SUPPRESSIVE EFFECT OF ACTIVE HEXOSE CORRELATED COMPOUND (AHCC) ON THYMIC APOPTOSIS INDUCED BY DEXAMETHASONE IN THE RAT

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Objective. Mushroom extracts are known to have immunomodulating and antitumor effects in humans as well as in animals. In the present study Active Hexose Correlated Compound (AHCC), an extract obtained from several kinds of basidiomycetes was examined for its suppressive effect on thymocyte apoptosis induced by dexamethasone.

Method. Thymic apoptosis was evaluated by gel electrophoresis and by flow cytometry at 3 h after injection of dexamethasone to rats.

Results. When given to rats at 4 % concentration in drinking water for more than 4 days, AHCC suppressed the internucleosomal DNA fragmentation in the thymus induced by dexamethasone. Flow cytometry also revealed that thymic apoptosis induced by dexamethasone was prevented by pretreatment with AHCC. Dexamethasone increased the caspase 3-like activity within 3 h after its treatment and AHCC pretreatment suppressed the increased enzyme activity only slightly. No apparent increase in serum levels of melatonin and interleukin 1β was observed after AHCC treatment.

Conclusions. These results suggest that AHCC exhibits immuno-modulating effects at least partially by regulating thymic apoptosis.

Key Words: AHCC – Thymic apoptosis – Dexamethasone – DNA fragmentation – Flow cytometry

Polysaccharide peptides found in many different species of mushrooms have been widely used as biological response modifiers (BRM) in Asian countries (YANG et al. 1993; MIZUNO and KAWAI 1995). They have been shown to increase resistance to infections and suppress tumorigenesis. Their antitumor activity is thought to be an indirect enhancement of immune response rather than a direct cytotoxic effect on tumor cells.

Active Hexose Correlated Compound (AHCC™, Amino UP Chemical Co. Ltd., Sapporo, Japan) is an extract from several kinds of mushrooms belonging to *Basidiomycetes* after being cultured in a liquid medium. It has been reported that this extract shows various beneficial effects in humans as well as in experimental animals acting as a BRM or an antioxidant. For example, AHCC inhibits cancer growth and metastasis of rat mammary adenocarcinoma (MATSUSHITA et al. 1998). It is also shown that AHCC suppresses the onset of diabetes induced by streptozotocin in the rat (WAKAME 1999). The main active component of AHCC is postulated to be an oligosaccharide whose molecular weight is approximately 5,000 (MATSUSHITA et al. 1998). The oligomer of glucose in AHCC has N-acetylated α-1,4-linkage structures and some esterized hydroxy groups. AHCC also contains other polysaccharides including β-glucans, amino acids and minerals. Both α-glucans and β-glucans are thought to be responsible for the an-

titumor effects of *Basidiomycetes* (Mizuno and Kawai 1995).

Apoptosis, or programmed cell death, is a physiological process that ensures the elimination of unwanted or damaged cells from multicellular organisms (Wyllie et al. 1980; Ellis et al. 1991). Apoptosis is now recognized as a crucial biological process for development and homeostasis of all multicellular organisms (Thompson 1995). It is an essential physiological process in the differentiation and maintenance of thymocyte population. Thymic apoptosis can also be induced by unphysiological stimuli such as excess glucocorticoids, gamma-irradiation, immunosuppressants (e.g. cyclosporin A) and oxidants (Cohen et. al. 1992; Buttke and Sandstrom 1994). Immature cortical thymocytes are exquisitely sensitive to these treatments and thymic apoptosis could lead to immunosuppression and thymic atrophy. Medullary thymocytes and peripheral lymphocytes are more resistant to glucocorticoids than cortical thymocytes (Schwartzman and Cidlowski 1994). DNA fragmentation in the thymus can be detected as early as 2 h after glucocorticoid treatment in vivo and this effect is receptor dependent (Compton and Cidlowski 1986). Increased thymocyte apoptosis is thought to play a role in the pathogenesis of autoimmune diseases and age-related events such as tumorigenesis (Ogawa et al. 1995).

There are only a few published, well-controlled studies of the effect of AHCC on the immune function. Therefore, we thought it of interest to test AHCC for its immunomodulating effects in animal models. The present study deals with the protective effect of AHCC on thymic apoptosis in the rat induced by dexamethasone (Dex), one of the most potent synthetic glucocorticoids.

