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AN IMMUNOMODULATORY PROTEIN, LING ZHI-8, FACILITATES CELLULAR INTERACTION THROUGH MODULATION OF ADHESION MOLECULES

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Ling Zhi-8 (LZ-8), a novel immunomodulatory protein, markedly enhanced the expression of CD11b, but not CD11a, CD13, CD14, CD18, CD33 or HLA-DR, on the U937 cell line in a dose-dependent fashion. It also induced ICAM-1 expression on vascular endothelial cells and significantly augmented γ-interferon-induced cellular binding between vascular endothelial cells and U937. Furthermore, LZ-8 increased the expression of CD2, but not VLA4, VLA5 or LFA3, on MOLT4 and enhanced rosette formation between human T cells and sheep red blood cells. These data suggest that LZ-8 exerts its pharmacological effect by modulating adhesion molecules on immunocompetent cells.

LZ-8, a polypeptide consisting of 110 amino acid residues, is isolated from the fungus Ling Zhi (Ganoderma lucidum) (1). It has a molecular mass of 12,420 Da and an isoelectric point of 4.4, showing considerable similarity to the immunoglobulin V_H region in its amino acid sequence (1,2). LZ-8 is capable of hemagglutinating sheep red blood cells and is mitogenic to murine spleen cells in vitro (1). In vivo, LZ-8 prevents systemic anaphylaxis in mice and the occurrence of insulitis in non-obese diabetic (NOD) mice (2, 3). However, the mode of action of LZ-8 remains to be clarified. We focused our

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attention on the hemagglutinating capacity and mitogenicity of LZ-8 and speculated that LZ-8 might exert its immunomodulatory effect by modulating adhesion molecules on immunocompetent cells. Here we report that LZ-8 facilitates cellular interaction through augmentation of adhesion molecules.

Materials and Methods

Cultures Human histiocytic lymphoma cell line U937, human promyelocytic leukemia cell line HL-60 and human T cell leukemia cell line MOLT4 were all cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cord veins within 12 h after delivery as previously reported by Jaffe et al. (4). The cells were grown to confluence in RPMI-1640 supplemented with 10% FCS, 30 μg/ml of endothelial cell growth supplemented (ECGS)(Collaborative Research, Belford, MA) and 10 U/ml of porcine mucosal heparin in gelatin-coated plastic dishes, and harvested (5).

Purification of LZ-8 LZ-8 was purifed from G. lucidum mycelia by gel filtration followed by ion-exchange chromatography as previously described, and LZ-8 of greater than 97% purity, as determined by Tricine-SDS polyacrylamide slab gel electrophoresis and Coomassie brilliant blue R staining (2), was used throughout this study.

Cytofluorometric analysis The cells were reacted with 10 μg/ml of various fluorescein-labeled monoclonal antibodies for 30 min at 4° C and analyzed by an Epics-Profile flow cytometer (Coulter Electronics, Hialeah, FL, USA) as previously reported (6). Monoclonal antibodies including Mol (CD11b), My4 (CD14), My7 (CD13), My9 (CD33), I3 (HLA-DR), T11 (CD2) and B6(CD23) were obtained from Coulter Immunology (Hialeah, FL). Anti-LFA-1α (CD11a), anti-LFA-1β (CD18) and anti-Leu44 (CD44) were obtained from Beckton-Dickinson Immunocytometry Systems (San Jose, CA) and anti-VLA4 (CDw49d), anti-VLA5 (CDw49e) and anti-LFA3 were from Immunotech S. A. (Marseille, France).

Cell-ELISA for measurement of ICAM-1 expression HUVECs were cultured with 200 pg/ml of recombinant interleukin 1β (Otsuka Co., Tokyo, Japan) or 10 IU/ml of recombinant γ-interferon (γ-IFN; Shionogi Pharmaceutical Co., Ohsaka, Japan) for 48 h on a gelatin-coated Petri dish in the presence or absence of LZ-8, and ten thousand cells were seeded into 96-well culture plates and fixed with 3% paraformaldehyde/8% saccarhose/phosphate buffered saline (PBS). Nonspecific binding was blocked by the addition of 2x blockace (Yukijirushi, Sapporo, Japan)/PBS and 5% goat serum/PBS for 1 h sequentially. Anti-ICAM-1(CD54) monoclonal antibody (British Bio-technology Ltd., Oxon, England) and alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Tago Inc., Burlingame, CA) were used as the first and second antibodies, followed by the addition of phosphatase substrate (Sigma Chemical Co., St. Louis, MO). The optical density of each well was determined by a microplate reader (Bio-rad Laboratories, Richmond, CA) at 405 nm.

Cellular binding assay HUVECs were stimulated with 10 IU of γ-IFN as described above and plated into 96-well culture plates. U937 cells were labeled with 10 μM 2',7'-bis(carboxyethyl)-5(6') carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM; Dojindo Laboratories, Kumatomoto, Japan) as previously reported (7), and the binding of U937 cells with HUVECs was determined after 1 h incubation by using an automated microplate fluorometer (Nihon Bunko, Tokyo, Japan). Rosette formation with sheep red blood cells (SRBCs) and peripheral blood mononuclear cells (PBMCs) was performed as previously described (8). In brief, a 1% (v/v) suspension of SRBCs was incubated with PBMCs or purified T cells for 1 h at 4° C in the presence or absence of LZ-8.

Statistical analysis Student's t-test was used to analyse the data.

Results

As shown in Fig. 1, 25 μg/ml of LZ-8 significantly increased the expression of CD11b, an α-chain of Mac-1, on U937 cells. This enhancement

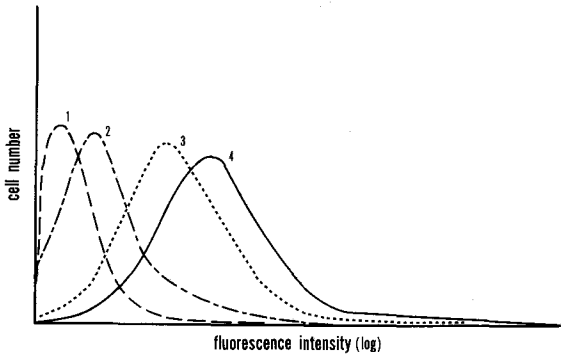

Fig. 1. Staining profiles of U937 cells with anti-CD11b monoclonal antibody in the presence or absence of LZ-8 (25 μg/ml). Curve 1: autofluoresence of U937 cells. Curve 2: the profile for irrelevant isotype-matched antibody in the presence or absence of LZ-8. Curve 3: the staining profile for anti-CD11b antibody in the absence of LZ-8. Curve 4: the profile for anti-CD11b antibody in the presence of LZ-8. An Epics-Profile flow cytometer was used to measure staining profiles of U937 cells.

reached maximum after 72 h incubation. CD11b expression on HL-60 and MOLT4 cells was also markedly induced by LZ-8 (data not shown). In contrast, the antigenic density of CD18, a β-chain of β_2 integrin, was not altered. In addition, no apparent change in the expression of CD11a, CD13, CD14, CD33 or HLA-DR was observed with LZ-8. The expression of ICAM-1 (CD54), a ligand of LFA-1 and Mac-1, on γ-IFN-treated HUVECs was also significantly augmented by LZ-8 in a dose-dependent manner (Fig. 2a). Furthermore, cellular binding between U937 and γ-IFN-stimulated HUVECs was markedly enhanced by LZ-8 in a dose-dependent fashion (Fig. 2b). Same mode of enhancement was also observed when γ-IFN-stimulated HUVECs was used (data not shown).

CD2 is universally expressed on human peripheral blood T cells and the receptor for LFA-3. LZ-8 strongly upregulated the expression of CD2, but not VLA4, VLA5 or LFA3 on both MOLT4 cells (Fig. 3a) and peripheral blood cells. Marked enhancement of rosette formation between human T cells and SRBCs was also observed; this enchancement was inhibited by anti-CD2 monoclonal antibody (Fig. 3b). Pretreatment of human T cells with LZ-8 resulted in a similar magnitude of augmentation in rosette formation, indicating that LZ-8 modifies CD2 expression on T cells but not LFA-3 expression on SRBCs. Finally, LZ-8 significantly increased cellular binding of MOLT4 cells with HUVECs (Fig. 4).

