Enhancement of CD93 expression and interleukin-8 (IL-8) production in the human monocyte-like cell line U937 in response to *Taimatsu* fermented rice germ gamma-amino butyric acid (GABA)

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Abstract

We examined the modulation of CD93 (receptor for complement component 1, subcomponent q phagocytosis; C1qRp) expression in the human monocyte-like cell line U937 cultured with *Taimatsu* fermented rice germ gamma-amino butyric acid (GABA) using CD93 monoclonal antibodies (mAbs), a glutaraldehyde-fixed cellular enzyme immunoassay (GCEIA), and flow cytometry. The expression of CD93 in the U937 cells cultured with *Taimatsu* fermented rice germ GABA was significantly enhanced ($P<0.01$), compared with that in the control cells (cultured without *Taimatsu* fermented rice germ GABA) at 24 hrs. This enhancement of CD93 expression was dose-dependent and time-dependent. The enhancement of CD93 expression was markedly blocked in the presence of the protein kinase C (PKC) inhibitor Go6976. In addition, the U937 cells cultured with *Taimatsu* fermented rice germ GABA significantly enhanced ($P<0.01$) the production of interleukin-8 (IL-8) in the culture supernatants. Together, these findings strongly indicated that *Taimatsu* fermented rice germ GABA has immunopotentiator effects on some human immune responses.

Key words : CD93, interleukin-8 (IL-8), *Taimatsu* fermented rice germ GABA, U937 cells.

Introduction

Gamma-amino butyric acid (GABA) was originally identified as a principal inhibitory neurotransmitter in mammalian brain, but it subsequently became clear that GABA and GABA receptors exist in many non-neuronal peripheral tissues [1]. In the developing rat embryo, GABA has been found to play an important role in the morphogenesis and maturation of many tissues outside the nervous system. A previous study indicated that GABA and glutamate decarboxylase (GAD), including its two isoforms (GAD65 and GAD67), were expressed in chondrocytes on the epiphyseal growth plate of rats and were mainly localized in the maturation zone, rather than the reserve zone or proliferating zone [2]. This previous report suggested that GABA plays certain functional roles in the differentiation of chondrocytes during the growth of the skeleton and participates in the regulation of cell differentiation and maturation.

On the other hand, several reports have shown that the expression of GABA and its synthetic enzyme, GAD, significantly increased in neoplastic tissues, such as colorectal carcinoma, breast cancer and gastric cancer, compared with that in normal tissues [3]. In addition, GABA is also known as an immune stimulator that activates immune-related cells and antibody production. For example, when GABA was orally administered, IgA antibody was effectively secreted in the saliva [4].

Several GABA-containing materials have been identified, studied and developed for use as supplements

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in the health food, medical and pharmaceutical industries. However, detailed analyses involving the modulation of cell surface antigens, particularly CD93 (receptor for complement component 1, subcomponent q phagocytosis; C1qRp) [5], and cytokine production have yet to be performed.

Recently, we succeeded in establishing a form of GABA produced by fermented rice germ, designated as *Taimatsu* fermented rice germ GABA, by developing and using the latest productive systems. In this study, we investigated some of the immunological actions of this material, such as its ability to modulate CD93 expression and to produce a soluble factor, interleukin-8 (IL-8), using the human monocyte-like cell line U937.

Materials and Methods

Reagents

Taimatsu fermented rice germ GABA was prepared by Taimatsu Foods Co. (Niigata, Japan) using the latest biological culturing and preparative techniques and is currently available commercially as a health food supplement. Phorbol myristate acetate (PMA) and the protein kinase C (PKC) inhibitor Go6976 were purchased from Sigma Chemical Co., St Louis, MO. (U.S.A.). An enzyme immunoassay (EIA) kit for human interleukin-8 (IL-8) was purchased from MBL Co. (Nagoya Japan).

Antibodies

The CD93 monoclonal antibody (mAb) (mNI-11, mouse IgG1) [6] was established in our laboratories. The CD93 mAb (R-3, mouse IgG1) [7] was purchased from eBioscience Laboratory (U.S.A.). A fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody and a horseradish peroxidase (HRPOD)-conjugated goat anti-mouse IgG were purchased from MBL Co. (Nagoya Japan).