Materials and Methods

Chemicals and enzymes. Ribonuclease A and proteinase K were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Wako Chemicals (Saitama, Japan), respectively. Dexamethasone (Decadron) was obtained from Banyu Pharmaceutical Co. Ltd. (Tokyo, Japan). AHCC was routinely prepared by Amino UP Co. Ltd. (Sapporo, Japan). Apo Tag Plus kits were purchased from Oncor Inc. (Gaithersburg, MD, USA). All other chemicals were of purest grade available.

Experimental animals. The studies in rats were approved by Animal Care and Use Committee, Dokkyo University School of Medicine. The animals were treated according to the guidelines for the Care and Use of Laboratory Animals of the Committee. Male Wistar rats of 8-10 weeks old were used throughout the experiments. They were housed in a room maintaining a temperature of 25±1 °C and on a 12-hr light/dark cycle (light on: 7.00-19.00 hr). The animals had free access to rat chow. AHCC at the concentration of 4 % in drinking water was given to the rats for one week, unless otherwise noted.

Thymic apoptosis was induced by Dex injected subcutaneously at a dose of 1 mg per kg body weight.

Isolation of thymocytes. Rats were killed by decapitation, their thymus was removed and placed in ice-cold PBS, pH 7.4. Single-cell suspensions were prepared by cutting the organs with scissors and pressing and then by passage through nylon mesh. The suspensions were centrifuged at 2,600 x g for 5 min at 4 °C. Cells were then resuspended in cold PBS.

Detection of apoptosis using gel electrophoresis. Thymic apoptosis was evaluated as reported previously (Burikhanov and Matsuzaki 2000). In brief, rat thymocytes were washed once with PBS and then lysed in a buffer containing 10 mmol/l Tris-HCl, pH 7.4, 10 mmo/l EDTA, 0.5 % Triton X100. After separating high molecular weight DNA from low molecular weight DNA by centrifugation at 18,000 x g, to both the supernatant and pellets was added digestion buffer containing 100 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 8.0, 25 mmol/l EDTA, 0.5 % sodium dodecyl sulfate (SDS) and proteinase K (10 mg/ml). After incubation at 37 °C for 16-20 h, ribonuclease A was added to each sample and incubated for 1 h at 37 °C. The samples were extracted three times with phenol/chloroform (1:1 vol/vol; plus isoamyl alcohol) followed by one chloroform extraction and precipitate by addition of 100 mmol/l NaCl and 2.5 vol ethanol at -70 °C. The DNA was resuspended in a buffer containing 10 mmol/l Tris, pH 8.0 and 1 mmol/l EDTA. The concentration of DNA was determined by measuring the absorbancy at 260 nm. Two μg DNA per lane was subjected to electrophore-

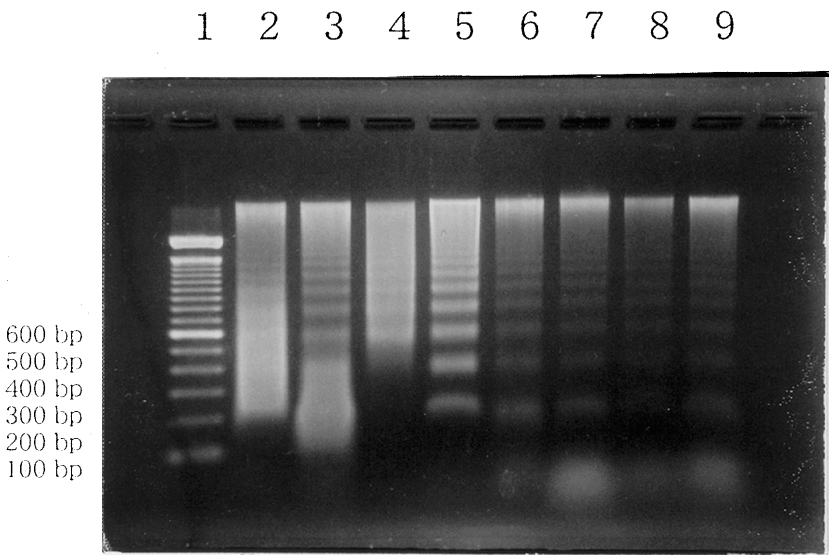

Fig. 1 Effect of AHCC on nucleosomal DNA fragmentation induced by dexamethasone (Dex). AHCC at 4 % in drinking water was given to rats for 1 week. Dex (1mg/kg body weight) was injected subcutaneously at 3 h before sacrifice. Lane 1 – DNA marker; Lane 2 – AHCC+Dex; Lane 3 – Dex alone; Lane 4 – AHCC + Dex; Lane 5 – Dex; Lane 6 – intact control; Lane 7 – AHCC alone; Lane 8 – intact control; Lane 9 – AHCC.

sis on 1.8 % agarose gels. The gels were stained with ethidium bromide (10 μg/ml), visualized by UV transillumination and photographed.

Flow cytometry. Thymic apoptosis was evaluated not only by DNA fragmentation, but also by DNA staining with propidium iodide and terminal dUTP nick end-labelling (Tunel) using Apo Tag kits (Oncor; Gaithersburg, MD). The detection and quantification of apoptosis were done at single-cell level according to manufacturer's protocol. Flow cytometry was carried out by analyzing 10,000 cells/test using a FACSCalibur (Becton Dickinson, Mountain View, CA).

Caspase assay. The activity of caspases in whole cell lysates was assayed using CaspACEtm Assay System, Fluorometric provided by Promega (Madison, WI, USA). The activity of caspase-1 and -3-like enzymes was measured separately by proteolytic cleavage of two different fluorogenic substrates, Ac-YVAD-fluorochrome 7-amino-4-methylcoumarin (AMC) and Ac-DEDV-AMC, respectively. AMC is released from these substrates upon cleavage by caspases. Free AMC produces a yellow-green fluorescence upon exposure to UV light, which is monitored by a fluorometer at 460nm (Thornberry 1994).

Melatonin and interleukin 1β assay. The serum levels of melatonin were measured by radioimmunoassay using melatonin assay kits (Buhlmann Laboratories AG, Switzerland) after C_{18} solid phase extraction. Rat interleukin (IL) 1β was assayed using commercial ELISA kits.

Statistical analysis. Statistical analysis was performed by Dunnett's multiple test to control and non-parametric method by Scheffe's multiple comparison test. All differences were considered significant at the 0.05 level.

Results

The apoptosis is usually accompanied by the internucleosomal degradation of chromosomal DNA (Compton and Cidlowski 1986; Wyllie et al. 1980). The characteristic feature of DNA fragmentation and laddering was observed at 3-12 h after Dex injection. Dex-induced apoptosis produced characteristic DNA ladder patterns on electrophoresis, with fragments equivalent to approximately 180 bp and their multiples (Fig. 1). When AHCC at 4 % in drinking water was pretreated for 7 days and then Dex was

