Expression of Recombinant Bovine L-Selectin in *Escherichia coli* and Insect Cells

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ABSTRACT. Bovine L-selectin was expressed in bacteria using pGEX vector and in insect cells infected with recombinant baculovirus in order to obtain recombinant protein for preparation of specific antiserum and its functional studies. In bacterial expression, L-selectin fusion protein with glutathione S-transferase was detected in the insoluble fraction with the expected molecular weight of 60 kDa by SDS-PAGE and reacted with anti-bovine CD62L monoclonal antibody in immunoblot analysis. In insect cells infected with recombinant baculovirus, a band corresponding to L-selectin was not observed in SDS-PAGE with protein staining, but they apparently reacted with anti-bovine CD62L monoclonal antibody in immunoblot analysis. Furthermore, the indirect immunofluorescence test revealed that bovine L-selectin was efficiently expressed on the surface of *Sf9* cells infected with recombinant baculovirus, and flow cytometric analysis showed that the percentage of CD62L positive cells in bovine PBMC was about 66 % and that most *Sf9* cells infected with recombinant baculovirus had specific immunofluorescence. —KEY WORDS: bovine, *Escherichia coli*, insect cell, L-selectin, recombinant baculovirus.

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L-selectin, known as a peripheral lymphocyte homing receptor, is a member of the selectin gene family of cell adhesion molecules and is expressed on leukocytes including neutrophils, monocytes, eosinophils, basophils and most lymphocytes [6, 8]. In rodents and humans, the specificity of lymphocyte homing is regulated by adhesive interactions of L-selectin on lymphocytes with vascular addressins on high-walled endothelial venules [4], and recent study has suggested that L-selectin is involved in the initial attachment of leukocytes to the endothelium [1, 7]. Further studies on cell surface antigens have revealed that detection of L-selectin and cluster of differentiation (CD) antigens such as CD45RA and/or CD45RO on CD4⁺ T cells makes it possible to identify the helper T cell subset in humans [9].

In bovine, the full length of L-selectin cDNA was cloned and sequenced, which was predicted to consist of lectin domain, epidermal growth factor domain, complement binding domain, transmembrane and cytoplasmic regions as reported in humans and rodents [2]. Although it was proposed that the functions of bovine L-selectin should be distinct from those of other species, there are few reports on the function and structure of bovine L-selectin and its distribution on the CD4⁺ T cell [2]. Therefore we attempted to produce bovine L-selectin by two different methods using *E. coli* and a baculovirus-insect cell system in order to obtain recombinant L-selectin for preparation of specific antiserum and its functional studies.

MATERIALS AND METHODS

Preparation of cDNA for bovine L-selectin: Bovine peripheral blood mononuclear cells (PBMC) were isolated and stimulated by concanavalin A (ConA). In brief, PBMC

were obtained after bovine peripheral blood was diluted with an equal volume of phosphate buffered saline without a divalent cation (PBS(-)) and centrifuged over NycobPrep 1.077 Animal (NYCOMED AS, Norway) at 800 g for 20 min at 4°C, and then stimulated by 5 µg/ml ConA for 24 hr at 37°C in a humidified 5% CO₂ atmosphere. The mRNAs were extracted by the QuickPrep *Micro* mRNA Purification Kit (Pharmacia, Sweden) as described by the manufacturer's instructions, and subjected to reverse transcriptional polymerase chain reaction (RT-PCR) in order to amplify cDNA for the L-selectin open reading frame (ORF) with or without signal peptide and transmembrane encoding regions. The primers were incorporated with *Bam*HI restriction sites at each terminus to facilitate subsequent DNA manipulations. The sequences of the primers were as follows: for recombinant baculovirus expression, 5'-GGATCCATGCTATGTCCATGGA-3' (forward primer) and 5'-GGATCCTCAACCATGCTTCTCCG-3' (reverse primer); for bacterial expression, 5'-GGATCCCCTGGAC TTACCATTAT-3' (forward primer) and 5'-GGATCCTC AGGGGTTATAATCACT-3' (reverse primer). Amplified cDNAs were cloned into pMOSBlue T-vector (Amersham, U.K.) and their sequences were verified by the dideoxynucleotide termination method.

