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CD72 polymorphisms associated with alternative splicing modify susceptibility to human systemic lupus erythematosus through epistatic interaction with *FCGR2B*

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We previously reported association of *FCGR2B*-Ile232Thr with systemic lupus erythematosus (SLE) in three Asian populations. Because polymorphism of *CD72*, another inhibitory receptor of B cells, was associated with murine SLE, we identified human *CD72* polymorphisms, tested their association with SLE and examined genetic interaction with *FCGR2B* in the Japanese (160 SLE, 277 controls), Thais (87 SLE, 187 controls) and Caucasians (94 families containing SLE members). Four polymorphisms and six rare variations were detected. The former constituted two major haplotypes that contained one or two repeats of 13 nucleotides in intron 8 (designated as *1 and *2, respectively). Although association with susceptibility to SLE was not detected, the *1 allele was significantly associated with nephritis among the Japanese patients ($P = 0.024$). RT–PCR identified a novel alternatively spliced (AS) transcript that was expressed at the protein level in COS-7 transfectants. The ratio of AS/common isoforms was strikingly increased in individuals with *2/*2 genotype when compared with *1/*1 ($P = 0.000038$) or *1/*2 ($P = 0.0085$) genotypes. Using the two Asian cohorts, significant association of *FCGR2B*-232Thr/Thr with SLE was observed only in the presence of *CD72*-*1/*1 genotype (OR 4.63, 95% CI 1.47–14.6, $P = 0.009$ versus *FCGR2B*-232Ile/Ile plus *CD72*-*2/*2). Minigene assays demonstrated that the 13-nucleotide repeat and 4 bp deletion within the same haplotype of intron 8 could regulate alternative splicing. The AS isoform lacks exon 8, and is deduced to contain 49 amino acid changes in the membrane-distal portion of the extracellular domain, where considerable amino acid changes are known in *CD72*^{c} allele associated with murine SLE. These results indicated that the presence of *CD72*-*2 allele decreases risk for human SLE conferred by *FCGR2B*-232Thr, possibly by increasing the AS isoform of CD72.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by production of an array of autoantibodies and immune complexes that leads to inflammation and damage of multiple organs. Although the precise mechanism of disease development is not clear, genetic epidemiological data indicate substantial contribution of genetic factors (1,2). Linkage studies identified multiple chromosomal regions linked with disease susceptibility

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and/or specific clinical manifestations, at least some of which are dependent on genetic or environmental background of populations (3).

In parallel with positional approach, several susceptibility genes have been identified through candidate gene approach. Candidate gene approach has higher detection power for disease associations when compared with positional approach (4), and is considered particularly advantageous in SLE where plenty of functional candidate genes are already available from studies of human SLE as well as mouse models (5).

Genes coding for molecules involved in B cell activation or regulation have been considered strong candidate susceptibility genes. By means of systematic polymorphism screening of genes such as *CD22* (6), Src-homology-2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1, *PTPN6*) (7), BCMA (*TNFRSF17*) (8) and BLyS/BAFF (*TNFSF13B*) (9), we previously identified association of dinucleotide repeat polymorphism within 3′-untranslated region (3′-UTR) of *CD19*, a positive regulator of B cell receptor (BCR) signaling (10) and also a SNP coding for an amino acid substitution, Ile232Thr, in the transmembrane region of an inhibitory receptor, *FCGR2B* (11). The latter association was confirmed in Thai and Chinese populations (12,13), but not in Caucasians and African-Americans (14–16). Instead, a promoter haplotype of *FCGR2B* has recently been shown to be associated with SLE in Caucasians (17,18), thereby suggesting that *FCGR2B* may be a susceptibility gene of SLE shared by multiple populations.

CD72 is a 45 kDa type II transmembrane protein containing a C-type lectin domain (19–22). CD72 is expressed as a homodimer in B cells of most developmental stages except for plasma cells, and also in dendritic cells and macrophages (19–21,23). CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail, one of which recruits SHP-1. CD72 functions as a negative regulator of BCR signaling (24–28). Importantly, CD72 has been shown to downregulate signaling of BCR containing IgG as well as IgM, whereas CD22 downregulates signals from IgM-BCR but not from IgG-BCR (24). Thus, if present, CD72 polymorphisms may be more relevant to SLE, where autoantibodies of IgG class play a pathogenic role. Recently, a model has been proposed that CD100, a ligand of CD72, turns off negative regulation of CD72 and leads to B cell activation both in human and in mouse (28–30). On the other hand, several lines of evidence indicate that CD72 also possesses potentials for positive signaling (31,32), which could be at least partially accounted for by the recruitment of Grb2 to the other ITIM, leading to association with B cell linker protein (BLNK, BASH, SLP-65) (33).

Human *CD72* gene is encoded on human chromosome 9p13.2, which has not previously been detected by linkage analyses. However, in a murine model of SLE, MRL/lpr, $CD72^c$ allele has been shown to be associated with systemic vasculitis phenotype in a recessive manner (34). Furthermore, the same $CD72^c$ allele has been shown to be responsible for the genetic background of murine SLE induced by immunization of a synthetic peptide representing an epitope of Sm autoantigen, through epitope spreading (35).

These observations led us to hypothesize that *CD72* polymorphisms may also be associated with SLE in humans. Thus far, systematic polymorphism screening of *CD72* has not been reported. In this study, we performed human *CD72* polymorphism screening and association study of *CD72* polymorphisms with SLE in Japanese, Thais and Caucasians. In addition, because both FcγRIIb and CD72 possess inhibitory potentials on BCR signaling, epistatic interaction between *FCGR2B* and *CD72* polymorphisms in relation to the susceptibility to SLE was examined. Furthermore, functional relevance of *CD72* polymorphisms was investigated.

RESULTS

New variations of human *CD72* gene

Variation screening of all exons with ~100 bp of flanking introns and promoter region (≤1 kb) was performed on genomic DNA from 10 Japanese SLE patients and 10 Japanese controls by direct sequencing. Ten variations were detected (Fig. 1A). Four of them, c.109A>G, c.338G>A, c.701C>T and c.728G>A, were located in the coding region and resulted in non-synonymous substitutions, Ile37Val, Cys113Tyr, Pro234Leu and Ser243Asn, respectively [mutation nomenclature is based on the work of den Dunnen and Antonarakis (36)].

