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A novel splice variant of human L-selectin encodes a soluble molecule that is elevated in serum of patients with rheumatic diseases

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ABSTRACT

L-selectin, a type I membrane protein, is a leukocyte adhesion molecule that mediates both lymphocyte homing to peripheral lymph nodes and leukocyte accumulation at sites of inflammation. L-selectin is rapidly shed from the cell surface after cellular activation, and the ectodomain thus released is thought to account for high levels of soluble L-selectin in serum. In this study, we report the identification of a novel, naturally occurring isoform of the human L-selectin gene. Sequence analysis revealed that this isoform is generated by an alternative splicing event: the 7th exon of the human L-selectin gene, which encodes the region containing the transmembrane domain, is excluded, predicting a soluble protein product. The mRNA for this splice variant was expressed in lymphoid organs, where conventional L-selectin mRNA was also expressed. Activating T cells increased the variant mRNA and its ratio to the membrane form. Soluble L-selectin translated from the variant mRNA was present in human serum, albeit at a much lower level than that arising from ectodomain shedding, and was markedly elevated in patients with various rheumatic diseases, including rheumatoid arthritis and systemic lupus erythematosus. These observations indicate that some of the soluble L-selectin present in human serum arises through alternative splicing, which may be upregulated during lymphocyte activation in patients with various clinical conditions.

1. Introduction

Adhesion molecules play important roles in the cellular interactions involved in immune surveillance and inflammatory responses. A number of cell surface adhesion molecules have been detected as soluble forms in human serum and other body fluids. The most common mechanism for generating soluble forms is a proteolytic cleavage that releases the protein from the cell surface. L-selectin (CD62L), a member of the selectin family of adhesion molecules expressed on leukocytes, mediates both lymphocyte homing to peripheral lymph nodes and neutrophil recruitment into sites of inflammation [1,2]. Upon leukocyte activation with diverse stimuli, L-selectin is rapidly shed from the cell surface by proteolytic cleavage [3]. Shed L-selectin is present in high levels in human serum, and retains functional activity [4]. Another mechanism by which soluble adhesion molecules are generated is through alternative splicing. Numerous cell surface adhesion molecules have been found to undergo alternative splicing, which may change the adhesion and/or signaling properties of the proteins [5].

Aberrations in cell adhesive interactions have been reported in patients with rheumatic diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SjS), polymyositis/dermatomyositis (PM/DM), and systemic sclerosis (SSc). These are often associated with early vascular alterations and cellular infiltration of the target tissues [6]. Consistent with these findings, the serum levels of soluble forms of some adhesion molecules involved in leukocyte–endothelial interactions are elevated in patients with some of these diseases. Soluble L-selectin is elevated in the serum of SLE [7] and SSc patients [8], as well as in the cerebrospinal fluid of SLE patients [9].

We previously reported that mouse L-selectin has two variant forms, each generated by the insertion of a cassette exon, predicting molecules that differ in their carboxy-terminal tails [10]. Although we did not find human orthologs for these variants, we found a novel splice variant, termed hL-selectin-s. While conventional L-selectin (termed hL-selectin-c) mRNA encodes a membrane

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protein, this splice variant lacks the 7th exon, which encodes the region containing the transmembrane domain, and thus it is predicted to encode a soluble protein. Indeed, we found that the soluble L-selectin derived from this splice variant is present in human serum, albeit at a much lower concentration than that of the shed form. These results suggest that soluble L-selectin found in human serum arises not only from ectodomain shedding, but also from alternative splicing. In addition, we report here that the production of this soluble variant was upregulated upon lymphocyte activation in vitro and its serum levels were markedly elevated in patients with various rheumatic diseases.

2. Materials and methods

2.1. Reagents

The anti-human L-selectin monoclonal antibody (mAb) 4G8, which recognizes an epitope in the extracellular domain of human L-selectin, and the sheep anti-human L-selectin antibody (Ab) were purchased from R&D Systems (Minneapolis, MN). Antiserum to the carboxy terminus of hL-selectin-s (anti-Cterm) was generated by immunizing rabbits with the peptide (KKSKRSMNDPY) conjugated to keyhole limpet hemocyanin (KLH) via a cysteine residue added to the amino terminus of the peptide (Scrum Inc., Tokyo, Japan). Antiserum to shed L-selectin (anti-shed) was also generated using the carboxy-terminal peptide of shed L-selectin (QKLDK) conjugated to KLH via a cysteine residue added to the amino terminus of the peptide through a polyethylene glycol spacer. The antisera were affinity-purified using sepharose beads coupled to the peptide, and processed to $F(ab')_2$ fragments using the Pierce $F(ab')_2$ Preparation Kit (Thermo, Rockford, IL).

