Original Article

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Cytokines as potential biomarkers of liver toxicity induced by *Dioscorea bulbifera* L.

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Summary **The present study is designed to search for the serum cytokine biomarker for liver injury induced by *Dioscorea bulbifera* L., which is a traditionally used herbal medicine in China. Mice were orally given various doses of ethyl acetate extract (EF) isolated from *D. bulbifera* for 12 days. The activity of serum alanine/aspartate transaminases (ALT/AST) was increased in EF (400 mg/kg)-treated mice. Histological assessment further confirmed EF (400 mg/kg)-induced liver injury. Results of a cytokine-antibody array demonstrated that there were 10 cytokines up-regulated and 1 cytokine down-regulated in EF (400 mg/kg)-treated mice. Enzyme-linked immunosorbent assay (ELISA) further confirmed the increased level of CD30 ligand (CD30L) and decreased level of interlukin-3 (IL-3) in EF-treated mice. In conclusion, our results demonstrate that the altered expression of CD30L and IL-3 may be potential biomarkers for hepatotoxicity induced by *D. bulbifera.***

Keywords: *Dioscorea bulbifera* L., hepatotoxicity, cytokines, CD30L, IL-3

1. Introduction

Dioscorea bulbifera L. is a native plant in Africa and Asia, and it is also an invasive species in many tropical areas, including Florida in the United States. *D. bulbifera* is generally used to treat thyroid disease (especially goiter) and cancer in clinics in China (*1*). However, during clinical practice *D. bulbifera* has been found to have hepatotoxicity, which has caused a great obstacle for its application in the clinic (*2*). Recently, study of the hepatotoxicity of *D. bulbifera* has attracted great interest. Our previous studies have already showed that an ethyl acetate extract (EF) isolated from *D. bulbifera* could induce oxidative stress liver injury (*3,4*). Meanwhile, our studies and other reports found that diterpenoids were the main hepatotoxic compounds in *D. bulbifera*, such as diosbulbin B and diosbulbin D (*3,5*).

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Drug-induced liver injury (DILI) has been a public health issue for many years, and it is the major cause for withdrawal of approved drugs from the market and leads to blockage of development of potential drugs. Generally, herbal medicines are regarded as having no toxicity or side-effects, and they are increasingly used worldwide as dietary supplements (*6,7*). However, there are increased reports about the hepatotoxicity induced by herbal medicines. For example, herbal medicines as dietary supplements caused about 9% of cases of DILI, while in Asia the percentage was about 20-55% cases of DILI (*8,9*). Thus, liver injury induced by herbal medicines needs to be paid more attention.

Currently, conventional widely used biochemical markers for evaluating liver injury are serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and cholestatic markers such as alkaline phosphatase (ALP), and γ-glutamyltransferase (γGT) (*10,11*). However, all of these biomarkers sometimes can not accurately reflect liver injury (*11*). Serum ALT activity has also been associated with toxicity of other organs and thus it leads to false positives due to other sources of serum ALT activity because it has specificity beyond the liver (*12*). Meanwhile, there is a report that serum ALT activity is variable in people, which will also lead to possible false positive or negative results

(*13*). Thus, searching for more simple and sensitive biomarkers as a supplementary index for DILI is an urgent problem that needs to be solved.

Cytokines are a diverse group of soluble proteins, peptides or glycoproteins, which have various biological functions. Recently, innate and acquired immunity mediated by cytokines has been reported to play a critical role in DILI, and thus cytokines have the potential value as biomarkers for DILI (*14,15*). In the present study, we employed a cytokine-antibody array to search for potential cytokine biomarkers for liver injury induced by *D. bulbifera*.

2. Materials and Methods

2.1. *Chemicals and reagents*

The rhizomes of *D. bulbifera* were collected in Qingyang, Anhui Province and authenticated by Prof. Shou-Jin Liu (Anhui College of Traditional Chinese Medicine, Anhui, China). The specimens were deposited in the herbarium of the Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine. The preparation of ethyl acetate extracts (EF) isolated from *D. bulbifera* was previously reported in our published papers, and the content of diosbulbin B is 13.72% (*16*). The kits for determining ALT/AST activity were obtained from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). RayBio™ Mouse Cytokine Antibody Array III was purchased from RayBiotech, Inc. (Norcross, GA, USA). ELISA kits were purchased from RapidBio (West Hills, CA, USA). Other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. *Experimental animals*

Specific pathogen free male ICR mice (18-22 g body weight) were purchased from the Chinese Academy of Sciences (Shanghai, China). The mice were fed a standard laboratory diet and given free access to tap water, kept in a controlled room temperature (22 ± 1°C), humidity (65 ± 5%), and a 12:12-h light/dark cycle. All mice received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

2.3. *Treatments of animals*

Mice were orally administered EF (100, 200, and 400 mg/kg, suspended in 0.5% CMC-Na, $n = 7$) for 12 days, and 0.5% CMC-Na was used as a vehicle control ($n = 8$). Animals were killed 24 h after the last administration. Blood was collected from the eyeball for measurement of ALT, AST, and cytokine analysis. Livers were collected for histological assessment.

