered water as control.

was measured every 3–4 days and the average diameter was estimated by the formula: (length + 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. The experimental protocol is shown in Figure 2. 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 μCi $Na^{51}CrO_4$, 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 (Corning 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 γ-counter (ARC-380). The percentage cytotoxicity was calculated by the following formula: % specific release=[(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 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 Slezak[21] with some modifications. Briefly, 1×10^6 SST-2 cells

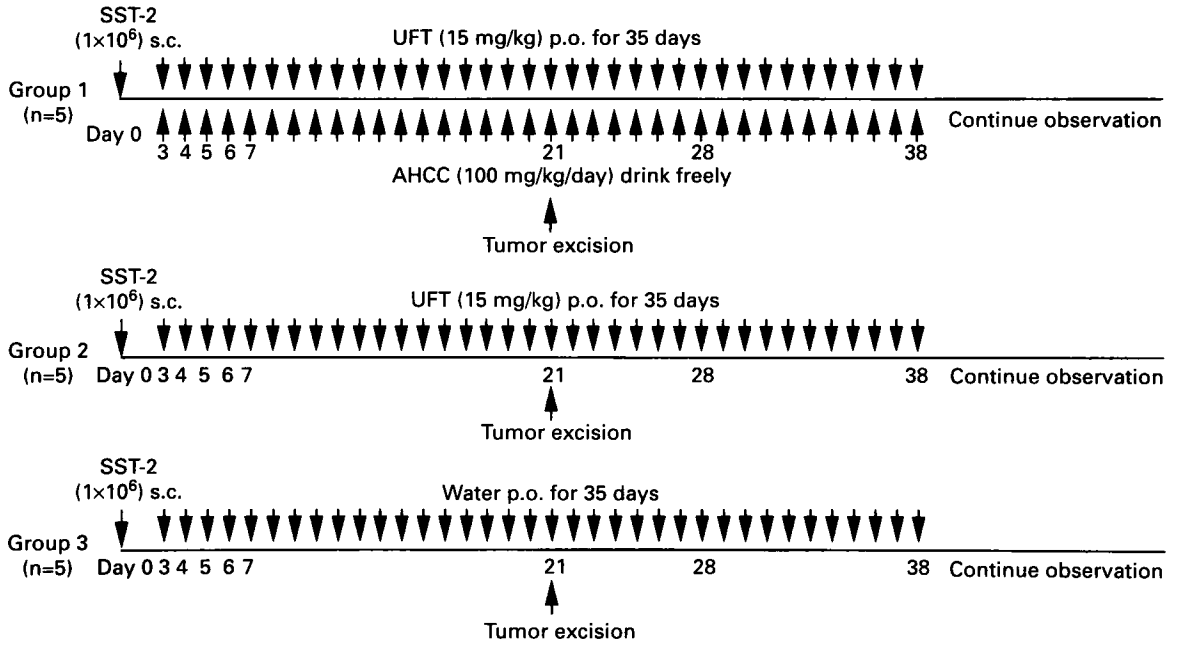

Figure 2. Protocol for observing death by metastasis. Rats were divided into three groups. Rats were administered UFT or UFT plus AHCC described as in Figure 1. Surgical tumor excision was performed on day 21. All the rats were kept under observation until they died.

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 (Corning Glass Works) were cultured for 16 h 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 μl propidium iodide (PI, 50 μg/ml) (Sigma, St Louis, MO) for 30 min on ice. The PKH-26 positive and PI negative cells ($PKHA^+$ 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 (%)=PKH^+ PI^+ cells/(PKH^+ PI^- cells + PKH^+ PI^+ cells) × 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 μl sample aliquots were mixed with an equal volume of Griess reagent (1% sulfanilamine/0.1% naphthylethylene diamine dihydrochloride/2% H_3PO_4; 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 macrophages on day 21 by the guanidine thiocyanate:phenol:chloroform method. Three micrograms of each RNA sample underwent cDNA synthesis in 50 μl of reaction mixture containing 75 mM KCl, 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreiotol, 0.5 mM per each dNTP, 2 μg/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 μl) was performed in 50 μl of a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 2.5 mM $MgCl_2$, 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′-ACCACCATGGAGAAGGCTGC-3′; reverse primer, 5′-CTCAGTGTAGCCCAGGATGC-3′; iNOs forward primer, 5′-AGAGTCCTTCATGAAGCACA-3′; reverse primer, 5′-CCAAATACCGCATACCTGAA-3′; tumor necrosis factor (TNF)-α forward primer, 5′-CAAGGAGGAGAAGTTCCCAA-3′; reverse primer, 5′-CGGACTCCGTGATGTCTAAG-3′; interleukin (IL)-1α forward primer, 5′-GTGGTGGTGTCAGCAACTTC-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-1α, 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 μl of each PCR sample was mixed with 1 μl 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 UFT on the s.c. growth and metastases of SST-2