2.2. Cell culture

Human peripheral blood mononuclear cells from healthy donors were purified by centrifugation on Ficoll–Paque PLUS (GE Healthcare, Uppsala, Sweden) and T cells were isolated using the Pan T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were cultured in RPMI 1640 containing 10% FBS and stimulated with plate-bound anti-CD3 (UCHT1; BioLegend, San Diego, CA) and anti-CD28 (CD28.2; BioLegend) or 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, St. Louis, MO) for 3 days. For expansion of T cells, cells were activated with plate-bound anti-CD3 and anti-CD28 for 24 h, then transferred to uncoated plates, and split every 2–3 days by adding fresh media containing 4 ng/ml IL-2 (R&D Systems). COS-7 cells were maintained in DMEM containing 10% FBS.

2.3. Cloning of L-selectin isoforms

Reverse transcription (RT) was performed from human thymus RNA (Clontech, Mountain View, CA) using Superscript III and oligo-dT primers (both from Invitrogen, Carlsbad, CA). L-selectin cDNA was amplified by PCR using the 5′ primer (5′-TGCGGATCCTCAAGAGGCTCAATGGGCTGCAGAAG-3′) and the 3′ primer (5′-TACGAATTCTAATATGGGTCATTCATACTTCTCTTG-3′). The amplified products were digested with *Bam*HI and *Eco*RI, subcloned into the pcDNA3.1 vector (Invitrogen) at the *Bam*HI and *Eco*RI sites, and sequenced.

2.4. Quantitative RT-PCR

Total RNA from various human tissues was purchased from Ambion (Foster City, CA). Total RNA was also isolated from T cells using the RNeasy Micro Kit (Qiagen, Valencia, CA). RT was performed as described previously [11]. PCR was performed in a final volume of 20 μl containing the cDNA, 1x LightCycler 480 Probes Master (Roche), 500 nM of each primer (Applied Biosystems, Carlsbad, CA), and 200 nM TaqMan probe (Applied Biosystems) using a LightCycler 480 (Roche). The primer pairs and probes are listed in Table S1. Eukaryotic 18S rRNA was amplified as an internal control.

2.5. Western blotting analyses

COS-7 cells were transfected with the expression plasmids for L-selectin isoforms using Lipofectamine 2000 (Invitrogen). After 4 h, the medium was replaced with AIM-V (Invitrogen), and the cells and culture supernatants were harvested 3 days after transfection. Transfected COS-7 cells or their culture supernatants were lysed or diluted, respectively, in SDS sample buffer. The samples were electrophoresed under non-reducing or reducing conditions, and the proteins were transferred to a membrane as described previously [11]. The membranes were probed with sheep anti-L-selectin Ab, followed by HRP-conjugated anti-sheep IgG (Chemicon International, Temecula, CA), or rabbit anti-Cterm or anti-shed, followed by HRP-conjugated anti-rabbit IgG (American Qualex, San Clemente, CA). Non-reducing conditions were used when the membrane was probed with sheep anti-L-selectin Ab, as this Ab hardly detected bands under reducing conditions. For other Abs, reducing conditions were employed.

2.6. ELISA

Total soluble L-selectin levels were measured by DuoSet ELISA (R&D Systems). Briefly, 96-well microtiter plates were coated with the 4G8 mAb overnight. The plates were blocked, washed, and incubated with standards, serially diluted culture supernatants, or serum samples diluted 1:800. All samples were filtered through a 0.22-μm filter before adding to the plates. After washing, the plates were incubated with biotinylated sheep anti-L-selectin Ab, and then with streptavidin-HRP. The plates were developed with 3,3′,5,5′-tetramethylbenzidine. To determine the hL-selectin-s levels, $F(ab')_2$ fragments of anti-Cterm Ab were used as the capture antibody, and serum samples diluted 1:40 were used. To test the influence of rheumatoid factor (RF) on these assays, control RF (Sysmex, Kobe, Japan) was added to the test serum up to 100 units/ml, which did not affect the ELISA results.

