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Immunosuppressive Properties of Catfish Bile from *Silurus asotus*: Inhibition of T Cell Activation in Mouse Splenocytes

Seong Soo Joo

Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea

Abstract Concentrated catfish *Silurus asotus* bile (SAB) containing high amounts of ursodeoxycholic acid (UDCA) and taurocholic acid may have immunosuppressive properties. To investigate the putative immunosuppressive properties of SAB, the anti-proliferation and suppression of early T cell activation markers, and the inhibition of cytokines induced by T cells in response to anti-CD3 mAb activation in mouse splenocytes were examined. The suppression of these activation repertoires are the main properties of calcineurin inhibitors. It was found that SAB effectively suppressed the activation of T cells, and cytokines from T cell activation, at levels similar to cyclosporine A, a calcineurin inhibitor. Although the mechanism in which suppression occurs is not clear, we speculate that SAB from *Silurus asotus*, which has been known to switch their intake habits to zoophagy during an early adult stage, may explain the suppressive effect of SAB as a result of high amounts of functional UDCA in bile. Our results suggest that the treatment or intake of SAB, either in therapy or as a food supplement, may act as an adjuvant therapy for the prevention of transplant rejection, although further investigation is required before this treatment can be applied clinically.

Keywords: bile, ursodeoxycholic acid, immunosuppressant, T cell, catfish, splenocyte

Introduction

Bile produced by hepatocytes in the liver consists of bile acids, cholesterol, phosphatidyl choline, and bilirubin. In many species, including humans, bile has two main functions. First, bile acids are critical for digestion and for absorption of fats and fat-soluble vitamins in the small intestine. Second, many of the body's waste products are secreted into the bile and are subsequently eliminated in fecal material. Bile ducts and the biliary system participate in these functions, and in Asian countries, bile has long been used as an oriental medicine for treating chronic hepatitis, ascites from a hepatoma or cirrhosis as well as for sedating a convulsion. In the last few decades, more scientifically based medicinal uses of bile have been developed via a synthetic technology, thanks to one of its major compounds, ursodeoxycholic acid (UDCA), which is normally present in bile at low concentrations (1). Although UDCA is widely used for the treatment of cholestatic liver disease, it has also been experimentally used to suppress immune responses (2) and bile acids may decrease the degree of allograft rejection after liver transplantation by changing the expression of the major histocompatibility complex class molecules in bile duct epithelium and central vein endothelium (3). In other studies, UDCA was found to inhibit apoptosis by preventing cytochrome *c* release and by modulating mitochondrial membrane perturbation (4,5). In addition, UDCA is an efficient treatment for the putatively immune-mediated liver diseases, although the mechanism of this action is unknown (6).

Immunosuppressive drugs are used in a clinical setting to prevent the rejection of transplanted organs and tissues. Among the clinically available immunosuppressants, cyclosporine A (CsA), a calcineurin inhibitor, has long been used and was one of the most widely used immunosuppressive drugs. Cyclosporine has been recently substituted with newer calcineurin inhibitors such as tacrolimus and sirolimus due to its nephrotoxicity (7). When calcineurin inhibitors bind to the cytosolic protein, cyclophillin, of lymphocytes (i.e., T cell), the formation of this complex ultimately leads to the reduced function of effector T cells, which are the major immune cells involved in transplantation rejection. In a previous study, we reported that UDCA may control cell activation by suppressing inflammatory cytokines, likely due to blocking the activation of NF-κB (8). Since T cell activation is a major cause of transplantation rejection, inactivation of these immune cells can be a meaningful tool for transplantation. In the present study, we examined whether the bile from catfish, *Silurus asotus*, containing high amounts of UDCA and taurocholic acid (TCA) compared to other representative zoophagous fishes in domestic freshwater habitats, has immunosuppressive effects equivalent to the calcineurin inhibitor, CsA.

*Corresponding author: Tel: +82-43-261-3325; Fax: +82-43-271-3246
E-mail: ssjoo@cbnu.ac.kr
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Materials and Methods

Sample preparation Fish bile was isolated from a fully grown domestic catfish (*Silurus asotus*) (Gangwon, Korea) and lyophilized under aseptic conditions. Catfish bile (SAB) was weighed and then diluted with distilled water at a concentration of 0.1 g/mL as a stock solution of SAB and stored at −70°C until use.

