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Protective activity of 30 kDa phytoglycoprotein from glucose/glucose oxidase-induced cell death in primary cultured mouse thymocytes

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Abstract

Dioscorea batatas Decne (DBD) has been traditionally used as herbal agent in folk medicine. DBD glycoprotein with a molecular weight of 30 kDa consists of carbohydrate (83.75%) and protein (16.25%), and has a strong anti-oxidative activity. To understand the protection from thymocytes death, we evaluated the activity changes of pro-apoptotic factors [cytochrome *c*, caspase 3, poly(ADP-ribose) polymerase (PARP), AP-1, NF-κB and nitric oxide (NO)] by DBD glycoprotein from glucose/glucose oxidase (G/GO)-induced cell death in primary cultured mouse thymocytes. In the activity of the apoptotic related proteins [cytochrome *c*, caspase 3 and PARP], the results showed that DBD glycoprotein (200 μg/ml) has an inhibitory effect on cytochrome *c* release into cytosol, caspase 3 activation and PARP cleavage in thymocytes. In the transcription factors (AP-1 and NF-κB) activity and NO production, the activities of NF-κB and NO production significantly decreased after DBD glycoprotein (200 μg/ml) treatment for 4 h in G/GO-induced thymocytes, compared with the control. Therefore, we speculate that DBD glycoprotein is one of the natural compounds for the protection of thymocytes that can produce cytokines.

Keywords: *Dioscorea batatas* Decne (DBD) glycoprotein; Reactive oxygen species; Caspase 3; Thymocytes

1. Introduction

The reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are continuously being produced by exogenous factors and agents in aerobic organisms, whereas they are scavenged by endogenous antioxidants, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Yu, 1994). However, the imbalance between production of ROS and endogenous antioxidant defense system generates the excessive accumulation of ROS that can lead to oxidative stress and damage in cells (Halliwell, 2001; Ciriolo, 2005; Chandra et al., 2000; Buttke and Sandstrom, 1995).

Apoptosis is a series of molecular and morphological events that is essential for the development and maintenance of the immune system (Opferman and Korsmeyer, 2003; Hengartner, 2000). It is mainly induced by a variety of mediators including glucocorticoids, inflammatory cytokines (TNF, IL-2, etc.), ROS and nitric oxide (NO) etc. that are markedly increased during the response to sepsis. Of these, NO is an important messenger molecule that key roles in a wide variety of physiologic functions, including neuronal transmission, vascular relaxation, cytotoxicity and immune modulation. However, the reaction of superoxide anion radicals with NO generates peroxynitrite that can have direct cytotoxic effects through extensive protein tyrosine nitration (Beckman et al., 1990; Pryor and Squadrito, 1995; Beckman and Koppenol, 1996).

Oxidative stress plays an important role in the physiological and pathological functions of the immune system (Remans et al., 2004). In the immune system, the chronic oxidative stress can lead to T cell hyporesponsiveness (Cemerski et al., 2003; Remans et al., 2004). One critical problem at the differentiation stage of immune cells is that T cells are occurred to die via apoptotic process caused by the active radicals,

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consequently the number of T cells becomes smaller. Such an apoptosis caused by ROS consequently leads to the lowering immunity, and to the pathologic processes in a number of human diseases including rheumatoid arthritis (Cope, 2002) and HIV infection (Cayota et al., 1996). Therefore, many scientists have tried to find blocking agents from ROS attack to T cells such as plant originated antioxidants.

It has been reported that the consumption of dietary antioxidants plays a positive role in improvement of health status in human (Benzie, 2003; Scalbert et al., 2005). Particularly, many phytoglycoproteins isolated from various kinds of natural source have been known to enhance biological functions with anti-oxidative property (Zhou et al., 2000; Oh et al., 2007). *Dioscorea batatas* Decne has been widely distributed in North-eastern Asia, China and Korea and has been used to heal the lungs, and kidneys as traditional folk medicine (Akoruda, 1984; Farombi et al., 2000). Recently, we isolated the glycoprotein from *D. batatas* Decne (DBD glycoprotein) with an approximate molecular mass of 30 kDa, and found that it consists of carbohydrate (83.75% content) and protein (16.25% content). Also, we discovered that the DBD glycoprotein has strong scavenging activity against free radicals in various radical generating systems (Oh and Lim, 2007).

To know whether or not the DBD glycoprotein protects oxygen radicals-induced thymocytes death, the experiments in the present study were carried out to examine the pro-apoptotic signals [cytochrome *c*, caspase 3, PARP, transcriptional factor (NF-κB and AP-1) and NO production] in the primary cultured mouse thymocytes.

2. Materials and methods

2.1. Chemicals

Ovalbumin (OVA), 2′,7′-dichlorofluoresin diacetate (D6883), penicillin G (H0474) and streptomycin (H0447) were obtained from Sigma (St. Louis, MO, USA). RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest analytical grade.