Cell line

The U937 cells used in this study were supplied by the Japanese Research Resource Bank (JRRB) (Tokyo, Japan) and were cultured in RPMI 1640 medium (GIBCO) supplemented with 10mM HEPES buffer, 2 mM glutamine, and 10% fetal calf serum (FCS) (GIBCO) (subsequently referred to as complete medium).

Preparation of U937 cells cultured with *Taimatsu* fermented rice germ GABA

U937 cells ($1x10^5$) in complete medium were cultured in 96-well microplates (Sumitomo Co. ,Tokyo Japan) with or without *Taimatsu* fermented rice germ GABA at various dilutions in the presence and absence of the protein kinase C inhibitor Go6976 at an optimal concentration; the plates were incubated at 37℃ for 24 to 120 hrs. In an additional experiment, U937 cells were cultured with PMA (100ng/mL) under the same conditions. After culturing, the cells were harvested and the modulation of CD93 was examined using CD93 mAb (mNI-11 or R-3), a glutaraldehyde-fixed cellular enzyme-immunoassay (GCEIA), and flow cytometry. Some culture supernatants under the same conditions were used for the interleukin-8 (IL-8) assay.

Glutaraldehyde-fixed cellular enzyme immunoassay (GCEIA)

The U937 cells cultured with or without *Taimatsu* fermented rice germ GABA (various dilutions) were fixed with 0.25% glutaraldehyde phosphate-buffered saline (PBS) solution for 60 min at room temperature. After fixation, the cells were washed three times with PBS and blocked with PBS containing 2% dried milk and 100mM glycine (blocking buffer) for 60 min at room temperature. CD93 mAb (mNI-11 or R-3) (diluted 0.2% dried milk-PBS) was added to each well and the samples were incubated for 60 min at room temperature with shaking. After washing three times with PBS, HRPOD-conjugated goat anti-mouse IgG (1:5000) was added to each well and the samples were further incubated for 60 min at room temperature with shaking. After washing with PBS, the substrate-chromogen (TMB) was added to each well, followed by 15 min of incubation at room temperature. The reaction was stopped by the addition of 0.5-N HCl, and the optimal density (OD) was read at 450 nm using a multichannel EIA microplate reader. The expression index was calculated using the following

formula: (Expression index) = (OD value of positively stained cells for CD93 mAb cultured with *Taimatsu* fermented rice germ GABA)/(OD value of positively stained cells for CD93 mAb cultured without *Taimatsu* fermented rice germ GABA). The experiments were repeated five times.

Flow cytometry

The cells were washed in cold PBS containing 0.1% NaN_3 (subsequently referred to as the washing buffer) and were then incubated in PBS containing 25% normal goat serum, 1 mg/mL of normal human IgG, and 0.1% NaN_3 for 10 min on ice to block the Fc receptor (FcR) for IgG. The cells were then incubated with an optimal concentration of the CD93 mAb (mNI-11) for 40 min at room temperature. After washing with the washing buffer, the cells were incubated with an FITC-conjugated goat anti-mouse IgG for 20 min at room temperature. Following a final wash with the washing buffer and resuspension in PBS containing 2% FCS and 0.1% NaN_3, the percentages and mean fluorescence intensities (MFIs) of positively stained cells for CD93 mAb (mNI-11) were determined using a FACScan (Becton Dickinson). The experiments were repeated three times.

Assay for interleulin-8 (IL-8) in the culture supernatants

The detection of interleukin-8 (IL-8) in the culture supernatants of the U937 cells cultured with or without *Taimatsu* fermented rice germ GABA (1:40 or 1:20) was performed using an EIA kit. The experiments were repeated three times.

Statistical analysis

The statistical analysis was performed using the Student *t*-test. Differences were considered significant when the *P* value was less than 0.05.