Other six variations were located in introns. One of them encoded a repeat polymorphism constituted of one or two repeats of 13 nucleotides, IVS8 + 50(AGGGACTCAGA GC)$_{1-2}$ (for simplicity, hereafter designated as IVS8VNTR), and another was a four-nucleotide deletion, both within intron 8. Among the variations, IVS4 + 14T>A and IVS8V NTR had already been registered in the dbSNP (http://www.ncbi.nlm.nih.gov/entrez/) and JSNP databases (http://snp.ims.u-tokyo.ac.jp/) under rs 2095858 and IMS-JST082335, respectively. The remaining eight variations had not been previously described, and have been registered under accession numbers AB180952–AB180959.

Association of *CD72* polymorphisms with susceptibility to lupus nephritis

We next carried out case–control association analyses of the detected *CD72* variations. Initially, 98 Japanese patients and 95 controls were genotyped to estimate allele frequencies and linkage disequilibrium. Among the variations, four of them, −223C>T, IVS4 + 14T>A, IVS8VNTR and IVS8(−23)_(−20)delCTCA, met the criteria of polymorphisms (major allele frequency <99%). The others, including all non-synonymous substitutions, were rare variations (Supplementary Material, Table S1). Alleles at four polymorphic sites were in strong linkage disequilibrium, constituting two major haplotypes that contained one or two repeats of IVS8VNTR (designated as *CD72*-*1 and -*2, respectively) (Fig. 1B and C). Thereafter, we considered IVS8VNTR as a haplotype-tagging polymorphism, and all remaining subjects were genotyped for this polymorphism.

Tables 1 and 2 show the results of case–control association study in the Japanese and Thais. The control genotypes were not deviated from Hardy–Weinberg equilibrium. Significant association was not observed between SLE and controls. However, when the patients were divided into subgroups in accordance with the presence of lupus nephritis,

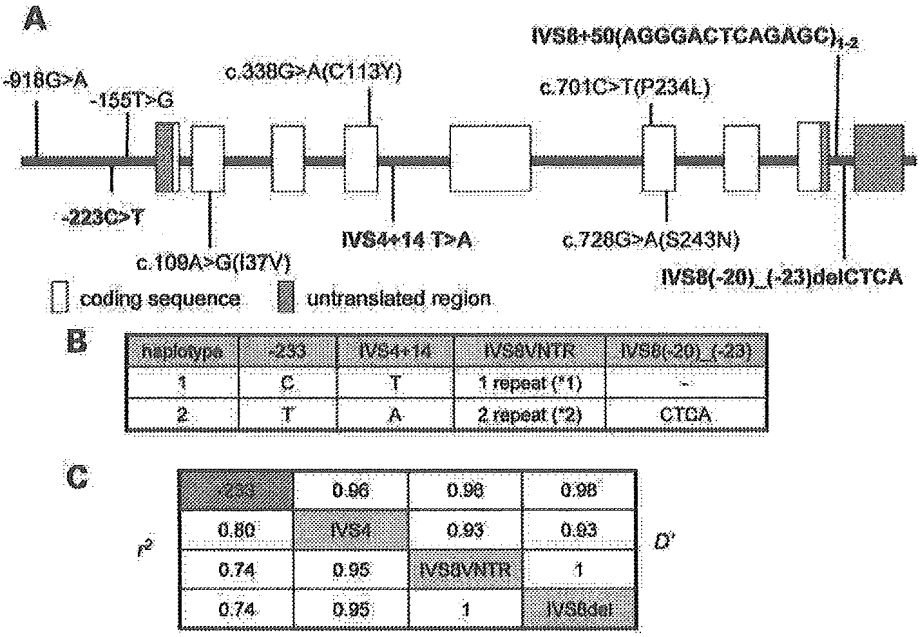

Figure 1. Human *CD72* gene structure, detected variations and haplotypes. (**A**) Exon–intron structure of *CD72* gene. Detected variations are shown in accordance with the nomenclature proposed by den Dunnen and Antonarakis (36). Variations shown in bold are polymorphisms, and others are rare variations. Note that only ~100 bp of exon-flanking regions were screened in introns. The size of each exon and intron is not to scale. (**B**) Major haplotypes constituted by the four polymorphisms. The VNTR alleles in intron 8 were designated as *1 and *2 and were used as the haplotype-tagging polymorphisms. (**C**) Linkage disequilibrium parameters among four polymorphic sites. D' and r^2 were calculated from genotyping data of 98 healthy Japanese individuals. The results were essentially the same as in SLE.

Table 1. Case–control association studies of *CD72* in the Japanese subjects

	Total SLE ($n = 160$)	SLE with nephritis ($n = 92$)	SLE without nephritis ($n = 65$)	Healthy controls ($n = 277$)
Genotype frequency				
*1/*1	51 (31.9)	34 (37.0)	16 (24.6)	98 (35.4)
*1/*2	82 (51.3)	47 (51.1)	33 (50.8)	125 (45.1)
*2/*2	27 (16.9)	11 (12.0)	16 (24.6)	54 (19.6)
Allele frequency				
*1	184 (57.5)	115 (62.5)	65 (50.0)	321 (57.8)
*2	136 (42.5)	69 (37.5)	65 (50.0)	233 (42.2)
Allele carrier frequency				
*1	133 (83.1)	81 (88.0)	49 (75.4)	223 (80.5)
*2	109 (68.1)	58 (63.0)	49 (75.4)	179 (64.6)

SLE with nephritis versus SLE without nephritis. Genotype frequency: $\chi^2 = 5.08$, $P = 0.024$ (Armitage's test for trend); allele frequency: $\chi^2 = 4.87$, $P = 0.027$; carrier frequency of *1 allele: $\chi^2 = 4.29$, $P = 0.038$. Clinical information with respect to nephritis was not available in three patients.

Table 2. Case–control association studies of *CD72* in the Thai subjects

	Total SLE ($n = 87$)	SLE with nephritis ($n = 48$)	SLE without nephritis ($n = 34$)	Healthy controls ($n = 187$)
Genotype frequency				
*1/*1	29 (33.3)	19 (39.6)	9 (26.5)	54 (28.9)
*1/*2	42 (48.3)	21 (43.8)	18 (52.9)	97 (51.9)
*2/*2	16 (18.4)	8 (16.7)	7 (20.6)	36 (19.3)
Allele frequency				
*1	100 (57.5)	59 (61.5)	36 (52.9)	205 (54.8)
*2	74 (42.5)	37 (38.5)	32 (47.1)	169 (45.2)
Allele carrier frequency				
*1	71 (81.6)	40 (83.3)	27 (79.4)	151 (80.7)
*2	58 (66.7)	29 (60.4)	25 (73.5)	133 (71.1)

Statistically significant difference was not observed. Clinical information with respect to nephritis was not available in five patients.

significant increase was observed in the allele and allele carrier frequencies of *CD72*-*1 in the patients with nephritis in the Japanese, and a similar tendency was observed in the Thais.

In contrast, association of *CD72* polymorphisms with susceptibility to SLE or lupus nephritis was not observed in 94 Caucasian SLE families using transmission/disequilibrium test (total SLE: *1 allele transmitted 35, non-transmitted 31; SLE with nephritis: *1 allele transmitted 7, non-transmitted 10). The distribution of genotype frequencies among the Caucasian parents was *1/*1, 41 (26.3%); *1/*2, 83 (53.2%); *2/*2, 32 (20.5%); thus, the frequency of *1/*1 genotype was slightly lower than in the Japanese.

Identification of a novel splicing isoform of *CD72*

Because polymorphisms encoding 13-nucleotide repeat and four-nucleotide deletion were found in intron 8, we investigated a possibility that these polymorphisms were associated with

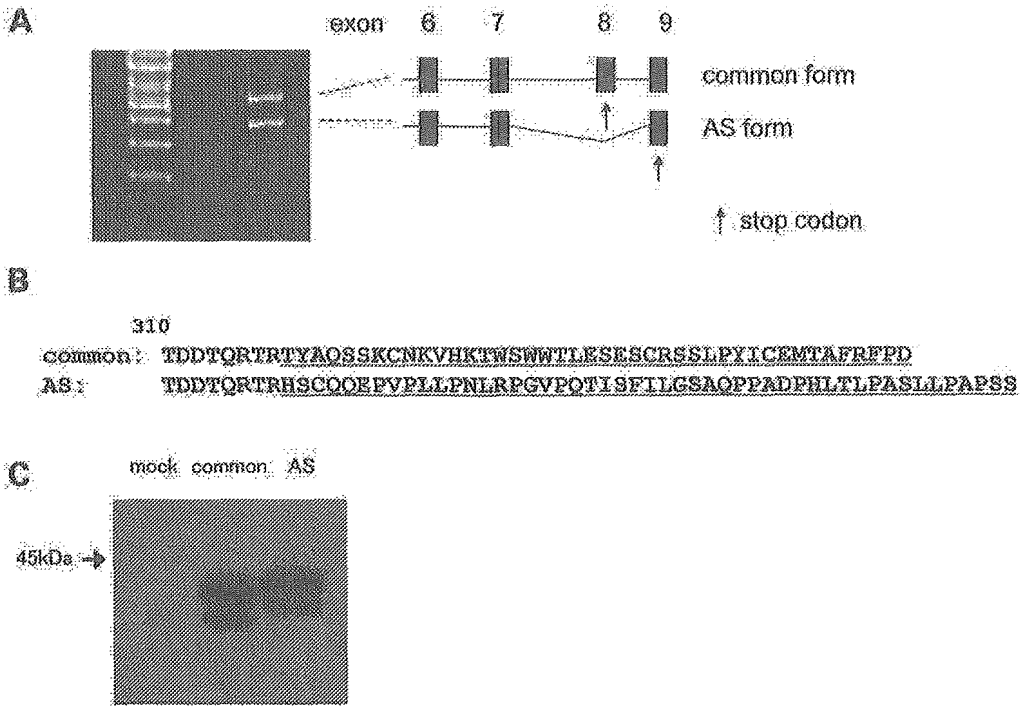

Figure 2. A novel alternative splicing isoform of *CD72*. (**A**) Two *CD72* fragments detected by RT–PCR. Sequence analysis revealed that the shorter fragment was the product of exon 8 skipping [alternatively spliced (AS) isoform]. In the AS isoform, the stop codon which normally appears in exon 8 is skipped, and alternative stop codon appears in exon 9 (arrows). Left lane: molecular weight marker (100 bp ladder). (**B**) Amino acid sequences of the C-terminal regions in the common and AS isoforms. In the AS form, 49 amino acids shown by underline are deduced to replace 42 amino acids of the common form. (**C**) Expression of alternatively spliced CD72 protein. COS-7 cells were transfected with full-length *CD72* cDNA coding for the common or alternatively spliced form by lipofection. Expression of CD72 protein was examined by western blotting with anti-human CD72 antibody directed against the cytoplasmic domain (H-96).

alternative splicing. RT–PCR of RNA derived from peripheral blood mononuclear cells (PBMCs) using primers placed within exon 6 and exon 9 revealed two amplification products (Fig. 2A). By sequencing, the longer product was found to be the normal *CD72* mRNA (common form), whereas the shorter band encoded alternative splicing isoform caused by exon 8 skipping (AS form). In the common form, termination codon appears within exon 8, and exon 9 constitutes 3′-UTR. In the AS form, exon 9 sequence was deduced to be translated into polypeptide until the new termination codon appears. As a result, C-terminal 42 amino acids in the membrane-distal portion of extracellular region of normal CD72 protein are thought to be replaced with new 49 amino acids in the AS form. Thus, alternative form of CD72 with exon 8 skipping paradoxically codes for a longer polypeptide of 366 amino acids, rather than 359 amino acids of the common isoform (Fig. 2B).

Full-length cDNAs coding for the common and AS *CD72* isoforms were both readily amplified from PBMC cDNA, thereby confirming the presence of the transcripts of the isoforms. Sequence analysis of multiple cDNAs revealed no other variations in the AS mRNA. Because an anti-human CD72 antibody that recognizes the extracellular domain of the AS isoform is not currently available, we confirmed the expression of the CD72 isoforms by western blotting with an antibody against the intracellular domain of CD72. Figure 2C shows the expression of the common and the AS isoforms in COS-7 cells. Both isoforms were detected at around the expected migration positions. The AS isoform displayed a slightly retarded mobility when compared with the common isoform, which could be ascribed to different molecular mass and/or to different post-translational modification including glycosylation.

Association of *CD72* genotypes with relative quantity of alternatively spliced isoform

We next investigated the expression of common and AS mRNA isoforms in PBMCs from 32 healthy individuals with different *CD72* genotypes (13 with *1/*1, 11 with *1/*2, 8 with *2/*2), using semi-quantitative RT–PCR. To standardize the difference in the numbers of CD72 positive cells among individuals, the quantity of AS isoform was normalized by that of the common form in each subject. As shown in Figure 3, the ratio of AS/common isoforms was strikingly increased in individuals with *2/*2 genotype ($n = 8$) when compared with *1/*1 ($n = 13$, $P = 0.000038$) or *1/*2 ($n = 11$, $P = 0.0085$). The ratio of AS isoform in heterozygotes was significantly higher than in *1/*1 homozygotes ($P = 0.0049$), indicating that the expression of AS isoform is dependent on the dosage of *2 allele.

Contribution of intron 8 polymorphisms to differential expression of alternative splicing products

To directly test whether the intron 8 polymorphisms influence the difference in the expression of splicing isoforms, minigene

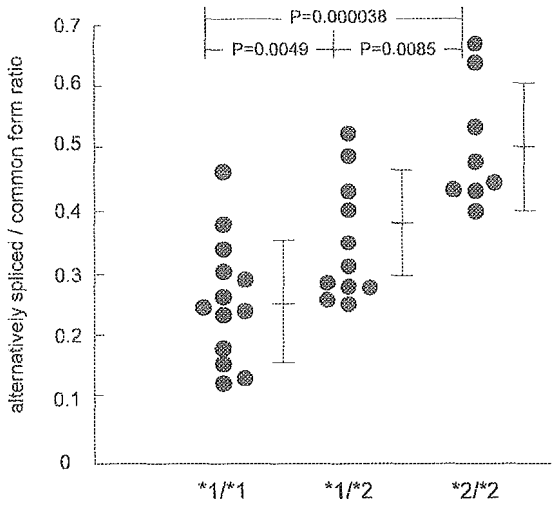

Figure 3. Association of relative proportion of AS isoform with *CD72* genotype. RNA purified from PBMCs of 32 healthy individuals (13 with *CD72*-*1/*1, 11 with *1/*2, 8 with *2/*2 genotype) was semi-quantitatively examined by RT–PCR. Mean ± SD of each genotype group is shown. Statistical difference was examined using Student's *t*-test.

assays were conducted. Minigene constructs containing genomic DNA segments derived from exon 7 through exon 9 of *CD72* were prepared from individuals carrying *1/*1 and *2/*2 genotypes, representing one or two repeat alleles of IVS8VNTR, respectively. Because of the linkage disequilibrium, only the former minigene construct contained CTCA deletion of intron 8. This construct was designated as *1/del. On the other hand, the latter did not contain the four-nucleotide deletion, and was designated as *2/+. In addition, to gain further insight into the individual contribution of these two polymorphic sites, chimeric constructs containing each of the VNTR and deletion were prepared (*1/+ and *2/del) (Fig. 4A). These four minigene constructs were introduced into COS-7 cells. COS-7 cells were used because these cells do not express endogenous CD72, and also because previous studies have successfully employed COS-7 cells for minigene assays (37). RT–PCR was performed using primers placed within exon 7 and exon 9.

As shown in Figure 4B, two mRNA isoforms with and without exon 8 were detected in the minigene-introduced COS-7 cells. Unlike in PBMCs, the exon 8-excluded isoform was more abundant in all COS-7 transfectants. Such a difference was probably related to the cellular environment and structure of the introduced genes. Nonetheless, clear difference was observed among the constructs. Exon 8 exclusion from mRNA was strongly increased in *2/+ construct when compared with *1/del, thereby confirming that intron 8 polymorphisms can regulate alternative splicing.

When the difference between *1/del and *1/+ was compared with that between *1/del and *2/del, it was suggested that although VNTR seems to play a greater role, each of the VNTR and the deletion possesses an independent effect on splicing, and both act in an additive manner to achieve full activity on exon 8 exclusion in the haplotype 2 (*2/+) (Fig. 4C). This was also supported from the comparison of *2/+, *1/+ and *2/del.

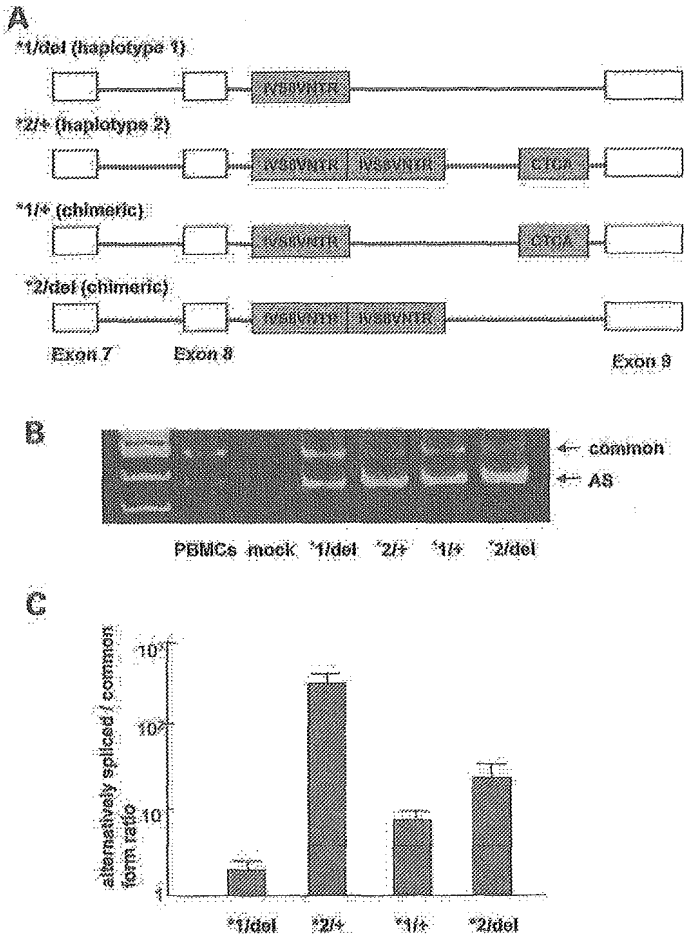

Figure 4. *CD72* minigene assay. (**A**) Four types of *CD72* minigene constructs. *1/del contains one repeat of 13-nucleotide repeat polymorphisms (IVS8VNTR) and CTCA deletion at −20 to −23 bp upstream from the splice acceptor site of intron 8, and corresponds to haplotype 1 (Fig. 1B). *2/+ contains two repeat allele of IVR8VNTR and no deletion of CTCA, and corresponds to haplotype 2 (Fig. 1B). *1/+ and *2/del are artificially engineered chimeric minigenes. (**B**) Representative RT–PCR gels of the minigene-introduced COS-7 cells. Two *CD72* splicing isoforms were detected in each transfectant. Exon 8 was excluded from the shorter isoform. Lane 1: molecular weight marker (100 bp ladder). Lane 2: human *CD72* isoforms derived from PBMC mRNA as a positive control. (**C**) AS/common ratio in the minigene transfectants. In this system, the quantity of the AS isoform was constitutively greater than the common isoform. The *Y*-axis is shown in a logarithmic scale. Mean and SD in three experiments are shown. The differences were statistically significant in all comparisons: *1/del versus *2/+, $P = 0.024$; *1/del versus *1/+, $P = 0.0091$; *1/del versus *2/del, $P = 0.0062$; *2/+ versus *1/+, $P = 0.025$; *2/+ versus *2/del, $P = 0.028$. Although VNTR appears to play a dominant role, both polymorphisms contribute to the splicing regulation in an additive manner.

Epistatic interaction between *CD72* and *FCGR2B* polymorphisms in conferring susceptibility to SLE

Finally, we examined whether genetic interaction between *CD72* and *FCGR2B* polymorphisms plays a role in conferring susceptibility to SLE, because both molecules are involved in negative regulation of BCR signaling. The analysis was done in the Japanese and Thai cohorts, in which significant association of *FCGR2B*-232Thr/Thr genotype had been already demonstrated (11,12). As shown in Tables 3 and 4, the risk

Table 3. Epistatic interaction between *FCGR2B* and *CD72* for the susceptibility to SLE in the Japanese population

FCGR2B	*CD72*-*1/*1			*CD72*-*1/*2			*CD72*-*2/*2		
	SLE (n = 51)	ctr (n = 98)	OR (95%CI)	SLE (n = 82)	ctr (n = 125)	OR (95%CI)	SLE (n = 27)	ctr (n = 54)	OR (95%CI)
Thr/Thr	6 (11.8)	3 (3.1)	3.20 (0.76–13.5)	9 (11.0)	8 (6.4)	1.80 (0.60–5.39)	1 (3.7)	2 (3.7)	0.80 (0.07–9.37)
Ile/Thr	18 (35.3)	38 (38.8)	0.76 (0.34–1.67)	29 (35.4)	39 (31.2)	1.19 (0.57–2.49)	6 (22.2)	20 (37.0)	0.48 (0.17–1.38)
Ile/Ile	27 (52.9)	57 (58.2)	0.76 (0.37–1.56)	44 (53.7)	78 (62.4)	0.90 (0.46–1.76)	20 (74.1)	32 (59.3)	1

See Table 4 for definitions.

Table 4. Epistatic interaction between *FCGR2B* and *CD72* for the susceptibility to SLE in the Thai population

FCGR2B	*CD72*-*1/*1			*CD72*-*1/*2			*CD72*-*2/*2		
	SLE (n = 25)	ctr (n = 47)	OR (95%CI)	SLE (n = 39)	ctr (n = 86)	OR (95%CI)	SLE (n = 15)	ctr (n = 32)	OR (95%CI)
Thr/Thr	6 (24.0)	2 (4.3)	7.88 (1.51–41.1)	4 (10.3)	8 (6.4)	1.31 (0.31–5.59)	2 (13.3)	2 (6.3)	2.63 (0.33–20.8)
Ile/Thr	7 (28.0)	20 (42.6)	0.93 (0.28–3.00)	17 (43.6)	27 (31.2)	1.65 (0.60–4.54)	5 (33.3)	9 (28.1)	1.46 (0.37–5.68)
Ile/Ile	12 (48.0)	25 (53.2)	1.26 (0.43–3.66)	18 (46.2)	51 (59.3)	0.92 (0.35–2.46)	8 (53.3)	21 (65.6)	1

Proportions of the patients or controls with particular *FCGR2B* genotypes among the total patients or controls with each *CD72* genotype are shown in parentheses. Odds ratio (OR) and 95% confidence interval (CI) are calculated against *CD72*-*2/*2 plus *FCGR2B*-232Ile/Ile. In the Thais, eight SLE and 22 control samples could not be genotyped for *FCGR2B* due to DNA conditions, and were excluded from this analysis.

of *FCGR2B*-232Thr/Thr genotype was observed only in the presence of *CD72*-*1/*1 genotype in both cohorts. In the Caucasian samples, frequency of *FCGR2B*-232Thr was too low to be analyzed for epistatic interaction (14).

When the data from these two cohorts were combined by meta-analysis, significant association with the onset of SLE was observed only in *FCGR2B*-232Thr/Thr plus *CD72*-*1/*1 genotypes when compared with *FCGR2B*-232Ile/Ile plus *CD72*-*2/*2 genotypes (Table 5). In all comparisons, no heterogeneity was detected in meta-analysis. Taken together, these observations indicated that *CD72*-*2 allele, associated with preferential production of AS isoform, decreases risk of development of SLE conferred by *FCGR2B*-232Thr allele product in an epistatic manner.

Table 5. Meta-analysis of the combinatorial effect of *CD72* and *FCGR2B* polymorphisms for the susceptibility to SLE in the Japanese and Thai cohorts

FCGR2B	*CD72*		
	*1/*1	*1/*2	*2/*2
Thr/Thr	4.63 (1.47–14.6)	1.60 (0.67–3.86)	1.58 (0.32–7.90)
Ile/Thr	0.80 (0.41–1.55)	1.33 (0.73–2.42)	0.77 (0.26–2.34)
Ile/Ile	0.89 (0.49–1.61)	0.91 (0.52–1.58)	1

The data shown in Tables 3 and 4 were combined by meta-analysis under the random effects model. Odds ratio and 95% confidence interval of each genotype combination against *FCGR2B*-232Ile/Ile plus *CD72*-*2/*2 are shown. Although the *FCGR2B*-232Thr/Thr genotype had been shown to be associated with SLE in both populations (11,12), significant association was observed only in the presence of *CD72*-*1/*1 genotype ($P = 0.009$).

DISCUSSION

In the present study, we demonstrated that human *CD72* polymorphisms are associated with the relative quantity of a novel alternative splicing product, and also with the presence of nephritis among the patients with SLE. Of particular interest, when the genetic interaction between *CD72* and *FCGR2B* was examined, the previously detected SLE susceptibility conferred by *FCGR2B*-232Thr/Thr genotype (11–13) substantially decreased in the presence of *CD72* allele associated with increased quantity of the alternative splicing product. This alternative mRNA form was confirmed to be expressed at the protein level. These observations suggest that CD72 and FcγRIIB may directly or indirectly interact at the protein level to regulate B cell activation, and their inhibitory potentials are influenced by their polymorphisms.

Because CD72 is a type II membrane protein, C-terminal region constitutes the membrane-distal portion of extracellular region (20). In the deduced human AS isoform, a stretch of 42 amino acids out of 243 amino acids in the extracellular region is replaced with a totally different sequence of 49 amino acids. Nevertheless, this alteration is predicted to preserve C-type lectin structure (38), thereby suggesting that the AS isoform represents a functional polymorphism possessing differential properties. In mice, four alleles of *CD72* with amino acid substitutions are known, some of which are caused by differences in the mode of splicing (39–42). This implies that regulation of CD72 function by splicing may be conserved in evolution. Among murine alleles, *CD72*^{c} has been shown to be involved in the susceptibility to vasculitis in MRL/*lpr* mice (34), and also to SLE induced by an immunization of a synthetic peptide representing Sm B/B′ sequence (35). Of particular interest, this allele contains multiple amino acid substitutions as well as a deletion of seven amino acid stretch in the membrane-distal portion of the extracellular domain that is altered in human CD72 AS isoform. Functional difference in mouse *CD72* alleles has not been investigated, although

considerable structural difference has been suggested for *CD72*^{c} from the difference in the reactivity with monoclonal antibodies (43).

The binding sites on CD72 with putative ligands such as membrane Ig (24) or CD100 (28) have not yet been mapped. The protective effect of *CD72*-*2 allele might be explained if the AS product has stronger inhibitory potential on BCR signaling, for example through stronger affinity to BCR or weaker affinity to CD100. *FCGR2B*-Ile232Thr polymorphism has been shown to be associated with changes in the inhibitory potential, although the effect seems to be inconsistent in different experimental systems (15,44). The inhibitory potential of FcγRIIb is dependent on phosphorylation of ITIM through positive BCR signaling (45); thus, it is likely that differences in CD72 function may influence the potential for inhibitory signals mediated by FcγRIIb. Alternatively, it is also possible that positive signals from CD72 may be decreased in the AS isoform product, thereby rendering carriers of *2 allele protective for the development of SLE or nephritis.

The expression of AS isoform in PBMC is considered to be stable at least at the mRNA level, because substantial quantity of AS isoform was detected in virtually all PBMCs from 32 individuals (Fig. 3), and the full-length cDNA of the AS isoform was readily amplified from PBMCs. Because antibodies that can specifically detect the AS protein product are not currently available, we cannot examine whether the AS isoform is differentially distributed among subsets of B cells or dendritic cells. Such studies as well as functional consequences of CD72 AS expression should be addressed in future studies, some of which are underway in our laboratory.

CD72 polymorphisms *per se* were not associated with SLE, but were associated with the presence of nephritis in SLE. SLE is a heterogeneous disease, and difference in the genetic background with respect to clinical manifestations has frequently been reported (1,3). Notably, *FCGR2B*-232Thr was also preferentially associated with lupus nephritis (11,13). Furthermore, association with *FCGR2B*-232Thr is observed in the Asians, but not in the Caucasians (11–16), where allele frequency of *FCGR2B*-232Thr is low. Thus far, association of *CD72* polymorphisms with SLE has also been observed in the Asians, but not in the Caucasians. The frequency of *CD72*-*1/*1 appears to be slightly lower in the Caucasians. These observations raise the possibility that the effect of *FCGR2B*-232Thr and *CD72* polymorphisms on SLE susceptibility is dependent on each other. All these hypotheses should be addressed by further association studies on multiple populations and by functional analysis at the protein level. Specifically, it would be important to test the genetic interaction between *CD72* and *FCGR2B* in African-Americans, where the allele frequency of *FCGR2B*-232The is rather high (15). It would also be interesting to examine the interaction of *CD72* with *FCGR2B* promoter polymorphism, which was recently shown to be associated with SLE in the Caucasians (17,18).

The epistatic interaction between *FCGR2B* and *CD72* was slightly different from that between the Japanese and Thai cohorts. In the Japanese, the difference between *CD72*-*1/*1 and *1/*2 groups appeared to rest more in the controls than in the patients. In the patients, striking difference in the *FCGR2B* genotype distribution was observed between carriers (*1/*1 + *1/*2) and non-carriers (*2/*2) of *CD72*-*1 allele. On the other hand, in the Thai population, striking difference in the *FCGR2B* genotype distribution was observed between *CD72*-*2 absent (*1/*1) and present (*1/*2 + *2/*2) groups. Such a difference could perhaps be ascribed to rather small sample size of each population. By combining the data of the two cohorts using meta-analysis, statistically significant effect of *FCGR2B*-232Thr/Thr was observed only in the *CD72*-*1/*1 genotype (Table 5).

Although the gender ratio was substantially different between cases and controls in the Asian cohorts, adjustment for gender ratio did not alter the results, because the genotype distributions were not different between male and female controls. The patient populations were older than the controls, but because of the low prevalence of SLE, difference in age distribution should not affect the results significantly, and even if it did, it should have made the analyses more conservative. It is possible that some of the patients classified as 'without nephritis' may later develop nephritis; however, in most of the patients, disease duration was >1 year at the time of the study, and because the proportion of the patients with nephritis (59% in both populations) was similar to general prevalence of nephritis in SLE, misclassification with respect to the presence of nephritis is not considered to pose a substantial problem.

Recently, a number of observations indicated physiological or pathological significance of alternative splicing and its role in the genetic background of diseases (46). In the immune system, alternative splicing products are known for important genes such as CD28 (47), CTLA-4 (CD152) (48,49) and CD40 (50). Notably, it was recently reported that genomic polymorphism of human CTLA-4 is significantly associated with expression of alternatively spliced variants and with susceptibility to Grave's disease, autoimmune hypothyroidism and type 1 diabetes (48). Our present observations further emphasize the role of genomic polymorphism in the regulation of alternative splicing and its relevance in complex diseases. Our knowledge on the mechanism of alternative splicing is still limited (45,51). In the case of *CD72*, our present observations suggested that both VNTR and four-nucleotide deletion act in combination as *cis*-acting intronic splicing enhancer or silencer.