Thin layer chromatograpy (TLC) fingerprint The components (UDCA/TCA) of lyophilized crude bile were identified by TLC. TLC separation was performed on silica gel F254 plates (10×20 cm) (Merck AG, Darmstadt,

Germany) (9,10), and the plate was developed with chloroform:methanol:water at a ratio of 7:3:1 at a distance of 6 cm.

Spleen cell culture Mouse spleens were aseptically isolated from Balb/c mice at sacrifice and single primary splenocytes were prepared by mechanical dissociation in cold phosphate buffered saline (PBS) at pH 7.2. Erythrocytes were depleted by red blood cell lysis buffer (eBioscience, San Diego, CA, USA) containing ammonium chloride, which lyses red blood cells with minimal effect on lymphocytes, and were cultured in 10% fatal bovine serum (FBS) RPMI complete medium containing anti-CD3 mAb (eBioscience), with or without graded concentrations of stocks from lyophilized SAB. Splenocytes were cultured at 5×10^6 cells/mL in 96-well microtiter plates or at 1×10^7 cells/mL in 24-well plates, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell-free supernatants were collected from well plates between 24 to 72 hr of culture and stored at −70°C until further use.

Cell proliferation assay The T cell proliferation in response to anti-CD3 mAb stimulation was investigated at 72 hr of culture using a cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The system uses WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo phenyl)-2H-tetrazolium, monosodium salt], which, upon bioreduction in the presence of the electron carrier 1-methoxy-phenazine methosulfate, produces a water-soluble colored formazan (11). The reaction plates were measured at 450 nm and the data from triplicate cultures were expressed as the mean±standard deviation (SD).

Flow cytometry Flow cytometry was performed using FACScan (Becton Dickinson, Mountain View, CA, USA) accompanied with argon ion laser excitation at 488 nm and the gate set around the lymphocytes ($CD45^+$) was used to exclude other cells from analysis. A total of 5,000 splenic lymphocytes were evaluated in this study. Flow cytometric analysis was used to assay the activated antigen expression on the surface of T lymphocytes. Following harvest at 24 hr, cells were washed once in FACS buffer (2% FBS, sodium azide, pH 7.4) and incubated for 30 min on ice in 300 mL of cold PBS with 2% FBS containing phycoerythrin-conjugated anti-CD25 or anti-CD69 mAb. Cells were simultaneously stained with fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 mAb to assess T cell purity. All data were expressed as the percentage of double stained-positive bright cells for total T cells.

Reverse transcript-polymerase chain reaction (RT-PCR) The total RNA from mouse splenocytes stimulated by anti-CD3 mAb were prepared by the Trizol method (Invitrogen, Carlsbad, CA, USA), and the cDNA was synthesized from RNA by the reverse transcription of 1 μg of total RNA using the Improm-II reverse transcription system (Promega, Madison, WI, USA) and oligo dT primers for a total volume of 20 μL. PCR amplification was performed using the following primers (Bioneer, Deajeon, Korea): Interleukin-2 (IL-2) sense, 5'-AGA TGA ACT TGC ACC TCT GCG G-3', antisense, 5'-GGG CTT GTT GAG ATG ATG CTT TG-3'; Interferon-γ (IFN-γ) sense, 5'-AGG TCA ACA ACC CAC AGG TCC A-3', antisense, 5'-CCA GAT ACA ACC CCG CAA TCA C-3'; β-actin sense, 5'- TGA CCG AGC GTG GCT ACA GC-3', antisense, 5'-ACC GCT CAT TGC CGA TAG TG-3'. The PCRs were carried out at the preheating condition of 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 57-62°C for 30 sec, extension at 72°C for 30 sec for 28 cycles and final extension at 72°C for 10 min. The PCR products were analyzed on a 1.2% agarose gel containing ethidium bromide (11). The band intensities of the amplified DNAs were compared to the gel documentation system (MiniBIS Pro, Jerusalem, Israel).

Enzyme-linked immunosorbant assay (ELISA) IL-2 and IFN-γ levels in the supernatants of 72 hr cultures from anti-CD3-activated T cells were measured by standard sandwich ELISA using paired anti-IL-2 and anti-IFN-γ antibodies. Both levels in each sample were determined using a standard curve constructed with recombinant IL-2 and IFN-γ. Data were expressed in ng/mL and compared with the control (activated with anti-CD3 mAb in complete medium alone).

Statistical analysis The statistical analyses employed to determine the differences between the groups included a one-way ANOVA with a Dunnet's *post-hoc* test, which were performed using the SPSS software (v. 13). Statistical significance was set *a priori* at $p<0.05$.

Results and Discussion

A TLC fingerprint was used to identify the presence of UDCA and TCA in SAB (Fig. 1). The distance traveled by the spot (substance) shows that SAB has abundant TCA; and a low concentration of UDCA at a given spot. Although the UDCA movement was barely visible in the plate shown (Fig. 1), we observed a very weak band for the

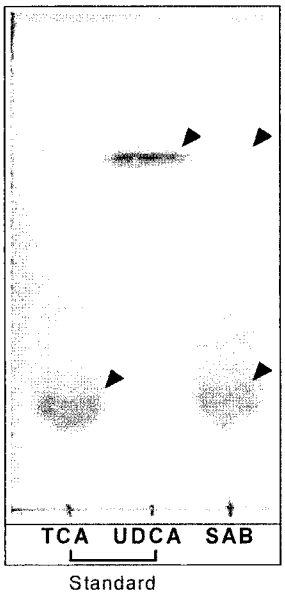

Fig. 1. TLC fingerprint of lyophilized crude bile with chloroform:methanol:water (7:3:1). The arrows indicate the separation of spots from SAB and the standard forms of UDCA and TCA. TCA, taurocholic acid; UDCA, ursodeoxycholic acid; SAB, catfish bile.

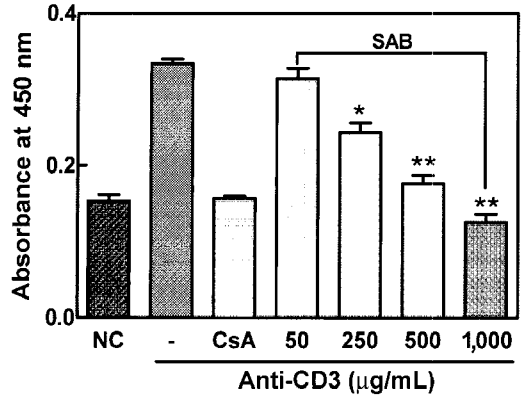

Fig. 2. Cell proliferation assay. Cells were plated in 96-well plates at 1×10^4 cells per well and cultured in the 2% FBS RPMI medium containing anti-CD3 mAb for 72 hr, with or without SAB. The cell numbers in triplicate wells were measured as the absorbance (450 nm) of reduced WST-8. NC, normal control; CsA, cyclosporine A. *p<0.05, **p<0.01 vs. control.

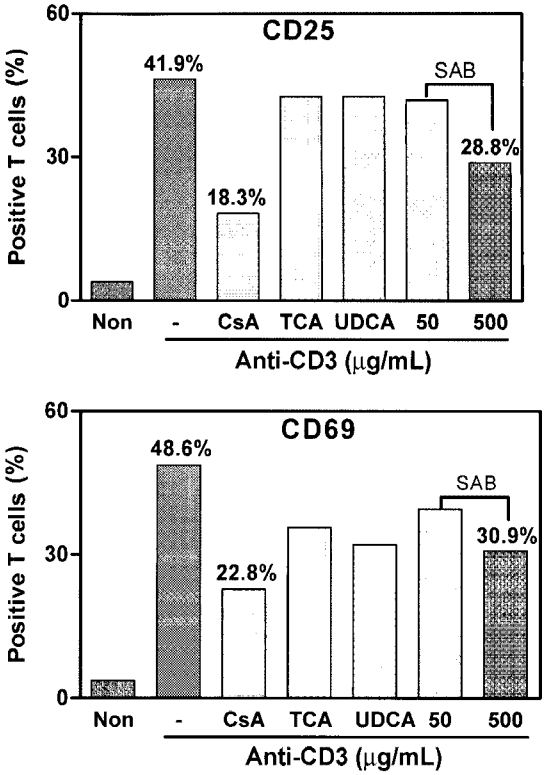

Fig. 3. Flow cytometric analysis of the changes in CD25/CD69 expression. Splenocytes were stimulated with anti-CD3 mAb in the presence or absence of SAB. SAB was treated at 50 and 500 μg/mL, CsA at 1 μg/mL, TCA at 100 μg/mL, and UDCA at 100 μg/mL. Following 24 hr of incubation, the percentages of CD25 and CD69 cell expression were determined by flow cytometry. Results are expressed as an individual % (mean±SD) to allow for a net comparison.