Results and Discussion

In this study, we investigated the immunological actions, at the cellular level, of *Taimatsu* fermented rice germ GABA. First, the modulation of CD93 on the U937 cells cultured with *Taimatsu* fermented rice germ GABA (various dilutions) was analyzed using GCEIA or flow cytometry. As shown in Fig. 1, the expression of CD93 on the U937 cells cultured with *Taimatsu* fermented rice germ GABA (1:100, 1:40, 1:20) was significantly enhanced ($P<0.05$, $P<0.01$ and $P<0.01$, respectively) at 24 hrs compared with control cells cultured without *Taimatsu* fermented rice germ GABA using the GCEIA system. On the other hand, phorbol myristate acetate (PMA) also significantly enhanced ($P<0.01$) CD93 expression by U937 cells (Fig. 1). PMA is a protein kinase C (PKC) activator which leads to activation of intracellular second messengers, and influences various human immune responses by regulation of the expression of several functional receptors in certain lymphoid cells, as previously described [6]. Furthermore, the flow cytometric analysis produced results similar to those obtained in the GCEIA analysis (Fig. 2), and the enhancement of CD93 expression recognized by the CD93 mAbs (mNI-11 or R-3) was dose-dependent and time-dependent (Figs. 1 and 3). This enhancement of CD93 expression in cells cultured with *Taimatsu* fermented rice germ GABA reached a maximal expression level at 96 hrs, and its expression gradually decreased at 120 hrs (Fig. 3).

CD93 is a receptor for complement component 1, subcomponent q phagocytosis (C1qRp) and was originally reported to be involved in the C1q-mediated enhancement of phagocytosis [8,9]. CD93 is selectively expressed on myeloid cells (granulocytes and monocytes), endothelial cells and stem cells, suggesting that this molecule is involved in some important biological functions in several immune responses [10]. The modulation of CD93 expression has been investigated in a variety of cells, particularly in granulocytes and monocytes, and the rapid up-regulation of this molecule's expression by the inflammatory peptide FMLP has been reported [11]. We also previously reported that CD93 expression on a human monocyte-like cell line (U937) was strongly up-regulated by exposure to a PKC activator, PMA; this up-regulation was controlled by a PKC delta isoenzyme [12]. Taken together, these results indicate that *Taimatsu* fermented rice germ GABA possesses PKC activity against U937 cells with

regard to the modulation of CD93 expression.

The enhancement of CD93 expression on the U937 cells cultured with *Taimatsu* fermented rice germ GABA was markedly blocked in the presence of an optimal concentration (3μM) of the PKC inhibitor Go6976 (Fig. 4). This finding clearly indicates that the enhancement of CD93 expression on the U937 cells by *Taimatsu* fermented rice germ GABA was mediated by the intracellular PKC activity. Therefore, *Taimatsu* fermented rice germ GABA may provide important information on cell-to-cell interactions in various human immune responses. In addition, the enhancement of CD93 cultured with *Taimatsu* fermented rice germ GABA may be related to the C1q-mediated enhancement of phagocytosis in innate immune responses.

We have also observed that the expression of CD54 (intercellular adhesion molecule-1; ICAM-1) was enhanced by *Taimatsu* fermented rice germ GABA (data not shown). CD54 is a member of the immunoglobulin (Ig) superfamily of adhesion molecules and is expressed on various cells such as lymphocytes, monocytes, granulocytes, and epithelial cells. It plays important roles as a ligand for CD11a (lymphocyte function-associated antigen-1α; LFA-1α) in various human immune responses [13].

To examine the biological function, we next measured IL-8 in the culture supernatants of U937 cells cultured with or without *Taimatsu* fermented rice germ GABA using an EIA kit. Figure 5 shows that *Taimatsu* fermented rice germ GABA strongly induced the release of IL-8 into the culture supernatants of the U937 cells at 24 hrs, and the difference between the supernatant from cells cultured in the presence or absence of *Taimatsu* fermented rice germ GABA was significant ($P<0.01$). IL-8 is one of the most potent chemoattractants for neutrophils and is released by several human cells stimulated by various substances, and is controlled mainly by protein kinases [14, 15]. Furthermore, the production of IL-8 also appears to depend on NF-κB activation and intracellular signaling mediated by Toll-like receptor-2 (TLR-2) [16]. Thus, *Taimatsu* fermented rice germ GABA may be used as a new reagent for NF-κB regulation in several biological fields.

Although GABA was originally identified as a principal inhibitory neurotransmitter in the mammalian brain [1], we have provided some evidence that *Taimatsu* fermented rice germ GABA also has immunoregulatory functions. The mechanism responsible for the effect of *Taimatsu* fermented rice germ GABA on immunoregulatory function as observed in this study is not well understood at present time. However, we have provided evidence that GABA is also an immune stimulator in addition to an inhibitory neurotransmitter. Furthermore, *Taimatsu* fermented rice germ also contains inositol hexaphosphate (IP6) in addition to the GABA element. Inositol hexaphosphate (IP6) or phytic acid is a naturally occurring hexaphosphorylated carbohydrate, ubiquitously present in most plants and mammalian cells. The basic carbohydrate moiety "inositol" in IP6 and its other phosphate derivatives (IP1-IP5) are physiologically interconvertible and regulate vital cellular functions [17]. This product is marketed as a dietary supplement owing to its antioxidant property and is known to have beneficial effects such as the prevention of kidney stones, cancer, high cholesterol levels, and heart and liver diseases [18-20]. Thus, *Taimatsu* fermented rice germ GABA may be a very useful material for the regulation of various human immune responses through the synergic effects of GABA and IP6.

In conclusion, *Taimatsu* fermented rice germ GABA significantly enhanced the expression of CD93 on U937 cells, ultimately leading to the production of IL-8. These findings strongly indicate that the action of *Taimatsu* fermented rice germ GABA is highly critical to immunological responses. Further analyses are needed to demonstrate the detailed mechanism(s) of this finding at the molecular level.

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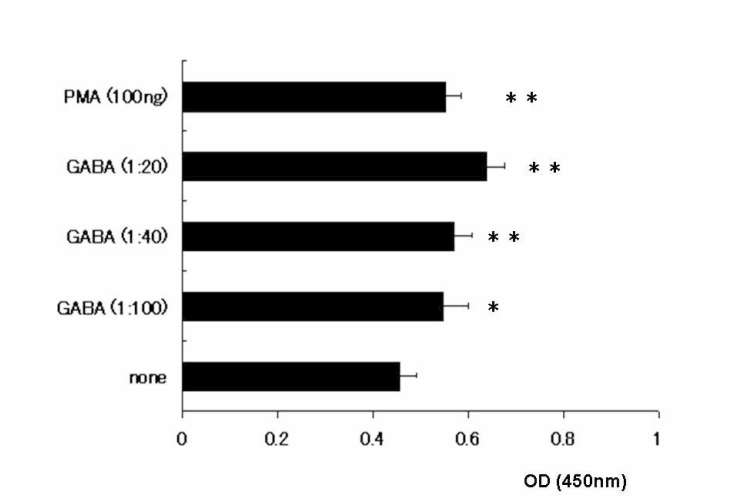

Figure 1. Enhancement of CD93 expression on U937 cells cultured with *Taimatsu* fermented rice germ GABA using GCEIA system. The U937 cells cultured with or without *Taimatsu* fermented rice germ GABA (1:100, 1:40, 1:20) or PMA (100ng/mL) at 24 hrs were fixed in 0.25% glutaraldehyde-PBS solution for 60 min at room temperature. After fixation, the reactivity of CD93 mAb (mNI-11) to these cells was examined using the GCEIA system (see Materials and Methods section for details). The experiments were repeated five times. **P*<0.05, none vs. *Taimatsu* fermented rice germ GABA (1:100). ***P*<0.01, none vs. *Taimatsu* fermented rice germ GABA (1:40 or 1:20, respectively) or PMA (100 ng/mL).

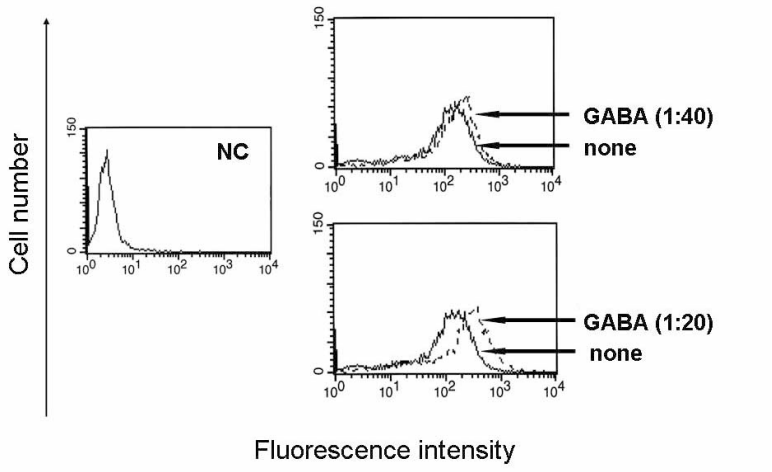

Figure 2. Enhancement of CD93 expression on U937 cells cultured with *Taimatsu* fermented rice germ GABA using flow cytometry. The U937 cells cultured with or without *Taimatsu* fermented rice germ GABA (1:40 or 1:20) were incubated with optimal concentrations of CD93 mAb (mNI-11) for 40 min at room temperature. After washing, the cells were incubated with an FITC-conjugated goat anti-mouse IgG for 20 min at room temperature. The percentages and mean fluorescence intensities (MFIs) of the cells that stained positive for CD93 mAb (mNI-11) were determined using flow cytometry (see Materials and Methods section for details). The experiments were repeated three times. NC: negative control (without CD93 mAb, mNI-11).

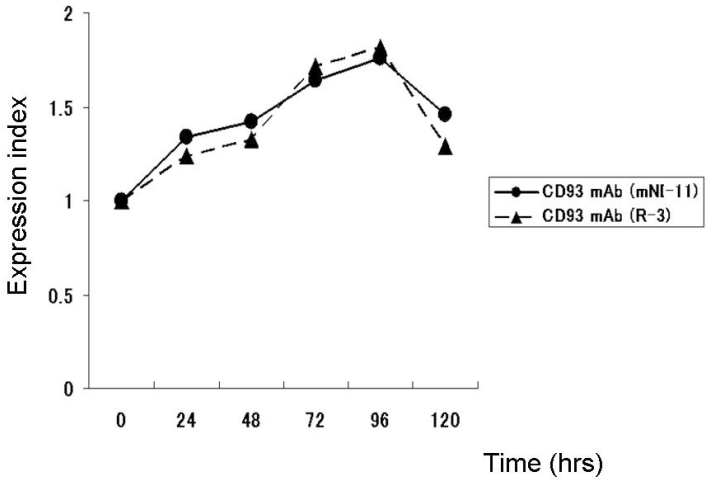

Figure 3. Time-dependent enhancement of CD93 expression on U937 cells cultured with *Taimatsu* fermented rice germ GABA. U937 cells cultured with or without *Taimatsu* fermented rice germ GABA (1:40) at 24 to 120 hrs were fixed in 0.25% glutaraldehyde-PBS solution for 60 min at room temperature. After fixation, the reactivity of CD93 mAb (mNI-11 or R-3) to these cells was examined using the GCEIA system (see Materials and Methods section for details). The expression index was calculated using the following formula: (Expression index) = (OD value of positively stained cells for CD93 mAb cultured with *Taimatsu* fermented rice germ GABA)/(OD value of positively stained cells for CD93 mAb cultured without *Taimatsu* fermented rice germ GABA). The experiments were repeated five times.