2.4. *Serum ALT/AST analysis*

Blood samples obtained from mice of all groups were allowed to coagulate for 2 h on ice. Serum was then isolated following centrifugation at 840 × g for 15 min. Serum ALT, and AST activity were measured with kits according to the manufacturer's instructions.

2.5. *Histological examination*

Portions of the liver were fixed in 10% formalin and embedded in paraffin. Samples were subsequently sectioned (5 μm) and stained with hematoxylin-eosin (HE) for further histological analysis.

2.6. *Cytokine antibody array*

Serum cytokines were assayed by Raybio Mouse Cytokine Antibody Array III according to the manufacturer's instructions. This array is capable of simultaneously detecting 62 different cytokines with high specificity. Chemiluminescence signals were visualized by exposure to light sensitive films. Films were digitized, and densitometric quantifications were analyzed with ScanAlyze software, and then the ratio of EF/Control was calculated. Differentially expressed cytokines were defined as over 2 fold alteration between the control and EF-treated groups.

2.7. *ELISA analysis*

Serum was used for ELISA analysis according to the manufacturer's instructions.

2.8. *Statistical analysis*

The results were expressed as Means ± SEM. SPSS 18.0 was used for statistical analysis. Significance difference between various groups was determined by One-Way ANOVA, and between two groups was evaluated by an independent-sample *t*-test. $p < 0.05$ was considered as indicating statistically significant differences.

3. Results

3.1. *EF induced liver injury*

As shown in Figure 1A, EF (400 mg/kg) increased serum ALT and AST activity ($p < 0.001$). Further, histological assessment showed normal liver shape and structure in control mice (Figure 1B). EF (400mg/kg)-treated mice exhibited severe liver damage indicated by intrahepatic hemorrhage, lymphocyte infiltration and the destruction of liver structure (Figure 1B).

3.2. *Distinct serum cytokine expression in normal and EF-treated mice*

To identify the cytokine biomarker for *D. bulbifera*-induced liver injury, we analyzed the content of 62 cytokines in serum by a cytokine antibody array in control and EF (400 mg/kg)-treated mice. Table 1 is the layout of the cytokine antibody array, and Figure 2 is the results of the array. After analyzing, as shown in Table 2, ten cytokines were up-regulated and one cytokine was down-regulated over 2-fold in EF (400 mg/kg)-treated mice as compared to control. The up-regulated cytokines are CD30L, fractaline, interferon inducible monokine (CRG2), granulocyte macrophage colony-stimulating factor (GM-CSF), thymus-expressed chemokine (TECK), monocyte chemoattactant protein 5 (MCP-5), IL-4, IL-