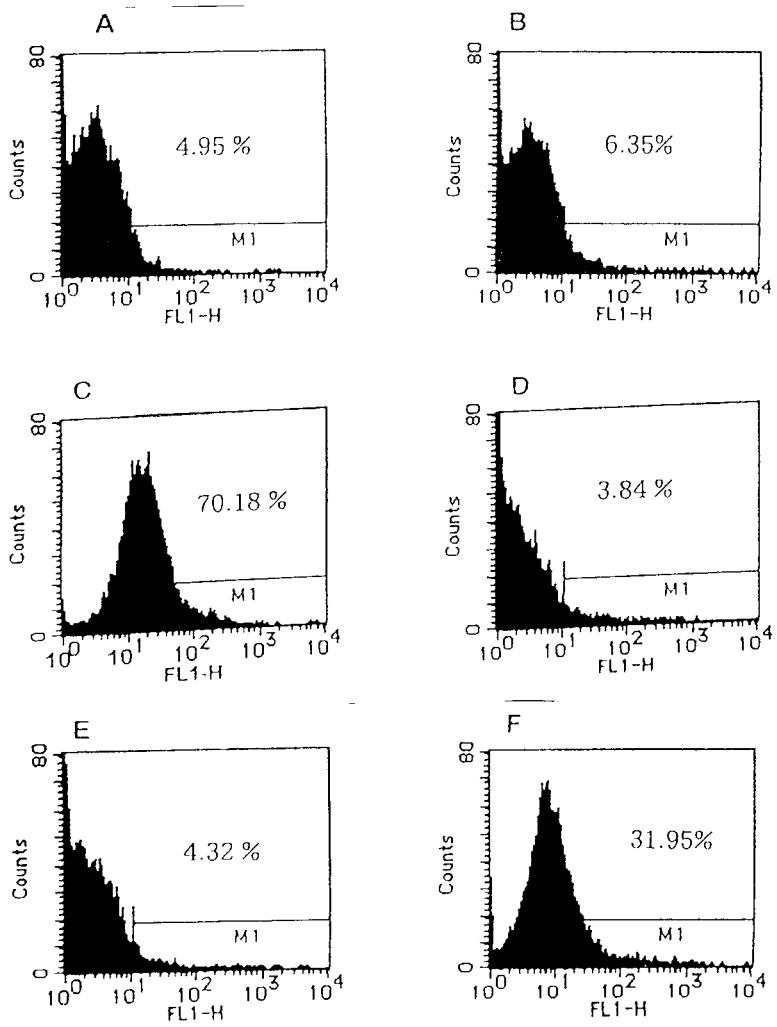

Fig. 2 Effect of various concentrations of AHCC on thymic apoptosis induced by Dex. AHCC at 1, 2 and 4 % in drinking water was given to rats for 7 days. Thymic apoptosis was induced by sc injection of Dex at a dose of 1mg per kg body weight. A - control; B - AHCC; C - Dex; D - 4 % AHCC + Dex; E - 2 % AHCC + Dex; F - 1 % AHCC Dex.

injected to rats at 3 h before sacrifice, no apparent apoptosis was observed in the thymus (Fig. 1). In AHCC treated groups, no DNA fragmentation in the thymus of thymocytes was induced by Dex and the percentage of apoptotic cells was nearly the same as that in intact control.

Thymic apoptosis induced by Dex was also demonstrated by flow cytometry as shown in Fig. 2. AHCC at 2 % as well as 4 % in drinking water was enough to suppress completely the thymic apoptosis induced by Dex (Fig. 2). Pretreatment with 1 % AHCC only partially suppressed Dex-induced apoptosis. The treatment with 4 % AHCC for at least 4 days was needed to suppress the thymocyte apoptosis (data not shown).

Dex administration resulted in a marked increase in the caspase-3-like activity but not caspase 1-like activity within 3 h. The suppressive effect of AHCC pretreatment on the caspase-3-like activity was slight but statistically significant (Fig. 3). The caspase-1-like activity of the thymus was not affected either by Dex or AHCC (data not shown).

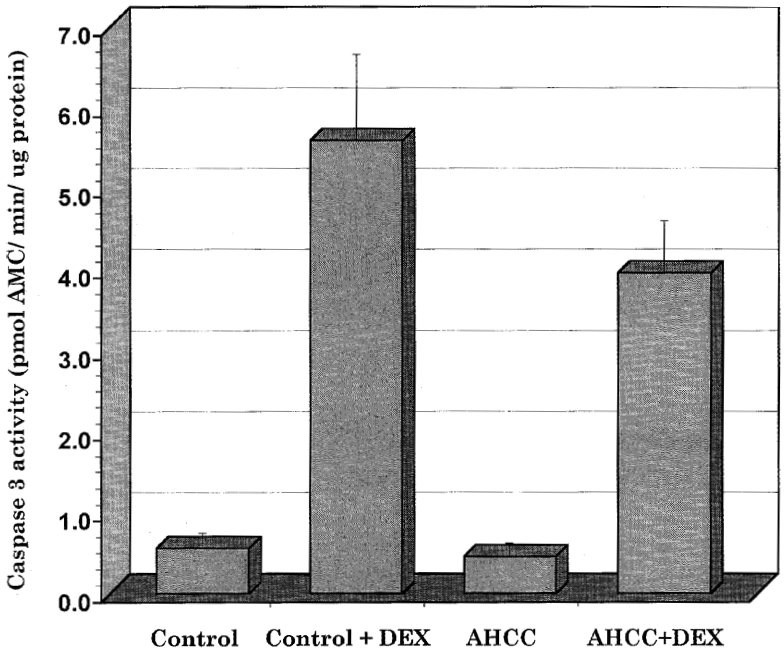

Fig. 3 Effect of AHCC on the activity of thymic caspase activated by dexamethasone. AHCC at 4 % in drinking water was given to rats for 7 days and Dex was injected sc at 3 h before sacrifice.

Oxidative stress-induced thymic apoptosis is suppressed by various antioxidants (Mc Gowan et al. 1996) including melatonin (Sainz et al. 1995). Circulating levels of melatonin were assayed to examine whether or not AHCC affects its secretion. No significant changes, however, were observed in the serum levels of melatonin from control and AHCC-treated groups during the daytime (Tab. 1).

Cytokines have been shown to regulate thymic apoptosis; IL-1 and IL-4 protect thymocytes from the apoptosis induced by glucocorticoids (Mc Conky et al. 1990; Migliorati et al. 1992, 1993). Serum levels of IL-1β were not affected by AHCC treatment (Tab. 1).