same movement in the UDCA standard. This pattern is similar to the quantified concentrations of UDCA and TCA in SAB, calculated from the standard curve (UDCA; 68 ng/mL, TCA; 633 ng/mL; data not shown).

Mitogenic anti-CD3 mAb was used to induce T cell activation in Balb/c mouse spleen cell cultures in the presence or absence of SAB (50-1,000 μg/mL), CsA (1 μg/mL), UDCA (100 μg/mL), or TCA (100 μg/mL). The T cell proliferation in response to activation by anti-CD3 mAB was dose-dependent and considerably attenuated at higher doses of SAB (>50 μg/mL), which was consistent with the suppressive effect observed for CsA (Fig. 2). The suppression of T cell proliferation by SAB might be an initial indication of its immunosuppressive effect. Early activation markers of T lymphocytes, CD25, and CD69, were screened by FACS (Fig. 3A and 3B). A decrease in the percentages of CD25 (28.8%) and CD69 (30.9%) at higher concentrations of SAB were observed compared to the control groups (41.9 and 48.6%, respectively). Despite this, the standard forms of UDCA and TCA, which are considered to be the major components in SAB, did not effectively control T cell activation at similar concentrations, whereas SAB decreased the percentage of CD25 and CD69 in a dose dependent manner. Moreover, Fig. 3C indicates that the expression of IL-2 and IFN-γ mRNA significantly decreased as a result of treatment with a higher concentration of SAB (500 μg/mL). Since IL-2 and IFN-γ are end products in response to T lymphocyte activation, the decreased expression of those mRNAs by SAB demonstrate that SAB can effectively control the activation of T lymphocytes as well as CsA, UDCA, and TCA.

The effect of SAB (50, 200, 400, and 800 μg/mL) on IL-2 and IFN-γ production in anti-CD3-activated spleen cell cultures was also studied. As shown in Fig. 4, the inhibition of IL-2 and IFN-γ production was dose dependent, with a maximum inhibition level of 800 μg/mL. In general, the polypeptide immunosuppressant, CsA, is a pro-drug binding to an intracellular immunophilin. Thus, this complex binds and inhibits the phosphatase activity of calcineurin, which interferes with the dephosphorylation of the members involved in the nuclear factor (i.e., NFAT) of activated T cells. NFAT interference, in turn, is involved in the production of genes encoding cytokines such as IL-2 and IFN-γ. Currently, widely used calcineurin inhibitors, such as Tacrolimus as well as other current immnosuppressants have usage limitations due to their side effects (12). Today, physicians face the important dilemma of selecting the most suitable drug for their patients, as well as at the correct dose in order to minimize the risks of infection, cancer, and other unwanted side-effects.

Although the emergence of new immunosuppressive agents has created a new era of success against transplantation rejection, T cell mediated acute rejection of transplanted organs is still a major impediment of the long-term success in transplantation and tolerance of an organ (13). In addition, these agents have limitations on their therapeutic benefits (14). UDCA, which is probably a major active compound in bile, has been shown experimentally to suppress immune response and been proven to be useful as a long-term treatment for individuals with limited chronic graft-vs.-host disease of the liver following allogeneic hematopoietic cell transplantation (15,16). The potential immunosuppressive properties of UDCA can be a clue in the development of a naturally-driven non-toxic and a further clinically adaptable substance, since UDCA has long been used as a safe drug. Despite this, an increase in the systemic quantities of UDCA, which can be used in the