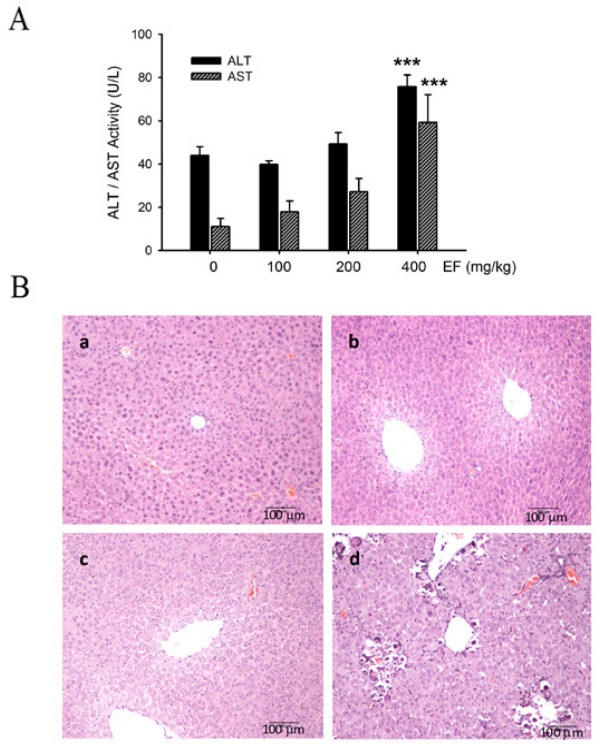

Figure 1. EF induced liver injury. (A) ALT and AST activity. Data are expressed as means ± SEM (n = 8 for control and n = 7 for experimental group). *** $p < 0.001$ compared with control. **(B)** Histological observation of EF-induced liver injury. **a**. Control; **b**. EF (100 mg/kg); **c**. EF (200 mg/kg); **d**. EF (400 mg/kg). Typical images were chosen from each experimental group. (original magnification: ×100).

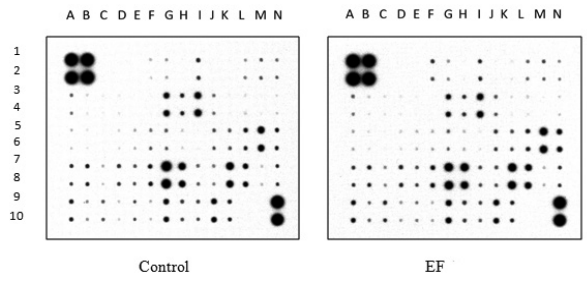

Figure 2. Results of cytokine antibody array. The images of control and EF (400 mg/kg)-treated mice in the cytokine antibody array. n = 8 for control and n = 7 for experimental group.

Table 1. The layout of cytokine antibody array

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
2	POS	POS	NEG	NEG	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
3	Eotaxin -2	Fas ligand	Fractalkine	G-CSF	GM-CSF	IFNγ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1β	IL-2	IL-3	IL-3Rb
4	Eotaxin-2	Fas ligand	Fractalkine	G-CSF	GM-CSF	IFNγ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1β	IL-2	IL-3	IL-3Rb
5	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L Selectin
6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L Selectin
7	Ln/XCL-1	MCP1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3β	MIP-3α	PF-4	P Selectin	RANTES	SCF
8	Ln/XCL-1	MCP1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3β	MIP-3α	PF-4	P Selectin	RANTES	SCF
9	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	sTNFRI	sTNFRII	TPO	VCAM-1	VEGF	Blank	Blank	POS
10	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	sTNFRI	sTNFRII	TPO	VCAM-1	VEGF	Blank	Blank	POS

Table 2. List of differentially changed cytokines between control and EF group

Row	Column	Cytokine symbol (Used on array)	Full name	The ratio (EF/Cont)
1,2	F	Axl	Axl receptor tyosine kinase	3.96
1,2	H	CD30L	CD30 ligand	2.22
1,2	K	CRG-2	Interferon inducible monokine	2.17
3,4	C	Fractalkine	-	2.20
3,4	E	GM-CSF	Granulocyte macrophage colony-stimulating factor	3.42
3,4	M	IL-3	Interleukin-3	0.26
5,6	A	IL-4	Interleukin-4	2.39
5,6	B	IL-5	Interleukin-5	2.02
5,6	C	IL-6	Interleukin-6	5.63
7,8	C	MCP-5	Monocyte chemoattactant protein 5	2.05
9,10	D	TECK	Thymus-expressed chemokine	2.11

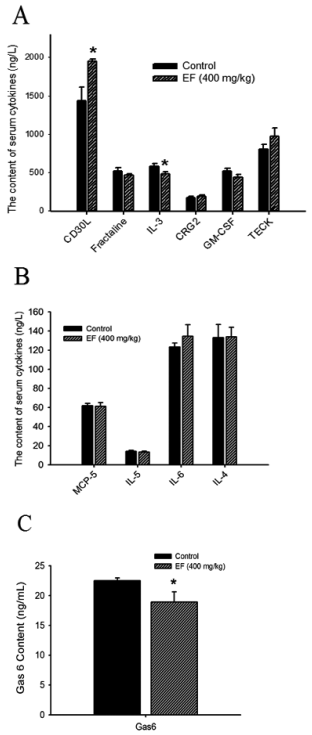

Figure 3. ELISA results. (A) The contents of serum CD30L, fractaline, IL-3, CRG-2, GM-CSF, TECK. **(B)** The contents of serum MCP-5, IL-5, IL-6, IL-4. **(C)** The content of serum Gas6. Data are expressed as means ± SEM (n = 8 for control and n = 7 for experimental group). * $p < 0.05$ compared with control.