It is estimated that at least half of the human genes are alternatively spliced (52,53), and this proportion is especially higher in genes coding for molecules in the immune system (52). Search for functionally relevant alternative splicing variants in a genome-wide manner (53) and their relationship with genomic polymorphisms as well as mechanisms of regulation of alternative splicing will provide important clues for the understanding of genetic background of complex diseases and new therapeutic approaches.

MATERIALS AND METHODS

Subjects

In the Japanese cohort, 160 patients with SLE (male 10, female 150, mean age ± SD 41.1 ± 13.9) and 277 controls (male 150, female 127, mean age ± SD 35.0 ± 10.0) were recruited at Juntendo Univerisity and The University of

Tokyo (Tokyo, Japan). In the Thais, 87 patients with SLE (male 11, female 76, mean age ± SD 30.0 ± 10.5) and 187 controls (male 103, female 84, mean age ± SD 37.4 ± 9.6) were recruited at the Siriraj Hospital, Mahidol University (Bangkok, Thailand). All individuals were unrelated Japanese living in Tokyo, or unrelated present-day Thais.

Association was also examined using genomic DNA from 94 Caucasian families recruited at University of California at Los Angeles, in which one or more members had SLE (14). In all families, both parents of the SLE patients were available for study. SLE was classified according to the American College of Rheumatology (ACR) criteria (54).

Presence of nephritis was defined in accordance with the ACR criteria (persistent proteinuria >0.5 g/day or 3+ or cellular cast). Patients with nephritis at the time of the study or with previous history of nephritis were classified into 'with nephritis' group. In most patients, disease duration at the time of study entry was >1 year.

The study was approved by the Research Ethics Committees of the Graduate School of Medicine, The University of Tokyo, Juntendo University and Mahidol University, and by the Human Subject Protection Committee of the University of California at Los Angeles.

Genomic DNA

Genomic DNA from patients and healthy individuals was purified from peripheral blood using the QIAamp blood Kit (Qiagen, Hilden, Germany).

Variation screening of human *CD72*

Variations of human *CD72* gene were screened using direct sequencing. Genomic DNA samples from 10 healthy individuals and 10 SLE patients were used for screening. All exons with ~100 bp of flanking introns and ~1 kb of promoter region of human *CD72* gene were amplified using specific primer sets designed based on the nucleotide sequences NM_001782 and NT_008413 (LocusLink, http://www.ncbi.nlm.nih.gov/locuslink/). PCR was carried out using GeneAmp reagents and AmpliTaq Gold DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA). The amplification conditions consists of initial denaturation at 96°C for 10 min, followed by 35 cycles of 96°C for 30 s, annealing temperature for 30 s and 72°C for 30 s in GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA). The direct sequencing analysis was performed using ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

CD72 genotyping

Genotyping of −918G>A, −223C>T, −155T>G, c.109A>G, c.338G>A, IVS4 + 14T>A, c.701C>T, c.728G>A and IVS8(−23)_(−20)delCTCA was done using PCR–single strand conformation polymorphism (SSCP). PCR was carried out as described earlier, followed by SSCP analysis using polyacrylamide gel (acrylamide/bis, 49 : 1) and constant temperature control system (AB1600 and AE6370, Atto, Tokyo, Japan). After electrophoresis, the separated fragments were visualized with silver staining (Daiichi Pure Chemicals, Tokyo, Japan). The primer sequences, annealing temperatures and SSCP conditions are listed in Supplementary Material, Table S2.

Genotyping of IVS8VNTR was done using PCR–simple sequence length polymorphism (SSLP). The primer sets and annealing temperatures are listed in Supplementary Material, Table S2. PCR–SSLP analysis was performed using 10% polyacrylamide gel (acrylamide/bis, 29 : 1). After electrophoresis, separated fragments were visualized with SYBER Gold (Molecular Probes, Eugene, OR).

FCGR2B-Ile232Thr genotyping

FCGR2B-Ile232Thr genotyping was done as previously described (11–13).

Semi-quantitative RT–PCR

The ratio of common and AS CD72 isoforms was estimated using semi-quantitative RT–PCR. PBMCs were isolated from 20 ml of venous blood samples of 13 healthy individuals with *CD72*-*1/*1, 11 with *1/*2 and 8 with *2/*2 genotypes by density-gradient centrifugation. Total RNA was extracted from PBMCs using the RNeasy kit (Qiagen). First-strand cDNA was synthesized using Oligo(dT) primer (Promega, Madison, WI, USA) and ReverTra Ace (Toyobo, Osaka, Japan).

RT–PCR to detect exon 8 skipping was performed using primers designed within exon 6 and exon 9 (5′-GGCAG GAGAGCCAAAAACAA-3′ and 5′-ACCCCATTCTACCAT GGGAA-3′). One microliter of template cDNA solution was placed into 25 μl of reaction solution, and amplification was done using AmpliTaq Gold DNA polymerase (Perkin-Elmer). To achieve linear amplification, 28 cycles were found to be optimal in preliminary experiments. The amplification conditions consisted of initial denaturation at 96°C for 10 min, followed by 28 cycles of 96°C for 30 s, 60°C for 30 s and 72°C for 1 min in GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). Quantitation of AS/common isoforms ratio was performed using Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, CA, USA). These experiments were repeated three times with essentially identical results.

Expression of the CD72 isoforms in COS-7 cells

Full-length cDNA encoding common and AS forms of *CD72* were amplified from first-strand cDNA derived from PBMCs with GeneAmp reagents and TaKaRa LA Taq (TaKaRa, Otsu, Shiga, Japan) and specific primer set (5′-GCAGAGCT GCTCAGGACCAT-3′ and 5′-ACCCCATTCTACCATGGG AA-3′) under conditions of initial denaturation at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s, 64°C for 30 s and 72°C for 1.5 min in GeneAmp PCR system 9700. These cDNAs were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen-Life Technologies, Carlsbad, CA, USA) and subjected to sequence analysis. The common or AS isoform without misincorporation of nucleotides was transfected into COS-7 cells by lipofection using Lipofectamine 2000 (Invitrogen-Life Technologies). After 36 h, the cells were lysed with boiling 2% SDS sample buffer and the lysates were subjected to SDS–PAGE followed by western

blotting with a rabbit polyclonal antibody against cytoplasmic region of human CD72 (H-96, Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase, and the signals were detected using ECL chemiluminescence system (Amersham Biosciences, Uppsala, Sweden).