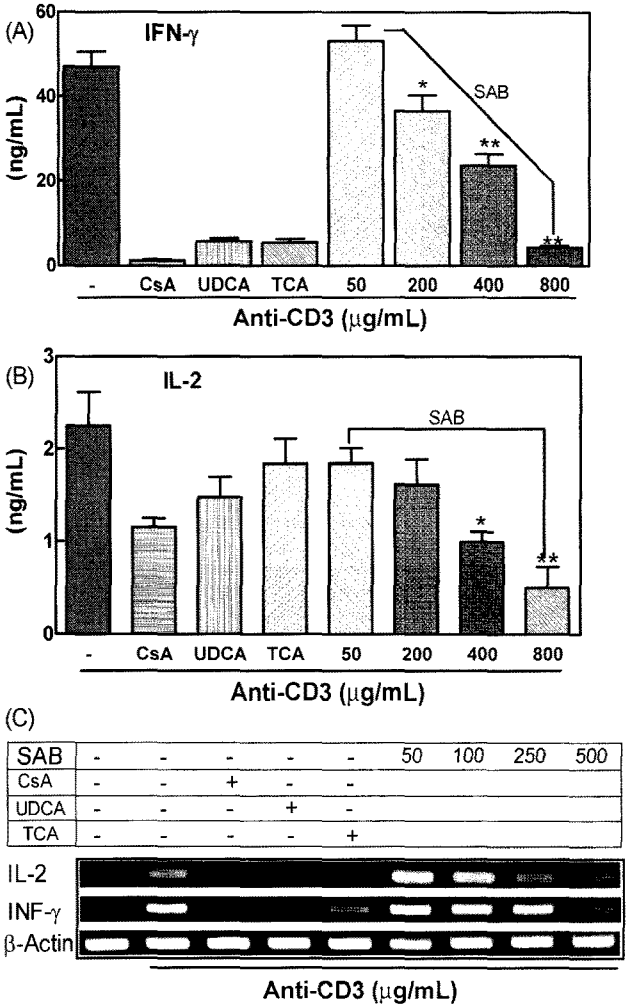

Fig. 4. IL-2 and IFN-γ expression in isolated mouse splenocytes. ELISA (A, B) and RT-PCR (C) were separately performed. Mouse splenocytes were cultured in 96-well (ELISA) or 12-well (RT-PCR) plates at 1.5×10^4 cells or 1×10^5 cells per well, respectively. Next, CsA (1 μg/mL), UDCA (100 μg/mL), TCA (100 μg/mL), and SAB (50-800 μg/mL) were assigned to a well and all wells were treated with anti-CD3 mAb for T cell activation. For the ELISA analysis, the cells were incubated for 72 hr, while cells for the RT-PCR analysis were incubated for 24 hr and β-actin was used as an internal standard. The data is expressed as the mean±SD. Cells were >90% for the trypan blue exclusion as observed under a phase-contrast microscope. *$p<0.05$, **$p<0.01$ vs. control.

digestive system, can prove to be toxic. Clinical adaptation of UDCA may serve as a limitation as a first drug of choice for immunosupression (17).

In this study, we found that fish bile, especially in zoophagous fishes, contains high amounts of UDCA and TCA, and that bile from *S. asotus* contains approximately 10× the amount of these compounds with respect to other zoophagous fish (i.e., *Siniperca scherzeri* and *Micropterus salmoides*; unpublished data). Moreover, SAB efficiently suppressed the important T cell activation markers, CD25 and CD69, as well as the cytokines IL-2 and IFN-γ, which are produced when T cells are activated via the T cell receptor (TCR) complex. As the regulation of the T cell growth factor cytokines involved in T cell activation and acute cellular rejection (18), such as IL-2 and IFN-γ, induce the suppression of the rejection of transplantation, SAB could be a good candidate for a potential immunosuppressant substance, either as an adjuvant drug or functional food agent, although further studies are required. One possible way in which SAB affects the suppression of T cell activation, is through its high functional UDCA content. In addition, similar to mammalian bile, fish bile contains bile salts, cholesterol, phospholipids, bile pigments, glycoproteins, and organic as well as inorganic ions (19). Thus, it may be possible that SAB could also substitute for bear bile, a commonly used folk medicine. These studies demonstrate that SAB has potent immunosuppressive properties *in vitro*, although these effects need to be further investigated before it can be safely and effectively used as a clinically useful agent or as a functional food source.

Acknowledgments

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