Discussion

The results of the present study have shown that AHCC suppresses thymic apoptosis in vivo induced by Dex in the rat. The suppressive effect of AHCC was detected only after 4 days of its treatment, suggesting that some functional changes may be induced by this compound. Since the sensitivity of thymocytes to glucocorticoid-induced apoptosis is regulated by some cytokines, the secretion of the cytokines which suppress thymic apoptosis may be increased following AHCC treatment. In fact, the programmed deaths of thymocytes can be inhibited by IL-1 (Mc Conkey et al. 1990) and other cytokines (Migliorati et al. 1992, 1993). AHCC is reported to increase circulating levels of tumor necrosis factor (TNFα), interferon-gamma (IFN-gamma) and IL-1β in patients with malignant tumors (Yagita et al. 1998). Our results, however, showed there was no significant difference

Table 1
Effect of AHCC treatment on serum levels of melatonin and interleukin 1β. AHCC at 4 % in drinking water was given to rats for 7 days. Animals were killed between 10 a.m. and 3 p.m. Data are shown as mean±S.E. with the number of determinations in parentheses.

Treatment	Melatonin (pg/ml)	Interleukin 1β (pg/ml)
Control (12)	5.38±0.80	19.0±0.58
AHCC (9)	5.30±1.08	17.3±0.96

in serum levels of IL-1β between control and AHCC-treated rats. Further studies are needed to elucidate the involvement of cytokines in the protective effect of AHCC on thymic apoptosis.

It is well known that oxidative stress can serve as a mediator of apoptosis (Buttke and Sandstrom 1994). Free radicals are known to initiate apoptosis signaling pathways in thymocytes (Wolfe et al. 1994). Oxidative stress-induced apoptosis can be prevented by various antioxidants (Mc Gowan et al. 1996). It is possible that AHCC exerts its effect by acting as an antioxidant, because onset of streptozotocin-induced diabetes is prevented by AHCC (Wakame 1999). This experimental diabetes is thought be induced by free radicals formed from streotozotocin (Kawada 1992). Being a potent antioxidant, the pineal neurohormone melatonin protects thymocytes from glucocorticoid-induced apoptosis (Sainz et al. 1995). These findings raise the possibility that AHCC itself acts as an antioxidant to suppress thymic apoptosis or that it stimulates melatonin secretion. AHCC pretreatment for 1 week induced no significant change in the secretion of melatonin during daytime. One cannot exclude the possibility that the secretion of other hormones which protect thymic apoptosis (Delgado et al. 1996) is regulated by AHCC. It is not known yet whether AHCC functions as an antioxidant directly or some free radical scavenging enzymes e.g. superoxide dismutase, are induced in vivo. Our preliminary experiments showed that AHCC had no suppressive effect on thymic apoptosis in vitro when added to culture media.

Apoptosis is mediated by members of the caspase family of proteases, and eventually causes the degradation of chromosomal DNA. Caspases, or interleukin-converting enzyme (ICE/CED-3), family of cysteine aspartic acid-specific proteases have been shown to play important roles in apoptosis (Thornberry et al. 1992; Nicholson et al. 1995; Tewari et al. 1995; Fernandez-Alnemri et al. 1994). All caspases are first synthesized as inactive proenzymes that can be activated upon apoptotic stimulation (Erhardt and Cooper 1996). Activation of caspases occurs during apoptosis and activated caspases participate in a cascade of cleavage events. It is widely accepted that the caspase-dependent cleavage of structural proteins is responsible for the various hallmarks of apoptosis such as nuclear fragmentation, cytoplasmic membrane blebbing, and DNA fragmentation. It was shown that activation of DNase is mediated by the caspase-specific cleavage of its associated inhibitor (Liu et al. 1997; Enari et al. 1998; Sakahira et al. 1998). Like in many other cells caspase-3-like activity in thymocytes is activated upon apoptosis induced by glucocorticoids (Mann et al. 2000). The caspase-3-like activity was activated at 3 h after Dex treatment, while the inhibitory effect of AHCC on the increased activity was only slight. Thus, the slight decrease in caspase-3-like activity alone would not be sufficient to explain the suppressive effect of AHCC on thymocyte apoptosis.

Dysregulation of apoptosis is closely associated with altered immune function and the development of autoimmunity (Ogawa et al. 1995) and tumorgenesis (Wyllie et al. 1980). Physiologically the glucocorticoids regulate the selection and maturation process occurring in the thymus during development of the immune system (Cohen et al. 1992; Mann et al. 2000). However, overdoses of glucocorticoids impair the immune system by inducing excess thymic apoptosis and lympholysis.

In conclusion, AHCC possibly maintains the homeostasis of the immune system at least partially by protecting excess thymic apoptosis induced by glucocorticoids under pathological conditions.

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