2.7. Human subjects

Fifteen patients with SLE, 15 patients with RA, 15 patients with SjS, 10 patients with PM/DM, 10 patients with SSc, and 10 healthy controls were included. All SLE, RA, and SSc patients fulfilled the American College of Rheumatology criteria. PM/DM patients fulfilled the Bohan and Peter's criteria. Laboratory test results for C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and RF were obtained from 14 RA patients. This study was approved by the Ethics Committee of Kyoto University.

3. Results

3.1. Identification of a novel splice variant for human L-selectin

Our previous finding that mouse L-selectin has two splice variants that encode proteins with different carboxyl-terminal tails [10] prompted us to search for splice variants for human L-selectin. RT-PCR was performed from human thymus RNA using a 5′ primer located in the 5′-untranslated region and a 3′ primer located in the carboxyl end of the coding region of the known human L-selectin,

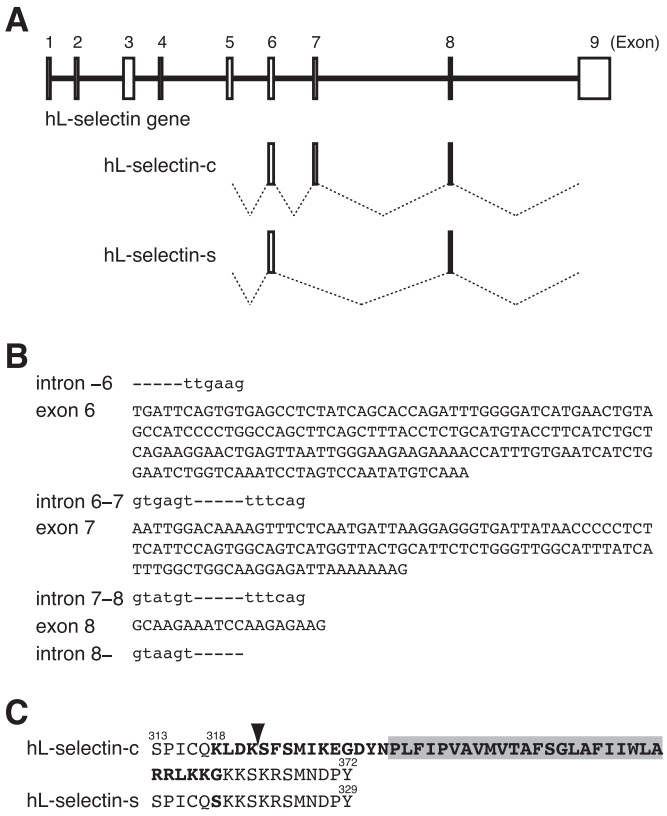

Fig. 1. Schematic representation of the human L-selectin gene and the two isoforms. (A) Genomic organization of the human L-selectin gene and the two mRNA transcripts. (B) Nucleotide sequences of exon 7 and its surrounding regions. (C) Amino-acid sequences of the carboxy-terminal regions of the two isoforms. The 44-amino acid sequence in hL-selectin-c (bold letters) is deleted and replaced with one new amino acid (bold letter) in hL-selectin-s. The transmembrane region in hL-selecin-c is shaded. The putative cleavage site is indicated by an arrowhead.

hL-selectin-c. Nucleotide sequence analysis of the cloned PCR fragments failed to detect human orthologs of the mouse splice variants, but unveiled the presence of a novel transcript, termed hL-selectin-s, which lacked the 129-bp sequence corresponding to the 7th exon of the human L-selectin gene[1] (Fig. 1A, B). The predicted protein lacked a 44-amino acid sequence (residues 318–361) of the hL-selectin-c protein, which contains the transmembrane domain, and instead had one amino acid (residue 318) followed by an 11-amino acid carboxy-terminal sequence shared by the two isoforms (Fig. 1C). A hydrophilicity plot of the predicted protein suggested that it would be a soluble protein.

3.2. Expression of the two L-selectin mRNA species in human tissues and cells

The expression of mRNA for the two human L-selectin isoforms was examined by quantitative RT-PCR. The 3′ primer for hL-selectin-c was located in exon 7, and that for hL-selectin-s was designed to span the junction of exons 6 and 8 (Fig. 2A, Table S1). Specificity was verified using the plasmids for each isoform as templates. hL-selectin-c was expressed at high levels in lymphoid organs including the spleen, thymus, and lymph nodes (Fig. 2B). The hL-selectin-s transcript was also expressed in these tissues. The mRNA level of hL-selectin-s relative to hL-selectin-c ranged from 0.4% to 2.4% depending on the tissue, being relatively high in the thymus and low in the spleen.

Lymphocyte activation is known to affect the pattern of alternative splicing [5]. When peripheral blood T cells were stimulated with PMA or anti-CD3 plus anti-CD28, the mRNA level of both hL-selectin-c and hL-selectin-s decreased on day 1 and then increased after 2 days (Fig. 2C). The mRNA ratio of hL-selectin-s to hL-selectin-c also increased, suggesting that lymphocyte activation may affect the balance of transcription of the two L-selectin mRNA species. Expansion of T cells with IL-2 most prominently increased the mRNA level of hL-selectin-s with a concomitant increase in the ratio of L-selectin-s to hL-selectin-c (Fig. 2D). These results suggest that the expression of the two L-selectin isoforms is dynamically regulated during lymphocyte activation.

3.3. The hL-selectin-s protein is secreted

The identification of hL-selectin-s suggests that part of the soluble L-selectin in human serum may originate from this splice variant. We transfected COS cells with the hL-selectin-c or hL-selectin-s construct, and the cell lysates and culture supernatants were analyzed by Western blotting using a polyclonal sheep Ab directed against the extracellular domain of L-selectin. A band of about 55 kD was detected under non-reducing conditions in the cell lysate and culture supernatant from COS cells transfected with hL-selectin-c, while bands of smaller size, around 45 kD and 53 kD, were detected in the cell lysate and culture supernatant, respectively, from COS cells transfected with hL-selectin-s (Fig. 3A). The detection of hL-selectin-c in the culture supernatant is consistent with the previous finding that COS cells constitutively shed L-selectin to release a soluble protein into the medium, in a manner similar to that of activated leukocytes [12].

It has been shown that membrane-bound L-selectin is cleaved between Lys^{321} and Ser^{322} [12], producing a soluble protein with a carboxy terminus different from that of hL-selectin-s (Fig. 1C). To distinguish the hL-selectin-s protein from the shed form, a rabbit antiserum (anti-Cterm) was raised against an 11-residue peptide corresponding to a region in the carboxy terminus of L-selectin-s. This region is shared by the hL-selectin-c protein, but is lost from the shed L-selectin. In addition, a rabbit antiserum against a 5-residue peptide corresponding to the carboxy terminus of shed L-selectin (anti-shed) was generated. The anti-Cterm Ab reacted with the hL-selectin-s protein but not the shed L-selectin in the COS cell supernatant, and it reacted with hL-selectin-c and hL-selectin-s in cell lysates (Fig. 3B). In contrast, the anti-shed Ab reacted with the shed protein released into the medium of hL-selectin-c-transfected COS cells, but not with the hL-selectin-s protein (Fig. 3C). These results indicate that the hL-selectin-s protein is secreted and specifically detected by the anti-Cterm Ab.

To determine the amount of hL-selectin-s, a sandwich ELISA was developed. A commercially available ELISA development system for quantifying the total soluble L-selectin uses the 4G8 mAb as a capture Ab, and biotinylated sheep Ab against the extracellular domain of L-selectin as a detection Ab. This ELISA system detected both shed L-selectin and hL-selectin-s (Fig. 3D). Replacing the 4G8 mAb with the anti-Cterm $F(ab')_2$ fragments specifically detected hL-selectin-s in the COS cell culture supernatants but did not detect shed L-selectin, while shed L-selectin was detected by using the anti-shed $F(ab')_2$ fragments (Fig. 3D). A typical standard curve of the hL-selectin-s assay using the anti-Cterm $F(ab')_2$ fragments as a capture Ab is shown in Fig. 3E. The spike and recovery for the assay, determined by adding recombinant hL-selectin-s to a sample, confirmed that the range of recovery was 95–100%.

[1] The nucleotide sequence reported here has been submitted to the DNA Data Bank of Japan under accession number AB583235.

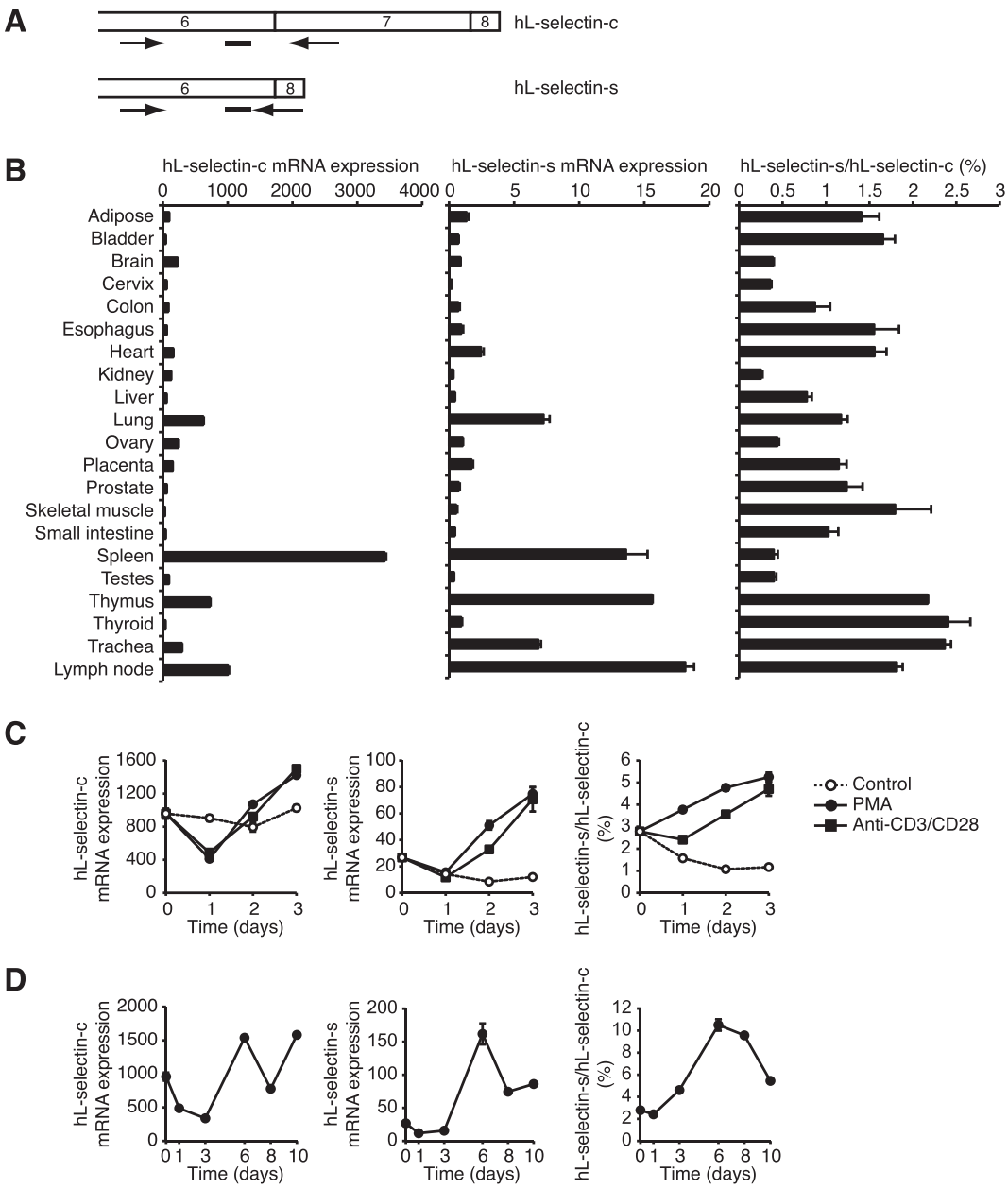

Fig. 2. Expression of the two mRNA transcripts of human L-selectin. (A) Schematic representation of the primers and probes used in quantitative RT-PCR. Primers and probes are shown as arrows and bold lines, respectively. (B) Expression of the two mRNA species and their ratio in various tissues. (C) Expression of the two mRNA species and their ratio in T cells activated with PMA or anti-CD3 plus anti-CD28. (D) Expression of the two mRNA species and their ratio in IL-2-expanded T cells. The results in B-D represent one of three independent experiments. The amount of each isoform was calculated using plasmid cDNA as a standard and normalized to 18S RNA. Expression of the two mRNAs is shown relative to the expression of hL-selectin-c mRNA in lymph nodes, which was set to 1000. The results are expressed as the mean ± SD of triplicate wells.

To examine whether lymphocytes could secrete the hL-selectin-s protein, the hL-selectin-s as well as total L-selectin in the culture supernatants of activated T cells was quantified using these ELISA systems. Both PMA and anti-CD3 plus anti-CD28 stimulation upregulated the total L-selectin and hL-selectin-s levels, and the highest amount was detected in the supernatants of IL-2-expanded T cells (Fig. 3F), in accordance with the increased mRNA expression of hL-selectin-s in these cells.

3.4. hL-selectin-s is upregulated in patients with various rheumatic diseases

Using the ELISA systems described above, the serum levels of total soluble L-selectin and hL-selectin-s were quantified in patients with various rheumatic diseases and in healthy controls. As shown in Fig. 4A, the total soluble L-selectin levels were higher in RA (1370 ± 411 ng/ml) than in controls (952 ± 148 ng/ml). Its levels in SLE (1220 ± 422 ng/ml), SjS (1120 ± 269 ng/ml), and PM/DM (1170 ± 320 ng/ml) also tended to be higher, although these increases did not reach statistical significance. In SSc, the total L-selectin level (946 ± 200 ng/ml) was comparable to that of healthy controls. In contrast to the rather modest increases in the total soluble L-selectin level, the hL-selectin-s level was markedly elevated in SLE (11.71 ± 7.78 ng/ml), RA (11.65 ± 5.21 ng/ml), SjS (8.50 ± 4.85 ng/ml), and PM/DM (9.54 ± 6.43 ng/ml) compared with healthy controls (2.06 ± 1.22 ng/ml) (Fig. 4B). Even in SSc, the hL-selectin-s level was significantly elevated (6.98 ± 2.89 ng/ml). These results

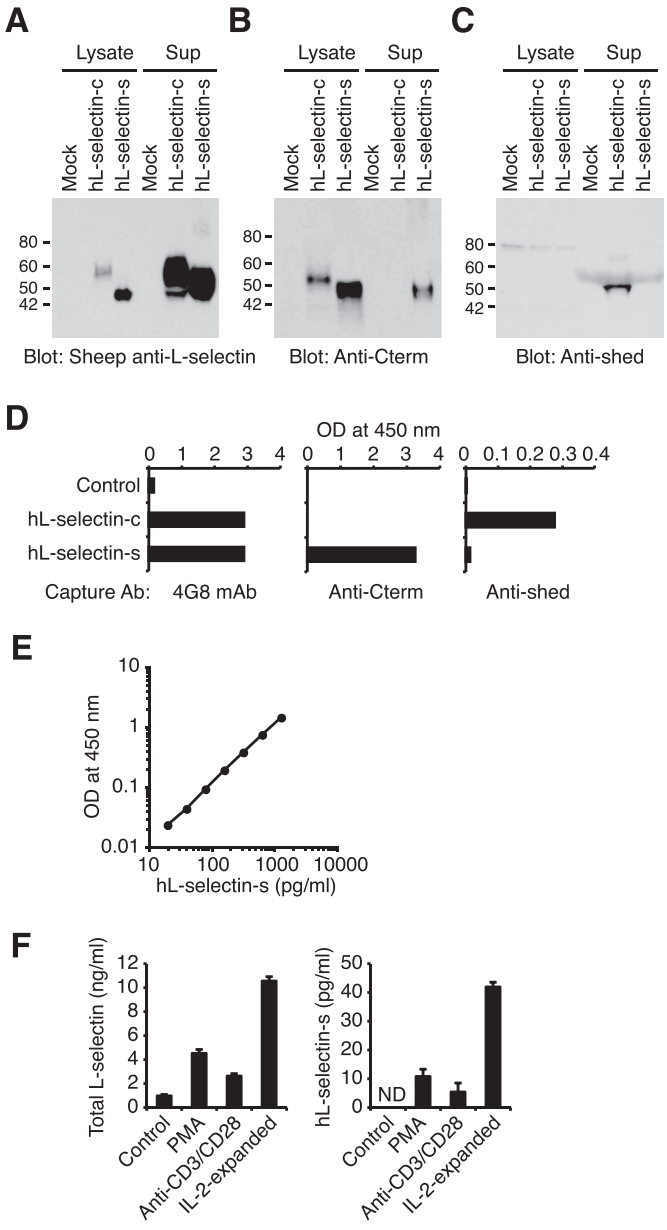

Fig. 3. hL-selectin-s is secreted. (A–C) Western blots of cell lysates and supernatants from COS cell transfectants. COS cell lysates and supernatants were analyzed by Western blotting with a sheep Ab to the extracellular domain of L-selectin (A), rabbit anti-Cterm Ab (B), or rabbit anti-shed Ab (C). The samples were electrophoresed under non-reducing conditions (A) or reducing conditions (B and C). (D) Specificity of the anti-Cterm Ab. The plates were coated with the 4G8 mAb, F(ab′)$_2$ fragments of anti-Cterm Ab, or F(ab′)$_2$ fragments of anti-shed Ab, and COS cell culture supernatants were added. Bound proteins were detected with biotinylated sheep anti-L-selectin Ab followed by HRP-conjugated streptavidin. (E) Standard curve of the ELISA used to quantify the hL-selectin-s protein. The recombinant hL-selectin-s protein expressed in COS cells was used as a standard. (F) Detection of total soluble L-selectin and hL-selectin-s in the culture supernatants of activated T cells. T cells were stimulated with or without PMA or anti-CD3 plus anti-CD28 for 3 days, or stimulated with anti-CD3 plus anti-CD28 for one day and expanded with IL-2 for an additional 5 days. The results are expressed as the mean ± SD of triplicate wells. The results in A-F represent one of three independent experiments.

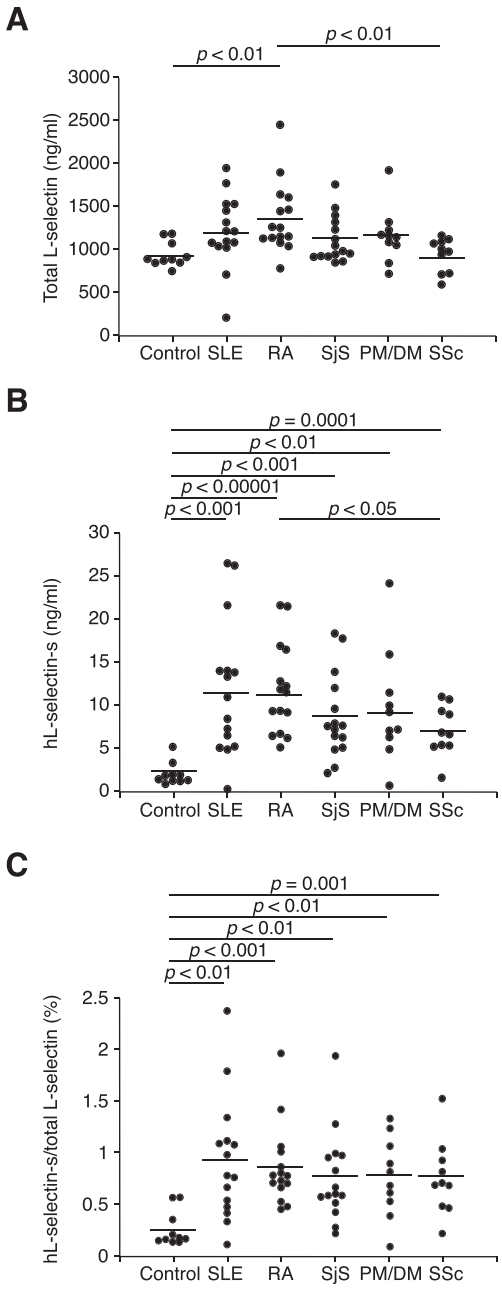

Fig. 4. Serum levels of total soluble L-selectin and hL-selectin-s in patients with various rheumatic diseases. Total soluble L-selectin (A) and hL-selectin-s (B) levels in serum were determined by ELISA. The percentage of hL-selectin-s in the total soluble L-selectin (C) is also shown. Each symbol represents the value for an individual subject and the mean of duplicate wells. Bars express the mean. *p* values correspond to independent *t*-tests between the groups indicated. The assay was repeated twice with similar results.

suggest that hL-selectin-s is a more sensitive marker for these diseases than total L-selectin.