2.2. Superoxide anion scavenging activity of DBD glycoprotein

D. batatas Decne glycoprotein was prepared, as described previously (Oh and Lim, 2007). The superoxide anion radicals were generated by a hypoxanthine/xanthine oxidase (HX/XO) system, and monitored by reduction of NBT. All procedures were carried out according to the method of Gotoh and Niki (1992). The reaction mixtures consisted of DBD glycoprotein (50–200 μg/ml), 100 μl of 30 mM EDTA (pH 7.2), 10 μl of 30 mM HX in 50 mM NaOH and 200 μl of 1.42 mM NBT, with the final volume being brought up to 3 ml with 50 mM phosphate buffer (pH 7.4). At 20 min after adding 100 μl of XO (0.5 U/ml) to the reaction mixtures, the absorption was measured at 560 nm with a spectrophotometer (S106, Seoulin Bioscience, Seoul, Korea). In this experiment, L-ascorbic acid was used as a positive control. The scavenging effects on the activity of the superoxide anion radicals were calculated as follows:

$$\text{Scavenging activity } (\%) = \left[\frac{(A_{560\,\text{control}} - A_{560\,\text{sample}})}{A_{560\,\text{control}}}\right] \times 100$$

2.3. Inhibitory effects of DBD glycoprotein on intracellular ROS induction in primary cultured mouse thymocytes culture

2.3.1. Primary culture

ICR mice (5-week-aged, female) were injected into their footpads with mixture of ovalbumin (100 μg) and aluminium hydroxide (alum) as an adjuvant. On 7th day, thymus collected from the OVA-immunized ICR mice under aseptic conditions in Hank's balanced salt solution. Collected thymus was minced using a pair of scissors and passed through a fine steel mesh (82 mesh) to obtain homogeneous cell suspension, and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation, the pelleted cells were washed with PBS and resuspended in RPMI 1640 supplemented 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C under 5% CO_2 atmosphere. The number of cells (1×10^6 cells/ml) was divided into 96-well flat bottom plates. Oxidative stress was induced using the G/GO system which involved the generation of hydrogen peroxide (H_2O_2) at a continuous rate (Rollet-Labelle et al., 1998) by placing the cells in serum-free medium, and then treated with 0.5% D-glucose and 50 mU/ml of glucose oxidase. Cell viability was determined using MTT assay (Mosmann, 1983). Briefly, the cells were treated with 50 mU/ml of glucose oxidase (GO), or co-treated with DBD glycoprotein (25–100 μg/ml) for 4 h. After incubation, the MTT stock solution (5 mg/ml) was added into each well, and the plates were incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. Acidic isopropanol was then added in the 96-well multi plates, and the plates were read at 560 nm using a SpectraCount™ (Packard Instrument Co. Downers, III, Meriden, CT, USA).

2.3.2. Determination of intracellular ROS in primary cultured mouse thymocytes

Amount of intracellular ROS was measured by using non-fluorescent 2′,7′-dichlorodihydrofluorescein (H_2DCF-DA). H_2DCF-DA is a fluorogenic freely permeable tracer specific for ROS assessment. It is deacetylated by intracellular esterases to the non-fluorescent 2′,7′-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by ROS. The thymocytes were pre-incubated with 10 μM H_2DCF-DA for 30 min at 37 °C, and then washed twice with PBS to remove the excess of H_2DCF-DA. The cells were treated with GO (50 mU/ml), or co-treated with DBD glycoprotein (50–100 μg/ml) in presence of GO (50 mU/ml) for 4 h. Finally, the fluorescence intensity was measured at excitation of 485 nm and emission of 530 nm using fluoresencence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA, USA). The values were calculated as relative intensity of DCF fluorescence, compared to the control.

2.4. Preparation of protein extracts for cell death signals

The protein extracts were prepared as previously described (Oh et al., 2007). For the immunoblotting of iNOS, the treated cells were rinsed twice with PBS after removing the medium and scraped in 300 μl of buffer A (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40, 25% glycerol) containing a protease inhibitor cocktail. For lysis, the cells were freeze–thawed, shaken at 4 °C, and centrifuged at 14,000 × *g* for 30 min. The supernatant was collected and used as whole cell lysates.

Cell pellets were resuspended in 300 μl of buffer B (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 250 mM sucrose) containing a protease inhibitor cocktail. The cells were homogenized by 10 strokes in a dounce homogenizer. To collect nuclei and debris, the homogenates were centrifuged twice at 750 × *g* for 5 min at 4 °C. The supernatants were centrifuged at 10,000 × *g* for 15 min to collect a mitochondria-enriched pellet. The resulting supernatants were centrifuged at 100,000 × *g* for 1 h and the final supernatants are referred to as cytosolic fractions.

For preparation of the nuclear extracts, the cells treated with DBD glycoprotein (50–200 μg/ml) in presence of 50 mU/ml GO were scraped in 500 μl of hypotonic buffer C (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40), followed by centrifugation at 3000 × *g* for 5 min. The pelleted nuclear proteins were then resuspended in 200 μl of NE buffer D (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim)