Activation of J774.1 murine macrophages by lactate dehydrogenase

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Abstract We have already reported that lactate dehydrogenase (LDH) activates lymphocytes in vitro and in vivo. In this paper, we report the activating effects of LDH on the macrophage-like cell line J774.1. LDH was found to enhance production of IL-6 and TNF- α by J774.1 cells in a dose-dependent manner. Transcription levels of IL-6 and TNF- α in J774.1 cells were also enhanced by supplementation with LDH. From immunoblot analysis, it was revealed that LDH enhances the phosphorylation level of JNK in J774.1 cells. Moreover, the JNK inhibitor SP600125 decreased production of IL-6 and TNF- α induced by LDH. NF- κ B translocation to the nucleus was also facilitated by LDH. These results were revealed that LDH enhances production of IL-6 and TNF- α by J774.1 cells via the increase of JNK phosphorylation

and NF- κ B translocation to the nucleus. Our data indicated that macrophages may be activated by LDH released from damaged tissues and cells in our body.

Keywords Lactate dehydrogenase · Cytokine · JNK · Macrophage · J774.1 cells

Abbreviations

AP-1	Activator protein-1
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HRP	Horseradish peroxidase
Ig	Immunoglobulin
I κ B	Inhibitor of NF- κ B
IL	Interleukin
JNK	C-Jun NH ₂ -terminal kinase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NaPB	Sodium phosphate buffer
NF- κ B	Nuclear factor- κ B
SD	Standard deviation
TBS-T	Tris-buffered saline with 0.1 % Tween 20
TLR	Toll-like receptor
TNF	Tumor necrosis factor

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Introduction

Bulbus arteriosus is an elasticity tissue located in the exit of the ventricle. Venous blood enters to the atrium

and is pushed to gills following the ventricle. At this time, bulbus arteriosus helps blood flow smoothly. We found that a water-soluble extract from bulbus arteriosus of tuna enhances immunoglobulin (Ig) production by the HB4C5 cells. As for the tuna bulbus arteriosus extract, lactate dehydrogenase (LDH) (EC 1.1.1.27), triosephosphate isomerase (EC 5.3.1.1), enolase (EC 4.2.1.11), and hemoglobin were identified as the active substances (Daifuku et al. 2012a). Because LDH showed the strongest Ig production-stimulating activity among these substances on primary spleen lymphocytes from mice, we focused on this enzyme. LDH is known to play a crucial role in maintaining aerobic metabolism by converting lactate to pyruvate in muscle and to exist ubiquitously not only in fish but also in all vertebrate organisms. We previously evaluated that the immunostimulatory effect of LDH is a novel function, which is independent of the enzymatic activity of the enzyme (Sugahara et al. 1998; Takenouchi and Sugahara 2002). In addition, oral administration of LDH increased the IgA level in serum from BALB/c mice (Daifuku et al. 2012b). Moreover, production of IgA and IgG by lymphocytes from the Peyer's patches and production of IgA, interleukin (IL)-4, IL-5, IL-10, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α by splenocytes was increased in the LDH-administered group. It was shown that LDH stimulates production of Igs and cytokines by lymphocytes in vivo.

Macrophages are phagocytic cells differentiated from monocytes in tissues and known as typical effector cells in innate immunity. Macrophages mainly play two crucial roles in the immune response. One is to exclude foreign bodies by phagocytosis, and the other is to present foreign bodies fragmented by phagocytosis to helper T cells as an antigen to connect between the innate and adaptive immune systems (Masuda et al. 2006). We previously reported that collagens from jellyfish and bovine Achilles' tendon activate cytokine production by the murine macrophage-like cell line J774.1 cells (Putra et al. 2012). Several plant extracts have also been reported to activate macrophages. For examples, an extract from *Astragalus membranaceus* increased cell migration and release of the immune response mediators such as TNF- α and granulocyte macrophage colony-stimulating factor by a murine macrophage cell line (Qin et al. 2012). An aqueous extract from *Phyllanthus niruri* enhanced phagocytosis, the lysosomal enzyme

activity, and TNF- α release by bone marrow-derived macrophages (Nworu et al. 2010). It was also reported that an endotoxin-free *Echinacea* extract stimulated production of IL-6, TNF- α , IL-12, and nitric oxide by toll-like receptor (TLR) 4-dependent and TLR4-independent mechanisms in murine peritoneal macrophages (Sullivan et al. 2008). These reports concluded that these plant extracts that activate macrophages may enhance both the humoral and cellular immune responses to promote the host defense. In this study, we focused on the effect of LDH on macrophages using J774.1 cells as model. We herein report that LDH has an ability to activate macrophages.

Materials and methods

Reagents

Lactate dehydrogenases from rabbit muscle was purchased from Oriental Yeast (Tokyo, Japan). A JNK inhibitor SP600125 was obtained from Enzo Life Sciences (Farmingdale, NY, USA). A TLR4 inhibitor VIPER was purchased from Imgenex (San Diego, USA). RPMI-1640 medium, penicillin, and streptomycin were products of Sigma (St. Louis, MO, USA). Aprotinin, Pefabloc SC, and complete EDTA-free protease inhibitor cocktail were purchased from Roche Diagnostics (Mannheim, Germany). Goat anti-actin antibody (sc-1616) and horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p38 (#9212), phosphorylated p38 (#4511), JNK (#9258), phosphorylated JNK (#4668), and NF- κ B p65 (#4764) and HRP-labeled anti-rabbit IgG antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cells and cell culture

The macrophage-like cell line J774.1 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin at 37 °C under humidified 5 % CO₂-95 % air.

Effect of LDH on cytokine production by J774.1 cells

J774.1 cells were cultured in 10 % FBS-RPMI-1640 medium containing various concentrations of LDH or 10 mM sodium phosphate buffer (NaPB) as control in culture plates at 37 °C. To examine the effect of inhibition of the JNK or TLR4 signaling pathway on LDH-induced cytokine production, various concentrations of the JNK inhibitor SP600125 dissolved in dimethyl sulfoxide or the TLR4 inhibitor VIPER dissolved in distilled water were added to the culture medium, respectively, before treating with LDH. Detailed procedures are described in the “Figure legends”. Then, the concentrations of IL-6 and TNF- α secreted into the culture medium were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits from eBioscience (San Diego, CA, USA). All assays were done in triplicate.

Immunoblot analysis

Whole cell lysates were prepared using a lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na₄P₂O₇, 2 μ g/mL of aprotinin, 5 mg/mL of Pefabloc SC, complete EDTA-free protease inhibitor cocktail, and Phosphatase Inhibitor Cocktail (Nakalai Tesque, Kyoto, Japan). Nuclear and cytosolic proteins were extracted using CellLytic NuCLEAR Extraction Kit (Sigma). Proteins in cell lysates were separated by a polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, Little Chalfont, UK). The membrane was incubated in 5 % skim milk for 1 h at room temperature. After washing with Tris-buffered saline with 0.1 % Tween 20 (TBS-T), the membrane was incubated with a primary antibody in 5 % bovine serum albumin-TBS-T at 4 °C overnight. After washing with TBS-T, the membrane was incubated with HRP-labeled anti-rabbit IgG antibody or HRP-labeled anti-goat IgG antibody in 5 % skim milk-PBS-T at room temperature for 1 h. After washing with TBS-T, blots were developed by ECL Western Blotting Detection Reagent (GE Healthcare) or ImmunoStar LD (Wako Pure Chemical Industries, Osaka, Japan). Bands were visualized using ChemiDoc XRS Plus apparatus (Bio-Rad Laboratories, Hercules, CA, USA), and the chemiluminescent intensity was

quantified using the Quantity One software (Bio-Rad Laboratories).

Statistical analysis

Data obtained were expressed as the mean $\pm$ standard deviation. One way ANOVA followed by Tukey's test was used to assess the statistical significance of the difference against control. Each value of * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ is considered to be statistically significant.

Results

Effect of LDH on cytokine production by J774.1 cells

The effect of LDH on the production of IL-6 and TNF- α by the macrophage-like cell line J774.1 was first examined. IL-6 and TNF- α are main cytokines produced by activated macrophages and related to activation of both the innate and adaptive immune systems (Murray and Wynn 2011). J774.1 cells were cultured in 10 % FBS-RPMI-1640 medium supplemented with LDH, and the concentration of cytokines secreted into culture medium was measured by ELISA. LDH was found to enhance production of IL-6 and TNF- α by J774.1 cells in a dose-dependent manner (Fig. 1). Production of IL-6 and TNF- α was increased 132-fold and 10-fold at 2.0 mg/mL of LDH, respectively. The increased levels of mRNAs for IL-6 and TNF- α were also confirmed in J774.1 cells by real-time RT-PCR (data not shown).

Effect of LDH on the signaling pathways in J774.1 cells

Immunoblot analysis was next conducted to examine the mechanism to activate J774.1 cells. Mitogen-activated protein kinases (MAPKs) such as JNK and p38 are related to activation of macrophages. JNK and p38 are activated by phosphorylation and enhance the activity of transcription factor activator protein-1 (AP-1), leading to increased transcriptional levels of cytokines in macrophages (Hommes et al. 2003). As shown in Fig. 2, the phosphorylation level of p38 in J774.1 cells was not affected by LDH. On the other hand, the phosphorylation level of JNK was enhanced

Fig. 1 Effect of LDH on production of IL-6 and TNF- α by J774.1 cells. J774.1 cells suspended in 10 % FBS-RPMI-1640 medium supplemented with various concentrations of LDH were inoculated at 5.0×10^5 cells/mL into a 24-well culture plate and cultured for 6 h at 37 °C. The amounts of IL-6 and TNF- α in each culture medium was then measured by ELISA. Control (Black circle) was treated with 10 mM NaPB instead of LDH. Results are expressed as the mean $\pm$ SD of three independent measurements

Fig. 2 Effect of LDH on the phosphorylation level of p38 and JNK in J774.1 cells. J774.1 cells suspended in 10 % FBS-RPMI-1640 medium supplemented with various concentrations of LDH were inoculated at 4×10^5 cells/mL into a 24-well culture plate and cultured for 1 h at 37 °C. Control was treated with 10 mM NaPB instead of LDH. The cells were then lysed and subjected to immunoblotting. A representative image is from two independent experiments

in a dose-dependent manner by LDH. To confirm whether activation of macrophages by LDH is related to the increased phosphorylation level of JNK, a JNK inhibitor SP600125 was used to examine the effect of LDH on induction of IL-6 and TNF- α production. The results showed that the JNK inhibitor decreased LDH-induced production of IL-6 and TNF- α by J774.1 cells

in a dose-dependent manner (Fig. 3). From these data, it was suggested that LDH would enhance JNK phosphorylation and then increase cytokine production.

NF- κ B also plays an important role related to macrophage activation (Nishikori 2005). NF- κ B is usually bound to the inhibitor of NF- κ B (I κ B) and locates in the cytosol; however, NF- κ B translocates to the nucleus to induce expression of IL-6 and TNF- α genes once I κ B is phosphorylated by the I κ B kinase complex and ubiquitinated to induce degradation of I κ B. The translocation of NF- κ B into the nucleus was found to increase by LDH (Fig. 4), suggesting that LDH activates J774.1 cells by increasing translocation of NF- κ B into the nucleus.

Effect of TLR4 inhibitor on cytokine production stimulated by LDH

To examine whether LDH works on TLR4 to activate J774.1 cells, a TLR4 inhibitor VIPER was used. As shown in Fig. 5, VIPER affected production of neither IL-6 nor TNF- α stimulated by LDH. In addition, the effect of VIPER on JNK phosphorylation induced by LDH was examined. Although lipopolysaccharide (LPS) enhanced JNK phosphorylation in J774.1 cells, LPS-induced phosphorylation of JNK was canceled by treating with VIPER (Fig. 6). By contrast, VIPER did not affect LDH-induced JNK phosphorylation in J774.1 cells. From these data, it was suggested that LDH works on a receptor other than TLR4 or a signaling factor.

Discussion

In this study, the immunoregulatory activity of LDH was evaluated using a commercially available, purified enzyme from rabbits. The nucleotide sequences encoding LDH are highly conserved between fishes and mammals (Tsuji et al. 1994). In fact, the homology of primary structures of LDH is fairly close to each other between *Oryctolagus cuniculus* and *Danio rerio* (identities = 75 %, similarities = 89 %). In addition, rabbit LDH exerts the immunostimulatory activity on lymphocytes as well as tuna LDH (Daifuku et al. 2012a). Thus, LDH from rabbits was used for the experiments described below.

Lactate dehydrogenase was found to enhance production of IL-6 and TNF- α by J774.1 cells. LDH

Fig. 3 Effect of JNK inhibitor on production of IL-6 and TNF- α by J774.1 cells induced by LDH. J774.1 cells suspended in 10 % FBS-RPMI-1640 medium supplemented with or without various concentration of SP600125 in the presence or absence of 2.0 mg/mL of LDH were inoculated at 4.0×10^5 cells/mL into

a 48-well culture plate and cultured for 3 h at 37 °C. The amounts of IL-6 and TNF- α in each culture medium were then measured by ELISA. Results are expressed as the mean $\pm$ SD of three independent measurements

Fig. 4 Effect of LDH on NF- κ B translocation in J774.1 cells. J774.1 cells were suspended in 10 % FBS-RPMI-1640 medium supplemented with various concentrations of LDH were inoculated at 4.0×10^5 cells/mL into a 24-well culture plate and cultured for 1 h at 37 °C. Control was treated with 10 mM NaPB instead of LDH. Both nuclear and cytosolic extracts were prepared and analyzed by immunoblotting. A representative image is from two independent experiments

also enhanced production of IL-6 and TNF- α by another macrophage cell line RAW264.7 cells (data not shown). Oral administration of LDH to BALB/c mice facilitated Ig and cytokine production by primary splenocytes in vivo (Daifuku et al. 2012b). LDH increased production of both Th1 cytokines such as IFN- γ and Th2 cytokines such as IL-4, IL-5, and IL-10 by primary spleen lymphocytes. Oral administration of LDH also increased TNF- α production that is mainly produced by monocytes such as macrophages, implying that LDH digested in the gastrointestinal tract still has an ability to stimulate macrophages. This

also indicated that macrophage activation is not caused by pyruvate, the metabolite produced by LDH. IL-6 production activity of splenocytes from mice was enhanced by oral administration of LDH. It was also confirmed that the immunomodulatory activity of LDH does not depend on its enzyme activity (Sugahara et al. 1998). Thus, it was thinkable that the protein structure of LDH stimulates lymphocytes (Takenouchi and Sugahara 2002). LDH ubiquitously and abundantly exists in most of the tissues in humans, especially in kidney, heart, skeletal muscle, pancreas, spleen, liver, and lung. Because cells do not actively secrete this enzyme, LDH concentration in blood is used as a marker of tissue damage, and release of LDH from damaged cells may enhance around tissues where cells have damages or cause inflammation. Because disrupted cells are removed by the phagocytes activated by detecting certain substances derived from the damaged cells, it is supposed that the phagocytes are stimulated by recognition of structure of LDH leaked from damaged cells, which might be present at a relatively high level in extracellular fluids. Macrophages are known as antigen-presented cells and induce both the innate immune response and the adaptive immune response by the release of several

Fig. 5 Effect of TLR4 inhibitor on production of IL-6 and TNF- α by J774.1 cells induced by LDH. J774.1 cells suspended in 10 % FBS-RPMI-1640 medium were inoculated at 4.0×10^5 cells/mL into a 24-well culture plate and pretreated with or without various concentrations of VIPER for 2 h at 37 °C. LDH

(2.0 mg/mL) was then added and cells were further cultured for 3 h. The amounts of IL-6 and TNF- α in each culture medium were measured by ELISA. Results are expressed as the mean $\pm$ SD of three independent measurements

factors such as cytokines (Murray and Wynn 2011). It was suggested that LDH might affect both lymphocytes and macrophages to enhance Ig and cytokine production by interacting each other in vivo.

Toll-like receptors recognize the pathogen-associated molecular pattern, a structural motif characteristically expressed by bacteria and viruses (Lu et al. 2008). TLRs are common upstream receptors of both of the MAPK and NF- κ B signaling pathways and expressed on cell membrane of monocytes such as macrophages. Among the TLR family, an effect of LDH on the TLR4 signaling pathway was examined. LPS, a ligand for TLR4, is a membrane component of gram-negative bacteria. Stimulation of TLR4 by LPS induces release of cytokines that are necessary to activate the potent immune response. The TLR4 inhibitor VIPER did not affect the production of IL-6 and TNF- α , even when macrophages were stimulated by LDH (Fig. 5). From these facts, it is suggested that LDH acts on a certain receptor on cell surface other than TLR4 or a signaling factor.

Immunoblot analysis showed that LDH enhances the phosphorylation level of JNK and translocation of NF- κ B to the nucleus in J774.1 cells. (Fig. 4) The JNK

Fig. 6 Effect of TLR4 inhibitor on the phosphorylation level of JNK in J774.1 cells. J774.1 cells suspended in 10 % FBS-RPMI-1640 medium were inoculated at 4.0×10^5 cells/mL into a 24-well culture plate and pretreated with 5 μ M VIPER for 2 h at 37 °C. LDH (2.0 mg/mL) or LPS (10 ng/mL) was next added and cells were further cultured for 1 h. The cells were then lysed and subjected to immunoblotting. A representative image is from two independent experiments

signaling pathway is essential for AP-1 activation caused by stress and exposure to various cytokines (Hommes et al. 2003). Two primary AP-1 transcription components phosphorylated by JNK are c-Jun and ATF-2. Activated JNK phosphorylates both c-Jun and ATF-2 and phosphorylated c-Jun and ATF-2 induce

transcription of downstream factors such as IL-6 and TNF- α . JNK has been reported to enhance c-Jun transcription (Turpaev 2006). Since LDH enhanced the phosphorylation level of JNK (Fig. 2), it was conceivable that activation of c-Jun and ATF2 might be also triggered. These data indicated that LDH itself is likely to provide a direct signal to cell surface receptor molecules, because activation of JNK and NF- κ B translocation normally result from the signal transduction from the cell surface receptors.

References

- Daifuku M, Nishi K, Okamoto T, Nakano H, Nishimoto S, Sugahara T (2012a) Immunostimulatory effects of water extract from *Bulbus arteriosus* in tuna in vitro. *J Funct Foods* 4:263–270
- Daifuku M, Nishi K, Okamoto T, Nishimoto S, Sugahara T (2012b) Immunomodulatory effects of lactate dehydrogenase in vitro and in vivo. *J Funct Foods* 4:972–978
- Hommes DW, Peppelenbosch MP, Van Deventer SJ (2003) Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* 52:144–151
- Lu YC, Yeh WC, Ohashi PS (2008) LPS/TLR4 signal transduction pathway. *Cytokine* 42:145–151
- Masuda Y, Kodama N, Nanba H (2006) Macrophage J774.1 cell is activated by MZ-Fraction (Klasma-MZ) polysaccharide in *Grifola frondosa*. *Mycoscience* 47:360–366
- Murray PJ, Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 11:723–737
- Nishikori M (2005) Classical and alternative NF- κ B activation pathways and their roles in lymphoid malignancies. *J Clin Exp Hematopathol* 45:15–24
- Nworu CS, Akah PA, Okoye FB, Proksch P, Esimone CO (2010) The effects of *Phyllanthus niruri* aqueous extract on the activation of murine lymphocytes and bone marrow-derived macrophages. *Immunol Invest* 39:245–267
- Putra ABN, Morishige H, Nishimoto S, Nishi K, Shiraishi R, Doi M, Sugahara T (2012) Effect of collagens from jellyfish and bovine Achilles' tendon on the activity of J774.1 and mouse peritoneal macrophage cells. *J Funct Foods* 4:504–512
- Qin Q, Niu J, Wang Z, Xu W, Qiao Z, Gu Y (2012) Astragalus membranaceus extract activates immune response in macrophages via heparanase. *Molecules* 17:7232–7240
- Sugahara T, Shimizu S, Abiru M, Matsuoka S, Sasaki T (1998) A novel function of enolase from rabbit muscle; an immunoglobulin production stimulating factor. *Biochim Biophys Acta* 1380:163–176
- Sullivan AM, Laba JG, Moore JA, Lee TD (2008) Echinacea-induced macrophage activation. *Immunopharmacol Immunotoxicol* 30:553–574
- Takenouchi S, Sugahara T (2002) Lactate dehydrogenase enhances immunoglobulin production by human hybridoma and human peripheral blood lymphocytes. *Cytotechnology* 42:133–143
- Tsuji S, Qureshi MA, Hou EW, Fitch WM, Li SS (1994) Evolutionary relationships of lactate dehydrogenases (LDHs) from mammals, birds, an amphibian, fish, barley, and bacteria: LDH cDNA sequences from *Xenopus*, pig, and rat. *Proc Natl Acad Sci USA* 91:9392–9396
- Turpaev KT (2006) Role of transcription factor AP-1 in integration of cellular signaling systems. *Mol Biol* 40:945–961