As shown in Figure 3, the difference of tumor sizes between three groups were 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 (Table 1).

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

As described above, UFT plus AHCC decreases the number of metastases. Because the metastases to lung 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. As shown in Table 2, 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. Survival curves of the three groups are shown in Figure 4.

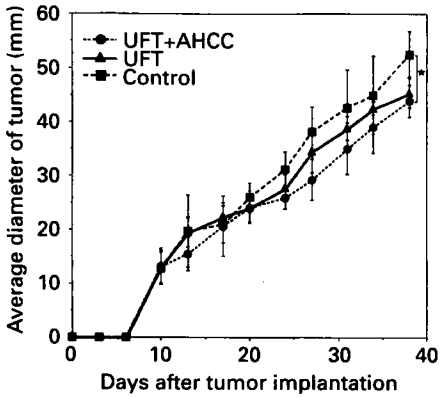

Figure 3. Growth curves of SST-2 tumors in SHR rats. SST-2 tumors were implanted s.c. on day 0. UFT (15 mg/kg) was administered p.o. every day from day 3 to 38. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 38. Each group consisted of five rats. Each point presents mean ± SD of average tumor diameter. *Statistically significant ($p<0.05$).

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 (Table 2 and Figure 4). 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. As shown in Figure 5, 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.

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	1.858±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	2.985±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. *$p<0.01$.

Enhancement of NO production and cytotoxicity of peritoneal macrophages by AHCC

As shown in Table 3, 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. As shown in Figure 6, 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-1α. 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

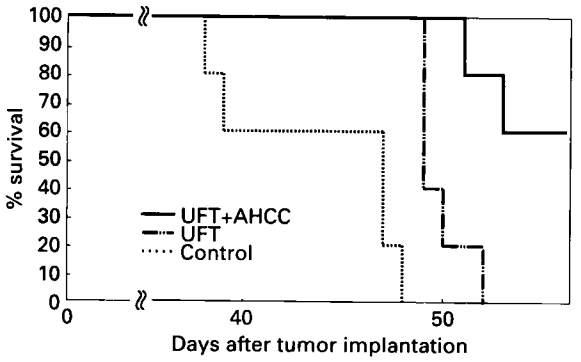

Figure 4. Survival curves of rats after surgical tumor excision on day 21. SST-2 tumors were implanted s.c. on day 0. UFT (15 mg/kg) was administered p.o. every day from day 3 to 38. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 38. Surgical tumor excision was performed on day 21. Each group consists of five rats. *Statistically significant ($p<0.05$) between the UFT plus AHCC treated group and the other two groups by the generalized Wilcoxon test.

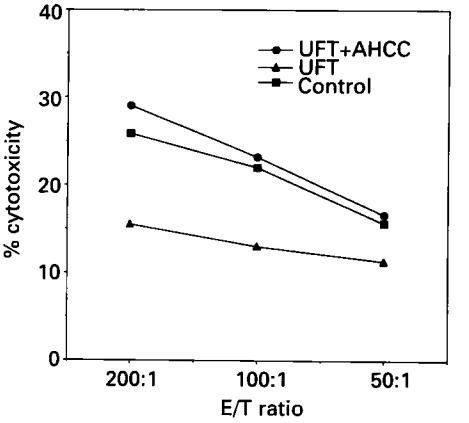

Figure 5. NK cell activity of spleen cells in SST-2-bearing rats. SST-2 tumors were implanted s.c. on day 0. UFT (15 mg/kg) was administered p.o. every day from day 3 to 21. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 21. On day 21, rats were sacrificed and spleens were aseptically removed from three rats in each group and mixed for cytotoxicity assay.

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	100:1
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).

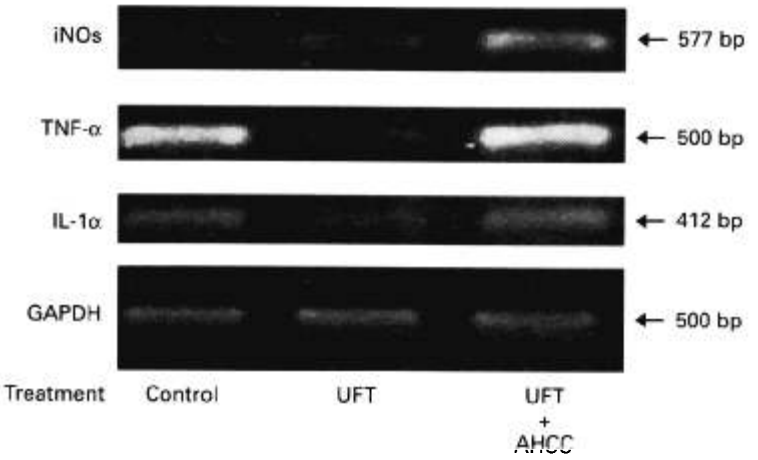

Figure 6. Expression of various cytokines and iNOs mRNA in peritoneal macrophages. SST-2 tumors were implanted s.c. on day 0. UFT (15 mg/kg) was administered p.o. every day from day 3 to 21. AHCC (100 mg/kg/day in 24 ml) was also administered from day 3 to 21. On day 21, rats were i.p. administered 1 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 guanidine 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.

between the UFT alone treated and control groups. Although lung metastases were not inhibited by the treatment with AHCC plus UFT, metastases to axillay 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 rat 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

We thank Ms M Yanome for preparing the manuscript.

