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Independence of tumor necrosis factor-α-induced adhesion molecule expression from sphingosine 1-phosphate signaling in vascular endothelial cells

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Adhesion of circulating leukocytes, especially monocytes, to activated endothelial cells, leading to the resultant transendothelial migration to the subendothelium, is an initial and important step in atherosclerosis [1]. Since this process is mediated by adhesion molecules, including E-selectin and vascular cell adhesion molecule (VCAM)-1, the regulation of expression of these molecules is related to the development of atherosclerosis [1]. The inappropriate expression of these adhesive proteins is induced by various inflammatory stimuli, of which the inflammatory cytokine tumor necrosis factor (TNF)-α is important [1,2]. Since sphingosine 1-phosphate (Sph-1-P) is abundantly stored in platelets and released extracellularly upon stimulation and since vascular endothelial cells express the G protein-coupled Sph-1-P receptors S1Ps, it is important to study the effect of Sph-1-P on endothelial cells from the viewpoint of platelet–endothelial cell interaction [3]. In this study, we examined the effect of Sph-1-P, in comparison with the inflammatory cytokines, on adhesion molecule expression; Sph-1-P was previously reported to mediate the response elicited by TNF-α [4].

In human umbilical vein endothelial cells (HUVECs), it was confirmed that TNF-α, as well as interleukin (IL)-1β, potently triggers the expression of E-selectin and VCAM-1 (Fig. 1A). Sph-1-P did induce adhesion molecule expression, but only very weakly (Fig. 1A). Similar results, i.e. strong responses induced by TNF-α and weak responses by Sph-1-P, were obtained when HUVEC plasminogen activator inhibitor-1 release was examined (data not shown). These findings are in contrast with the strong intracellular Ca^{2+} mobilization and migration responses induced by Sph-1-P, which have been established to be due to its extracellular actions via S1Ps [3,5]. Since NF-κB is an essential transcriptional factor for not only the expression of adhesion molecules but also that of PAI-1, it is likely that the ability of Sph-1-P to activate this transcriptional regulatory protein is weak. Our findings are consistent with the report that Sph-1-P fails to induce E-selectin expression or NF-κB activation [6], but not with one describing

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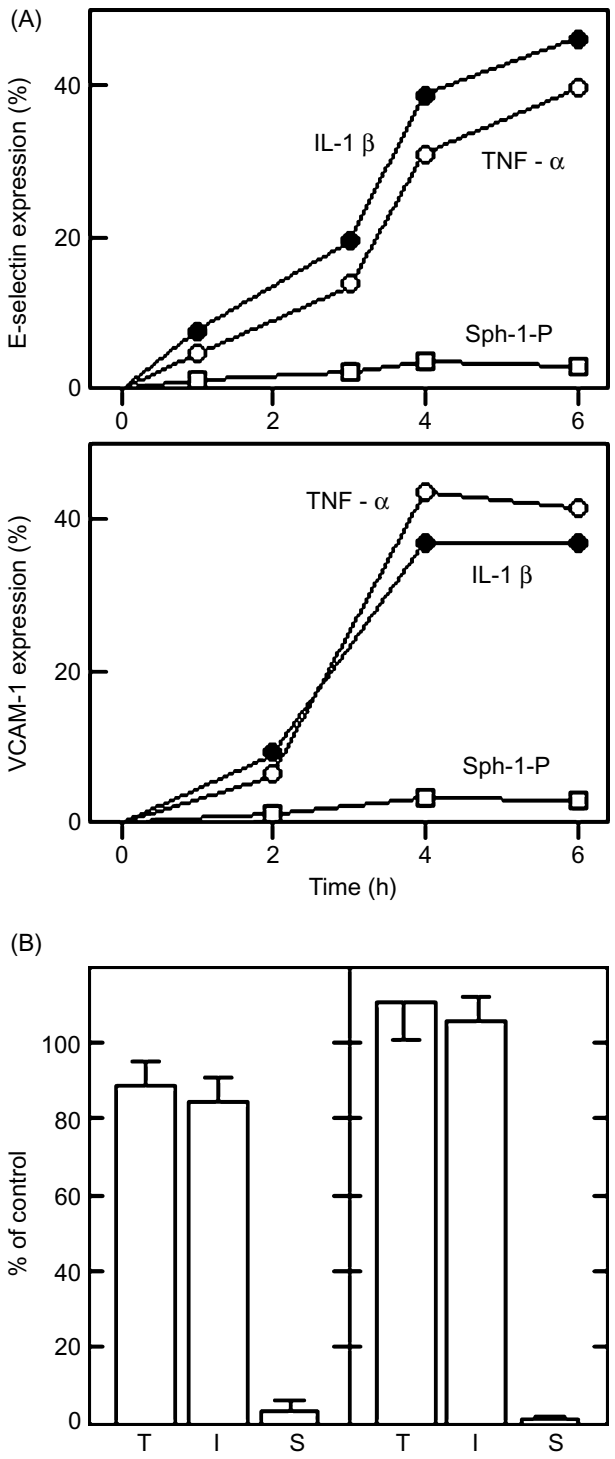

Fig. 1. Expression of E-selectin and vascular cell adhesion molecule (VCAM)-1 in human umbilical vein endothelial cells (HUVECs). To examine the expression of adhesion molecules, HUVECs, after the indicated reactions, were washed with PBS and incubated with anti-VCAM-1 (Berkeley Antibody Co., Richmond, CA, USA) or anti-E-selectin (Upstate Biotechnology, Lake Placid, NY, USA) monoclonal antibody for 30 min. Cells were then incubated with fluorescein isothiocyanate-conjugated goat antimouse IgG (Nippon Becton Dickinson, Tokyo, Japan) and fixed in 3% paraformaldehyde. The number of positive cells for the expression of cell-surface adhesion molecules was measured using a FACScan flow cytometer (Nippon Becton Dickinson), and expressed as a percentage of the total cell number. The stimulation-dependent expression was calculated as (positive cells upon stimulation) − (positive cells without stimulation). (A) HUVECs were stimulated with 100 U mL^{-1} of tumor necrosis factor (TNF)-α (○) or interleukin (IL)-1β (●), or 5 μM sphingosine 1-phosphate (Sph-1-P) (□), for various durations, and the cell surface expression of E-selectin (upper panel) or VCAM-1 (lower panel) was measured. (B) HUVECs were pretreated with 100 ng mL^{-1} of pertussis toxin for 16 h, and then stimulated with 100 U mL^{-1} of TNF-α (T) or IL-1β (I), or 5 μM Sph-1-P (S), for 4 h. Cell surface expression of E-selectin (left panel) or VCAM-1 (right panel) was measured. The results are expressed as a percentage of the control (without the toxin pretreatment). Columns and error bars represent the mean ± SD ($n = 3$).

that Sph-1-P can mimic the effect of TNF-α leading to the adhesive responses [4].

Sph-1-P has been shown to act not only as an extracellular mediator but also as an intracellular second messenger. Both types of Sph-1-P actions can even be observed within one system, and the HUVEC has been considered as one such example; Sph-1-P induces migration, proliferation, angiogenesis, and nitric oxide formation through the cell surface receptors $S1P_1$ and $S1P_3$ [3,5], while the Sph kinase pathway through the generation of intracellular Sph-1-P is critically involved in mediating TNF-α-induced endothelial activation [4]. Xia *et al*. reported that: (i) TNF-α induces a rapid Sph kinase activation and the resultant Sph-1-P generation; (ii) Sph-1-P, as well as TNF-α, potently stimulates NF-κB activation and adhesion protein expression; and (iii) the Sph kinase inhibitor dimethylsphingosine inhibits the TNF-α-induced responses [4]. Thus, it was postulated that intracellular Sph-1-P serves as a second messenger in mediating TNF-α-induced adhesion molecule expression [4]. However, it should be kept in mind that dimethylsphingosine inhibits not only Sph kinase but also protein kinase C and MAP kinase, and the data obtained using this non-specific inhibitor should be interpreted with caution [7].

In this study, we observed that: (i) the stimulatory effect of Sph-1-P on HUVEC adhesion molecule expression is much weaker than that of TNF-α (Fig. 1A); (ii) the Sph-1-P (but not TNF-α) response was abolished by pertussis toxin (Fig. 1B); and (iii) Sph conversion into Sph-1-P, reflecting Sph kinase activation, was not affected by TNF-α (data not shown). These present results suggest that Sph-1-P induction of adhesion molecule expression, although weak, is mediated by cell surface receptors, possibly one or more of S1Ps, and argue against the second messenger role of Sph-1-P. Further studies are needed to clarify the mechanism by which TNF-α induces adhesion molecule expression in vascular endothelial cells.

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More on: unusual expression of the F9 gene in peripheral lymphocytes hinders investigation of F9 mRNA in hemophilia B patients

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See also Green PM, Rowley G, Giannelli F. Unusual expression of the *F9* gene in peripheral lymphocytes hinders investigation of *F9* mRNA in hemophilia B patients. *J Thromb Haemost* 2003; **1**: 2675–6.

We read with interest the recent publication in this journal by Green *et al.*: 'Unusual expression of the F9 gene in peripheral lymphocytes hinders investigation of F9 mRNA in hemophilia B patients' [1]. The use of RNA from peripheral lymphocytes to examine exons g-h has been documented over many years [2,3]. Publication of data covering the remainder of the coding region of this eight-exon gene has, however, not been forthcoming.

Green *et al.* postulate that an incomplete transcript is produced in peripheral lymphocytes and other tissues, consisting only of exons g-h. Their results support our own findings, that F9 mRNA in lymphocytes is abnormal.

Of 54 hemophilia B families referred to this center for analysis, we have failed to identify a mutation in the genomic DNA of five index cases. RNA from whole blood from three of these five was extracted. In each case we successfully reverse transcribed RNA into cDNA and sequenced exons g-h. In 2/3 cases we have also been able to amplify and sequence exons a-b. We have been unable to obtain amplification products specific to exons c-f from patient or control lymphocyte RNA.

These results indicate that abnormal transcripts are produced in peripheral lymphocytes, but that these are not comprised exclusively of exons g-h, as suggested by the previous authors. While it may be that discrete transcripts are produced from the 5′ and 3′ ends of the gene, it is possible that partially processed transcripts are being produced, comprising exons a, b, g and h.

It is unlikely that a full-length F9 transcript will be produced from peripheral lymphocytes. It is not feasible, in patients with hemophilia B, to obtain RNA from the tissues successfully utilized by Green *et al.* Thus, in cases where no genetic abnormality can be identified by exon–exon sequencing, the only remaining option is analysis of the intronic regions.

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