L-selectin expression in *E. coli*: pGEX-5X-1 (Pharmacia, Sweden) which had *tac* (*trp-lac*) promoter and a glutathione S-transferase (GST) encoding region for making fusion protein was used as an expression vector. L-selectin cDNA with neither signal peptide nor transmembrane encoding region was released from T-vector by *Bam*HI digestion and subcloned into the *Bam*HI site of pGEX-5X-1 under the control of the *tac* promoter. *E. coli* strains containing the correct plasmids were identified, and GST fusion protein was expressed. In brief, 2xYT-G medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl and 20 g/l glucose) supplemented with 100 µg/ml ampicillin was inoculated with single colony of bacteria transformed with recombinant

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plasmid and incubated at 37°C as detailed by the manufacturer's instructions. Protein expression was induced by isopropyl- β -D-thiogalactoside (IPTG). After incubation, *E. coli* were sonicated in short bursts and the extracted proteins were respectively assessed by SDS-PAGE with coomassie brilliant blue (CBB) staining and immunoblot analysis using anti-bovine CD62L monoclonal antibody (Mab; VMRD, U.S.A.) as the primary antibody.

L-selectin expression in Sf9 cells: The cDNA for L-selectin ORF with signal peptide and transmembrane regions was subcloned into the *Bam*HI site of pAcYM1 under the control of the polyhedrin promoter. The pAcYM1 with L-selectin cDNA was co-transfected to Sf9 cells with linearized AcVAPK6 baculovirus DNA which contained an *E. coli* β -galactosidase (*lac Z*) gene in place of the polyhedrin gene of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) [5]. Briefly, the lipofectin mixture containing 8 μ l lipofectin and 4 μ l water was added dropwise to 12 μ l of the co-transfection mixture of 1 μ g of pAcYM1 with L-selectin cDNA and 50 ng of linearized DNA, and incubated for 15 min at room temperature. The co-transfection mixture was seeded into a 35 mm tissue culture dish containing 1.5×10^6 Sf9 cells in 1 ml of serum-free TC-100 and incubated for 1 hr at 28°C. One ml of TC-100 containing 10% FCS was then added to the dish, and the supernatant was harvested after 3 days incubation at 28°C. Recombinant baculovirus was selected and purified by plaque forming assay [3]. Plaques of white phenotype were obtained and purification was repeated until no contaminating blue plaques could be detected. For expression of L-selectin, 1.5×10^6 Sf9 cells were infected with purified recombinant baculovirus at a multiplicity of infection (MOI) of 10 and incubated for 3 days at 28°C unless otherwise indicated. Proteins were extracted in RIPA buffer by short sonication, and analyzed by SDS-PAGE with CBB staining and immunoblot methods.

Indirect immunofluorescence test: Sf9 cells infected with the recombinant baculovirus at an MOI of 10 were cultured for 48 hr, and then subjected to the indirect immunofluorescence test. Sf9 cells infected with or without baculovirus carrying no L-selectin cDNA as negative controls and fresh bovine PBMC obtained by discontinuous gradient centrifugation as described above were also used for this assay. After washing with PBS(-) containing 0.1% Na₃ 3 times, the cells were added with anti-bovine CD62L Mab as a primary antibody and incubated for 30 min at room temperature. Then, the cells were washed 3 times, added with rabbit anti-mouse IgG and IgM antibody conjugated with FITC as a secondary antibody (Biosource, U.S.A.), and incubated for 30 min at room temperature. After washing 3 times, the cells were examined under a fluorescence microscope and flow cytometric analysis was also performed using an EPICS® XL flow cytometer (Coulter, U.S.A.); fluorescence intensity was expressed on a logarithmic scale.

RESULTS

Expression of L-selectin in E. coli: The sequence of bovine L-selectin cDNA was compared with that reported by Bosworth *et al.* [2], and two bases were transited in the ORF without amino acid replacement (data not shown). Then, we attempted to express L-selectin as GST fusion protein in bacteria. GST protein was also expressed in bacteria introduced with only pGEX-5X-1. Figure 1A shows the result of SDS-PAGE analysis. The molecular weight (MW)s of GST and GST-L-selectin fusion protein were estimated as 26 kDa and 60 kDa as expected, respectively. The result in Fig. 1B shows the immunoblot analysis for bovine L-selectin. Although a non-specific reaction of anti-bovine CD62L Mab to GST was slightly observed, the bacterial fusion protein was apparently detected in the insoluble fraction of *E. coli* sonicate and was also faintly found in the soluble fraction.

Expression of L-selectin in insect cells: The result of SDS-PAGE analysis for L-selectin expressed in Sf9 cells is shown in Fig. 2A. Sf9 cells infected with or without AcVAPK6 baculovirus DNA were also analyzed as negative controls. The result showed that a band corresponding to the L-selectin was not observed on SDS-PAGE with coomassie brilliant blue staining. Figure 2B shows the result of immunoblot analysis for L-selectin in Sf9 cells. Although non-specific reactions were observed in the sonicates of Sf9 cells with and without AcVAPK6, the band for L-selectin was detected in the sonicate and culture supernatant of Sf9 cells with recombinant virus as indicated by the arrows. The immunoblot analysis also illustrated that L-selectin was slightly detected in the culture supernatant.

Indirect immunofluorescence test: The result of the indirect immunofluorescence test is shown in Fig. 3. Specific immunofluorescence was detected on the surface of bovine PBMC and Sf9 cells infected with recombinant baculovirus, whereas Sf9 cells and those with AcVAPK6 were not detected (data not shown). Flow cytometric analysis showed that the percentage of CD62L positive cells in bovine PBMC was about 66% and most Sf9 cells infected with the recombinant baculovirus had positive immunofluorescence (Fig. 4).

DISCUSSION

In this study, bovine L-selectin was expressed by two different methods using *E. coli* and a baculovirus-insect cell system in order to obtain recombinant L-selectin for preparation of specific antiserum and for further studies on its structure and function. The cloned cDNA for bovine L-selectin was sequenced and compared with the sequence data reported by Bosworth *et al.* [2]. Two bases in the cDNA sequence were transited, but were not changed in deduced amino acids. Therefore, the cDNAs were used for further experiments of expression. Although the transition might be caused by technical misreading during cDNA synthesis, these minor changes of cDNA sequence might be rather due to individual or breed differences.

Fig. 1. SDS-PAGE (A) and immunoblot (B) analysis of GST-L-selectin fusion protein. MW marker (lane 1), *E. coli* sonicate with only GST (lane 2), sediment (lane 3) and supernatant (lane 4) of *E. coli* sonicate with GST-L-selectin are shown. Arrows in lanes 2 and 3 indicate the bands for GST and GST-L-selectin fusion protein, respectively. The molecular weight of the fusion protein is estimated as about 60 kDa. Bacterial fusion protein is slightly recognized in the soluble fraction (B, lane 4).

Fig. 2. SDS-PAGE (A) and immunoblot (B) analysis of *Sf9* cells. MW marker (lane 1), sonicate (lane 2) and culture supernatant (lane 3) of *Sf9*, sonicate (lane 4) and culture supernatant (lane 5) of *Sf9* infected with AcVAPK6, sonicate (lane 6) and culture supernatant (lane 7) of *Sf9* infected with recombinant baculovirus are shown. The band corresponding to bovine L-selectin was not observed by coomassie brilliant blue staining. Arrows in lanes 6 and 7 (B) indicate the bands corresponding to bovine L-selectin.

Fig. 3. Indirect immunofluorescence test for bovine L-selectin. *Sf9* cells infected with recombinant baculovirus display significant fluorescence.

In bacterial expression, SDS-PAGE and immunoblot analysis showed that GST fusion protein with L-selectin was efficiently expressed in *E. coli*. The MW of the bacterial fusion protein was estimated as about 60 kDa by SDS-PAGE analysis. As the MW of GST itself is 26 kDa and the predicted MW of L-selectin without sugar chains is about 37 kDa, the GST-L-selectin was not glycosylated as seen in the bacterial expression of some glycoproteins. The fusion protein was recovered mostly in the insoluble fraction in spite of expression without the hydrophobic region as the signal peptide and transmembrane region. The different

Fig. 4. Flowcytometric analysis of L-selectin expression on fresh bovine PBMC treated with control (A) or anti-bovine CD62L Mab (B) and *Sf9* cells infected with the recombinant baculovirus treated with control (C) or anti-CD62L Mab (D). *Sf9* cells infected with or without control baculovirus showed the same pattern as negative control treatment (C).

solubility might be due to the different refolding and/or glycosylation processes between prokaryotic and eukaryotic cells. Immunoblot analysis using anti-CD62L Mab detected the GST-L-selectin fusion protein, suggesting that this GST gene fusion system was available for expression of bovine L-selectin in a short period.

In the insect cell expression system, the band for L-selectin synthesized in *Sf9* cells was not observed in SDS-PAGE analysis with protein staining. However immunoblot analysis using monoclonal anti-CD62L antibody detected the band corresponding to bovine L-selectin in the infected insect cells, and also slightly in the culture supernatant. Although recombinant L-selectin protein in this insect cell system was considered to be expressed at a low level, glycosylation and/or shedding from the cell surface might affect the faint band on SDS-PAGE with CBB staining. The MW of bovine L-selectin expressed in the insect cells was evaluated as about 50 kDa by immunoblot analysis. Since the predicted MW of L-selectin without sugar chains was estimated as about 37 kDa, recombinant L-selectin might be glycosylated in the insect cells. The immunoblot analysis also revealed that a small amount of L-selectin was detected in the culture supernatant, and it was thought that this glycoprotein was partly shed from the surface of *Sf9* cells into the supernatant as seen in human neutrophils [1]. To detect L-selectin in culture supernatant or sera will probably be of interest in clinical studies, especially in inflammation. Although anti-CD62L Mab might have faint cross-reactivity against structural protein in this analysis, this commercial antibody appears to be useful for structural and/or further analysis in combination with other monoclonal antibodies recognizing

different epitopes and/or polyclonal antisera. We are now investigating whether insoluble fusion protein in bacteria can be used as an antigen for preparation of antibody against L-selectin.

The indirect immunofluorescence test using monoclonal anti-bovine CD62L antibody was performed to observe whether bovine L-selectin was expressed on the surface of *Sf9* cells. Although specific immunofluorescence was not observed in *Sf9* cells infected with or without control baculovirus, *Sf9* cells infected with the recombinant baculovirus had specific fluorescence on the surface as seen in bovine PBMC. These results suggested that bovine L-selectin was efficiently expressed on the membrane of the insect cells infected with the recombinant virus. Since recombinant L-selectin was not only expressed on the cell membrane but also possibly detected in the culture supernatant, this expression system might be useful for a study on processing of bovine L-selectin molecules in eukaryotic cells.

As stated above, bovine L-selectin and GST-L-selectin fusion protein were respectively expressed in insect cells and bacteria. Preparation of murine antiserum against GST-L-selectin fusion protein is now in progress. We would like to attempt further experiments to research the functions of bovine T cells from the view point of both L-selectin and cytokine expressions and to assess the relations between CD4⁺ helper T cell subset and L-selectin expression.

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