References

1. Poste G, Fidler IJ. The pathogenesis of cancer metastasis. *Nature* 1980; **28**: 139-46.

2. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991; **64**: 327-36.
3. Kurebayashi J, Sonoo H. Antiproliferative and antimetastatic effects of UFT on MKL-4 human breast cancer cells transplanted into nude mice. *Gan To Kagaku Ryoho* 1995; **22**: 665-70.
4. Otani S, Maeta M, Oka A, *et al.* Long-term survival of 5 years following initial surgery for gastric cancer and simultaneous disseminated peritoneal metastasis: report of a case. *Surg Today* 1995; **25**: 959-61.
5. Pazdur R, Lassere Y, Rhodes V, *et al.* Phase II trial of uracil and tegafur plus oral leucovorin: an effective oral regimen in the treatment of metastatic colorectal carcinoma. *J Clin Oncol* 1994; **12**: 2296-300.
6. Li XB, Hamada J, Takeichi N, Hosokawa M, Kobayashi H. Timing of UFT administration combined with surgical operation in pulmonary metastasis of rat mammary carcinoma. *Gan To Kagaku Ryoho* 1989; **16**: 2381-5.
7. Komatsumoto M, Li S, Hamada J, *et al.* Inhibitory effect of UFT on postoperative lung metastasis of mammary adenocarcinoma in SHR rats. *Gan To Kagaku Ryoho* 1988; **15**: 1739-45.
8. Mihich E. Immunosuppression in cancer therapeutics. *Transplant Proc* 1975; **7**: 275-8.
9. Hersh E. Immunosuppressive agents. In Sartoli AC, Johns DG, eds. *Antineoplastic and immunosuppressive agent.* New York: Springer 1974: **1**: 577.
10. Bayon LG, Izquierdo MA, Sirovich I, van Rooijen N, Beelen RH, Meijer S. Role of Kupffer cells in arresting circulating tumor cells and controlling metastatic growth in the liver. *Hepatology* 1996; **23**: 1224-31.
11. Hrushesky WJ, Gruber SA, Sothern RB, *et al.* Natural killer cell activity: age, estrous- and circadian-stage dependence and inverse correlation with metastatic potential. *J Natl Cancer Inst* 1988; **80**: 1232-7.
12. Gruber SA, Hoffman RA, Sothern RB, *et al.* Splenocyte natural killer cell activity and metastatic potential are inversely dependent on estrous stage. *Surgery* 1988; **104**: 398-403.
13. Hasegawa J, Hosokawa M, Okada F, Kobayashi H. Inhibition of mitomycin C-induced sister-chromatid exchange in mouse bone marrow cells by the immunopotentiators Krestin and Lentinan. *Mutat Res* 1989; **226**: 9-12.
14. Mizushima Y, Yuhki N, Hosokawa M, Kobayashi H. Diminution of cyclophosphamide-induced suppression of antitumor immunity by an immunomodulator PS-K and combined therapeutic effects of PS-K and cyclophosphamide on transplanted tumor in rats. *Cancer Res* 1982; **42**: 5176-80.
15. Akiyama J, Kawamura T, Gotohda E, *et al.* Immunochemotherapy of transplanted KMT-17 tumor in WKA rats by combination of cyclophosphamide and immunostimulatory protein-bound polysaccharide isolated from basidiomycetes. *Cancer Res* 1977; **37**: 3042-5.
16. Hosokawa M, Mizukoshi T, Morikawa K, Xu ZY, Kobayashi H. The therapeutic effects of an immunopotentiator, PS-K, on 3-methylcholanthrene-induced autochthonous tumors in C57BL/6 mice in combination with the surgical removal of primary sites. *Cancer Immunol Immunother* 1986; **22**: 181-5.
17. Mokyr MB, Hengst JC, Dray S. Role of antitumor immunity in cyclophosphamide-induced rejection of subcutaneous nonpalpable MOPC-315 tumors. *Cancer Res* 1982; **42**: 974-9.
18. Matsuoka T, Takeichi N, Kobayashi H. Age-related changes of natural antitumor resistance in spontaneously hypertensive rats with T cell depression. *Cancer Res* 1987; **47**: 3410-3.
19. Takeichi N, Ba D, Koga Y, Kobayashi H. Immunologic suppression of carcinogensis in spontaneously hypertensive rats (SHR) with T-cell depression. *J Immunol* 1983; **130**: 501-5.
20. Koga Y, Hamada J, Takeichi N, Nakane A, Minagawa T, Kobayashi H. Activation of natural resistance against lung metastasis of an adenocarcinoma in T-cell depressed spontaneously hypertensive rats by infection with Listeria monocytogenes. *Cancer Immunol Immunother* 1985; **20**: 103-8.
21. Slezak SE, Horan PK. Cell-mediated cytotoxicity. A highly sensitive and informative flow cytometric assay. *J Immunol Methods* 1989; **117**: 205-14.
22. Yunoki S, Tanaka N, Hizuta A, Orita K. Enhancement of antitumor cytotoxicity of hepatic lymphocytes by oral administration of PSK. *Int J Immunopharmacol* 1994; **16**: 123-30.
23. Brunson KW, Ohashi M, Miller CA, Kitson RP, Basse PH, Goldfarb RH. Interleukin-2 (IL-2) activated natural killer (A-NK) cells: binding to microvascular endothelial cells and BRM enhancement of cytolytic activity. *In Vivo* 1994; **8**: 71-5.
24. Uchida A, Kariya Y. Autologous tumor killing (ATK) and tumor metastasis in human cancer patients. *Hum Cell* 1993; **6**: 15-20.
25. Wiltrout RH, Herberman RB, Zhang SR, *et al.* Role of organ-associated NK cells in decreased formation of experimental metastases in lung and liver. *J Immunol* 1985; **134**: 4267-75.
26. Hanna N. Regulation of natural killer cell activation: implementation for the control of tumor metastasis. *Nat Immun Cell Growth Regul* 1983; **3**: 22-33.
27. Sakurai T, Ohno N, Suzuki I, Yadomae T. Effect of soluble fungal (1→3)-beta-D-glucan obtained from *Sclerotinia sclerotiorum* on alveolar macrophage activation. *Immunopharmacology* 1995; **30**: 157-66.
28. Talmadge JE, Fidler IJ, Oldham RK. The NCI preclinical screen of biological response modifiers. *Behring Inst Mitt* 1984; **74**: 189-94.
29. Gallo-Hendrikx E, Copps J, Percy D, Croy BA, Wildeman AG. Enhancement of pancreatic tumor metastasis in transgenic immunodeficient mice. *Oncogene* 1994; **9**: 2983-90.

(Received 13 January 1998; accepted 29 January 1998)