To examine whether the increase in hL-selectin-s parallels that in the total L-selectin level, the correlation between total L-selectin level and hL-selectin-s level was calculated. There was some correlation in all groups, particularly in the RA and PM/DM groups (Table S2). However, the ratio of hL-selectin-s to total soluble L-selectin in serum was significantly elevated in all the disease groups compared to the control group (Fig. 4C), suggesting that the increase in hL-selectin-s is not merely due to the elevation of total L-selectin. In addition, in the RA group, RF titers significantly correlated with the hL-selectin-s level, but not with the total L-selectin level (Table S3), further suggesting that hL-selectin-s is a marker more significantly associated with the disease activity than total L-selectin.

4. Discussion

In this study, we identified a novel splice variant of the human L-selectin gene, termed hL-selectin-s, which was predicted to encode a soluble protein. The mRNA for this novel isoform was present in the cells and tissues that express hL-selectin-c. The soluble L-selectin arising from this variant was present in human serum, and was markedly upregulated in patients with various rheumatic diseases.

The novel splice variant is generated by the exclusion of the exon that contains the region encoding the transmembrane domain. Consequently, this variant was predicted to encode a soluble protein. This isoform is different from a previously reported human L-selectin isoform encoding a phospholipid-linked form of L-selectin [13]. Among the selectin family members, human P-selectin is reported to have two splice variants, one predicting a molecule containing eight short consensus repeat domains instead of the typical nine and the other predicting a soluble form [14,15]. The P-selectin variant predicting a soluble form is similar to the L-selectin variant identified here in that it is generated by the exclusion of the exon that encodes the region containing the transmembrane domain. This P-selectin isoform has been shown to be present in vivo as a circulating molecule [16].

Spliced variants for putative soluble isoforms have been reported for a number of adhesion molecules and membrane receptors [17,18]. It has been shown that alternative splicing events of numerous molecules show changes following cell activation [5]. The observation that activation of T cells changes the amount or balance of transcription of the two mRNA species may suggest that the expression of a novel variant is altered in situations or diseases in which leukocytes are activated. Indeed, we found that in various rheumatic diseases, the hL-selectin-s production was upregulated. These diseases are associated with the activation of T cells [19]. Although we observed that lymphocytes activated in vitro indeed secrete the hL-selectin-s protein, it has not yet been clarified which cells produce the hL-selectin-s protein under normal or pathological conditions in vivo.

The elevated levels of soluble adhesion molecules in the serum from patients with various inflammatory diseases may provide some useful diagnostic and prognostic information [20,21]. Serum L-selectin levels are reported to be elevated in a number of diseases and clinical conditions. We found that there was a correlation between the hL-selectin-s and total L-selectin levels in serum, but the ratio of hL-selectin-s to total L-selectin was elevated in all rheumatic diseases examined, rendering hL-selectin-s a more sensitive marker of inflammation in these diseases. In addition, we observed that the hL-selectin-s level in RA patients correlated with RF titers. RF is a well-recognized clinical marker for RA and higher titers are associated with a more severe disease [22]. A previous report showed that the levels of peripheral blood memory T cells correlate with RF titers, suggesting that T cell activation is associated with high RF titers [23]. Given that hL-selectin-s production is upregulated during lymphocyte activation, high serum hL-selectin-s levels in patients with rheumatic diseases may reflect lymphocyte activation in these patients.

In conclusion, our study shows for the first time a soluble splice variant of the human L-selectin gene. Its levels in serum are elevated in various rheumatic diseases, suggesting that it may serve as an inflammatory marker in combination with other markers. Further investigation will be required to clarify the mechanisms of the upregulated expression and the pathophysiological role of this isoform in various clinical conditions.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.002.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.002.

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