5, IL-6, and axl receptor tyosine kinase (Axl), while the down-regulated cytokine is IL-3.

3.3. *ELISA assay of changed cytokine*

Next, ELISA assays were used to validate the change of those cytokines in the cytokine antibody array. Figure 3A showed that CD30L was increased and IL-3 was decreased in EF (400 mg/kg)-treated mice ($p < 0.05$), which is consistent with the results of the cytokine antibody array. There were no obvious changes of other cytokines such as fractaline, CRG2, GM-CSF, TECK, MCP-5, IL-5, IL-6, or IL-4 (Figures 3A and 3B), which is inconsistent with the results of the cytokine antibody array. Growth arrest-specific 6 (Gas 6), the ligand for axl receptor tyosine kinase (Axl), was decreased in EF (400 mg/kg)-treated mice ($p < 0.05$) in the ELISA assay (Figure 3C).

4. Discussion

In China, *D. bulbifera* is traditionally used to treat thyroid disease. Recently, *D. bulbifera* has been found to have a good therapeutic effect for some solid cancers, and now is commonly used in cancer therapy in the clinic. However, with the increased application of *D. bulbifera* in the clinic, its induced hepatotoxicity has widely attracted attention. The present study shows that after treatment with EF isolated from *D. bulbifera* for 12 days, serum ALT and AST activities were increased. Further, histological assessment demonstrated liver destruction in EF-treated mice. All of these results indicate the potential hepatotoxicity of *D. bulbifera*, when used for a stage treatment in cancer therapy.

Inflammation is reported to contribute to progressive liver damage induced by exogenous chemical hepatotoxins including drugs like acetaminophen, cycloheximide **etc**. (*17-20*). Cytokines are a family of secreted proteins that promote inflammation and participate in both innate and adaptive immune responses which play important roles in DILI (*15,21,22*). To our knowledge the present study is the first study using a cytokine antibody array to identify potential serum biomarkers for DILI. In the study, the primary screen using cytokine antibody array demonstrated 11 cytokines differentially altered after treatment with EF. Further, ELISA results confirmed that 2 cytokines were significantly changed after treatment with EF, which are CD30L and IL-3. The other 9 cytokines were not changed when measured by ELISA, which may be due to the limitations of the cytokine antibody array. There is already a report demonstrating that a linear response between the chemiluminescent signal and quantity may not always exist, which may lead to the false results shown in the cytokine antibody array (*23*), so further verification experiments such as ELISA are necessary.

CD30L is the ligand for CD30, which represents the newest member of the tumor necrosis factor receptor (TNF-R) family (*24*). Up-regulation of CD30/CD30L is associated with several hematological malignancies such as Hodgkin's disease (HD), anaplastic large cell lymphoma (ALCL) and subsets of Non-Hodgkin's lymphomas (NHL's) (*25*). Recently, the interaction of CD30/CD30L was reported to play some role in the crosstalk between natural killer and dendritic cells, and is critical for humoral immunity (*26,27*). Meanwhile, some studies demonstrate that CD30L/CD30 might be useful as a novel biological therapy for allergic rhinitis, inflammatory bowel disease, autoimmune diabetes, and chronic inflammatory skin diseases like psoriasis or atopic dermatitis (*28-31*). However, there is no report about whether there is some role of CD30L in liver injury. The present study found that serum CD30L was increased in EF-treated mice, which suggests potential involvement of CD30L in *D. bulbifera*-induced liver injury. This result is the first report about the potential role of CD30L in liver injury, and the concrete mechanism needs further investigation.

IL-3 is a key cytokine which promotes survival, proliferation, differentiation and maturity of bone marrow-derived hematopoietic stem cells, and thus it is widely studied to treat different states of bone marrow failure or hematologic malignancies (*32,33*). In addition, recent studies demonstrate that IL-3 participates in the response of the organism to various types of stress (*34*). Although there are already reports about the change of IL-3 in hemorrhage or alcohol-induced liver injury, the role of IL-3 in liver injury is still not clear (*35,36*). Our results demonstrate the down-regulation of serum IL-3 in EF-treated mice, which suggest potential involvement of IL-3 in *D. bulbifera*-induced liver injury.

In conclusion, the present study demonstrates that serum CD30L and IL-3 levels were up-regulated and down-regulated respectively in EF-treated mice, which demonstrates that inflammation may contribute to the liver injury induced by *D. bulbifera*, and the concrete mechanism needs further investigation.

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