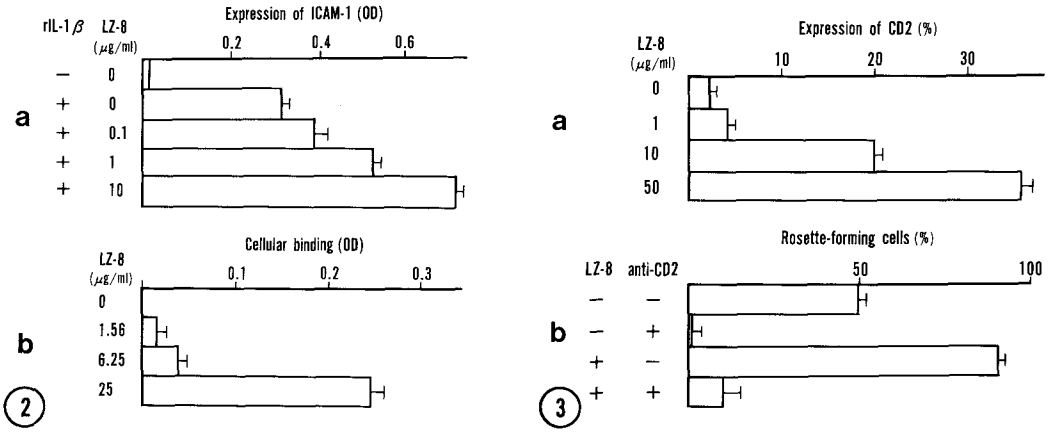

Fig. 2. (a) Induction of ICAM-1 on γ-IFN-stimulated human umbilical vascular endothelial cells (HUVECs). Expression of ICAM-1 on HUVECs was determined by cell-ELISA. (b) Augmentation of cellular binding between U937 cells and HUVECs by LZ-8. HUVECs were cocultured with BCECF-AM-labeled U937 cells in the presence or absence of LZ-8, and cellular binding was assessed with an automated microplate fluorometer. Each column is the mean of triplicate cultures and bars indicate standard errors.

Fig. 3. (a) Induction of CD2 on MOLT4 cells by LZ-8. MOLT4 cells were cultured in the presence or absence of LZ-8 for 72 h, and the expression of CD2 was determined by flow cytometry. (b) Increase in T-cell rosette-formation with SRBC as a result of LZ-8 (25 μg/ml) and its inhibition by anti-CD2 antibody (50 μg/ml). T cells forming rosettes with SRBC in the presence or absence of LZ-8 were enumerated after 1 h incubation at 4°C.

Discussion

LZ-8 is mitogenic toward both human and murine lymphocytes (1,2), and stimulates both IL-1 and IL-2 production with the same range of concentration as we used (unpublished data). It also has in vivo immunosuppressive activity

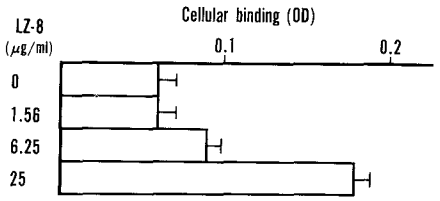

Fig. 4. Enhancement of cellular binding between MOLT4 and HUVECs by LZ-8. HUVECs were cocultured with fluorescein-labeled MOLT4 cells and cellular binding was measured with a microplate fluorometer as described in Materials and Methods.

such as the prevention of insulitis in NOD diabetic mice with an increased L3T4/Lyt-2+ ratio, suggesting that the suppressor pathway might be operated by LZ-8 (3). Recent studies show that multiple adhesion molecules are involved in cellular interactions in the immune system, where they act as receptors and ligands (8). It has been shown that blockade of CD11b/CD18 expression on macrophages prevents intra-islet infiltration by macrophages and T cells, and inhibits development of diabetes in NOD mice (9). We therefore examined the modulatory effect of LZ-8 on both the expression of adhesion molecules on macrophages, T cells and endothelial cells, and cellular interaction among them. Our data revealed that LZ-8 enhanced CD11b and ICAM-1 expression on U937 cells and HUVECs, respectively. In addition, LZ-8 augmented cellular binding between these cells, although multiple adhesion molecules other than CD11b and ICAM-1, e.g., those in the selectin family, might be involved in this interaction. Similarly, cellular binding between MOLT4 and HUVECs was also significantly upregulated. Furthermore, LZ-8 increased both CD2 expression on T cells and CD2-mediated rosette formation with SRBCs. Augmentation of rosetting was achieved in 1 h incubation with LZ-8, whereas the enhancement of CD2 expression took place after 48 h in flow cytometry, according to our preliminary data, suggesting that LZ-8 induces not only quantitative but also qualitative changes in CD2 expression. A similar finding has been reported in phorbol ester-induced LFA-1 activation, in which phorbol ester induced the avidity of LFA-1 to ICAM-1 without affecting its antigenic expression (9). LZ-8 may exert its immunomodulatory effect both in vitro and in vivo through quantitative and/or qualitative modulation of adhesion molecules, which in turn facilitates the cellular interaction that is defective in autoimmune diseases. We have not found any immunomodulating agents other than LZ-8 that have the capacity to modifiy the expression of adhesion molecules on immunocompetent cells. It is therefore possible that LZ-8 may be a new type of therapeutic agent for autoimmune diseases.

Acknowledgments

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