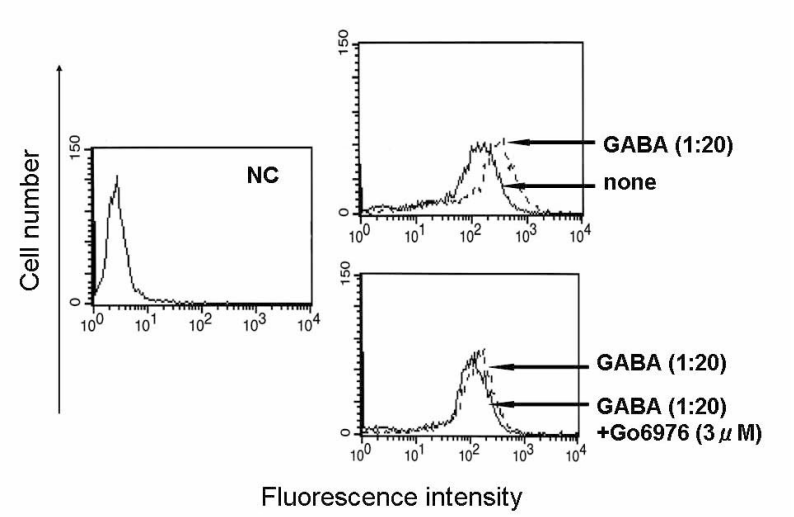

Figure 4. Enhancement of CD93 expression on U937 cells cultured with *Taimatsu* fermented rice germ GABA was markedly blocked by the PKC inhibitor Go6976. U937 cells were cultured with or without *Taimatsu* fermented rice germ GABA (1:20) in the presence or absence of the PKC inhibitor Go6976 (3 μM) at an optimal concentration. The cells were incubated with the optimal concentration of CD93 mAb (mNI-11) for 40 min at room temperature. After washing with the washing buffer, the cells were incubated with an FITC-conjugated goat anti-mouse IgG for 20 min at room temperature. The percentages and mean fluorescence intensities (MFIs) of the cells that stained positive for CD93 mAb (mNI-11) were determined using flow cytometry. The experiments were repeated three times. NC: negative control (without CD93 mAb, mNI-11).

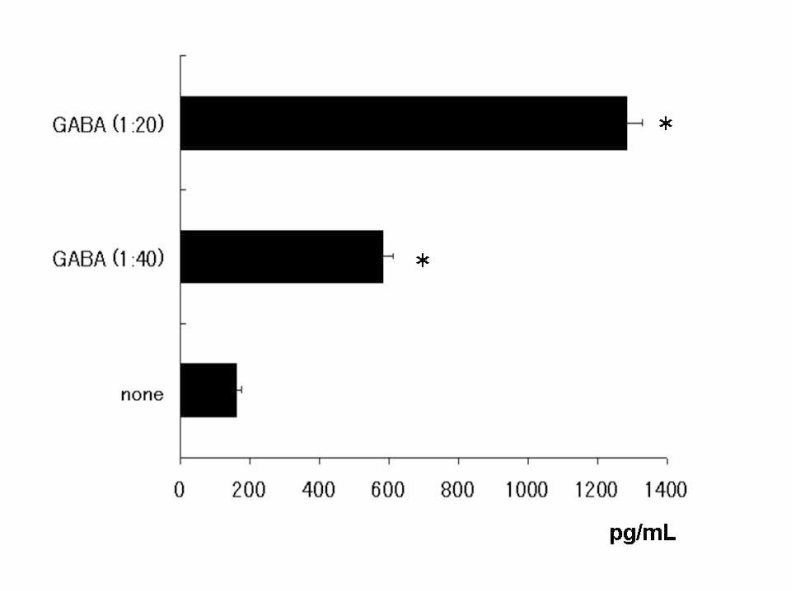

Figure 5. Enhancement of IL-8 production in U937 cells cultured with *Taimatsu* fermented rice germ GABA. IL-8 was detected in the culture supernatants from U937 cells cultured with or without *Taimatsu* fermented rice germ GABA (1:40 or 1:20) using an EIA kit. The experiments were repeated three times. *P<0.01, none vs. *Taimatsu* fermented rice germ GABA (1:40 or 1.20, respectively).

たいまつ米胚芽発酵GABAによるヒト単球系細胞株(U937)表面上のCD93の発現およびインターロイキン-８(IL-8)の産生増強

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要旨

たいまつ米胚芽発酵GABAによる単球系細胞株(U937)表面上のCD93抗原の発現をモノクローナル抗体を用いた酵素抗体法およびフローサイトメトリー法で解析した。その結果、たいまつ米胚芽発酵GABAで培養したU937細胞はCD93(C1qRp)の発現が有意に増強した。また、CD93の発現増強はprotein kinase C (PKC)阻害剤であるGo6976で抑制された。さらに、たいまつ米胚芽発酵GABAで培養したU937細胞はインターロイキン-８(IL-8)の産生も有意に増強した。以上の結果から、たいまつ米胚芽発酵GABAにはヒトの免疫応答を増強する作用があることがわかった。

キーワード：CD93、インターロイキン-８(IL-8)、たいまつ米胚芽発酵GABA，U937細胞
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