Minigene assay

CD72 genomic DNA segments containing 109 bp of intron 6, whole exon 7, intron 7, exon 8, intron 8 and 238 bp of exon 9 were amplified by PCR from genomic DNA derived from two donors, one with *CD72*-*1/*1 (also containing IVS8(−23)_(−20)delCTCA) and the other with *CD72*-*2/*2 genotype. PCR was conducted using TaKaRa LA Taq, GeneAmp reagents and specific primer set (5′-CATATATA CAGCCCCCTTGC-3′ and 5′-ACCCCATTCTACCATGGG AA-3′) under conditions of initial denaturation at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s, 63°C for 30 s and 72°C for 2 min in GeneAmp PCR system 9700.

PCR products were blunt-ended with T4 DNA polymerase (Takara Bio Inc.), digested with *Eco*RI restriction site in exon 7 and inserted between *Eco*RI and *Eco*RV sites of pcDNA3.1-Zeo(+) vector (Invitrogen-Life Technologies). Therefore, the final minigene constructs were composed of 68 bp of exon 7, whole intron 7, exon 8, intron 8 and 238 bp of exon 9 (Fig. 4A, *1/del and *2/+).

To differentially examine the effects of IVS8VNTR and CTCA deletion on splicing, the other two combinations, *1/+ and *2/del, were prepared. The minigenes, *1/del and *2/+, were first subcloned into pBlueScript SK(+) (Invitrogen-Life Technologies) lacking internal *Nco*I site. Subsequently, the 3′ portions encompassing the CTCA deletion but not the VNTR were cut out between two *Nco*I sites, and were exchanged between *1/del and *2/+. These new combinations were recloned into *Eco*RI and *Not*I sites of pcDNA3.1-Zeo(+) vector (Fig. 4A, *1/+ and *2/del).

After the sequences were confirmed, these four minigene constructs were introduced into COS-7 cells by lipofection using Lipofectamine 2000 (Invitrogen-Life Technologies). After 24 h of transfection, total RNA was extracted as described earlier.

Semi-quantitative RT–PCR was performed with specific primer sets placed within exon 7 and exon 9 (5′-CATATT GGACTGGCCTCAGC-3′ and 5′-ACCCCATTCTACCATG GGAA-3′). To achieve linear amplification, 27 cycles were found to be optimal in preliminary experiments. The amplification conditions consisted of initial denaturation at 96°C for 10 min, followed by 27 cycles of 96°C for 30 s, 60°C for 30 s and 72°C for 30 s in GeneAmp PCR system 9700. The quantitation was done as described earlier. These experiments were repeated three times with essentially identical results.

Statistical analysis

The frequencies of the *CD72* genotypes were compared between groups using the χ^2-test. In addition, Armitage's test for trend in proportions was performed according to Sasieni (55). The ratio of two mRNA isoform levels was compared between genotypes using Student's *t*-test. *P*-values <0.05 were regarded as statistically significant. Linkage disequilibrium parameters were calculated using EH program (56). Meta-analysis for the Japanese and Thais data was performed using χ^2-based *Q*-statistic (57). Heterogeneity was considered significant for $P < 0.10$. Study-specific data were combined using random effects model in general variance-based method. Statistical analyses were done using StatView J5.0 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Crucial Role of Inhibitor of DNA Binding/Differentiation in the Vascular Endothelial Growth Factor-Induced Activation and Angiogenic Processes of Human Endothelial Cells[1]

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Angiogenesis plays a pivotal role in the aggressive proliferation of synovial cells in rheumatoid arthritis. We have previously reported the overexpression of inhibitor of DNA binding/differentiation (Id) in the endothelial cells within the synovial tissues of rheumatoid arthritis. In this study, we investigated the role of Id in inflammation and angiogenesis in an in vitro model using HUVECs. Vascular endothelial growth factor (VEGF) and TGFβ induced the expression of Id1 and Id3 in HUVECs. Forced expression of Id induced proliferative activity in HUVECs accompanied by down-regulation of p16^{INK4a}. Overexpression of Id enhanced expression of ICAM-1 and E-selectin, and induced angiogenic processes such as transmigration, matrix metalloproteinase-2 and -9 expression, and tube formation. In contrast, knockdown of Id1 and Id3 with RNA interference abolished proliferation, activation, and angiogenic processes of HUVECs induced by VEGF. These results indicated that Id plays a crucial role in VEGF-induced signals of endothelial cells by causing activation and potentiation of angiogenic processes. Based on these findings, it was proposed that inhibition of expression and/or function of Id1 and Id3 may potentially be of therapeutic value for conditions associated with pathological angiogenesis. ***The Journal of Immunology*****, 2004, 173: 5801–5809.**

Angiogenesis (1), along with recruitment of inflammatory cells, production of proinflammatory cytokines (2), and aggressive proliferation of fibroblast-like synoviocytes (3), is critically involved in the process of chronic inflammation and joint destruction in rheumatoid arthritis (RA).[3] In the previous study, through a comprehensive analysis of mRNA in the synovial tissues, we identified overexpression of inhibitor of DNA binding/differentiation (Id) family proteins, Id1 and Id3, and their localization to the endothelial cells, within the synovial tissues of RA (4).

The Id family consists of four members, Id1 to Id4, which contain helix-loop-helix (HLH) domains, but not DNA binding domains (5). Id proteins were originally identified as dominant-negative antagonists of the basic HLH transcription factors such as MyoD (5) as well as non-basic HLH proteins such as Rb (6), and have been known to play a crucial role in developmental processes (7–11). They are predominantly expressed in fetal tissues and in some transformed cells (12), and are down-regulated during differentiation (13, 14). Id also controls cell proliferation and the progression of cell cycle (15). Id has been shown to repress p16^{INK4a} expression by directly inhibiting the binding of Ets1 and Ets2 transcription factors to p16^{INK4a} promoter (16), and to prevent exit from the cell cycle. Expression of Id3 is regulated by Ras-ERK MAPK cascade (17) and Smad1/5 signaling (18).

A previously unsuspected role of Id was recently disclosed. Id1$^{+/-}$Id3$^{-/-}$ mice were unable to support angiogenesis necessary for the progression of tumor xenografts (19), suggesting that Id1 and Id3 may also be required for the angiogenesis in adult tissues. We hypothesized that the localization of Id within the synovial endothelial cells in RA (4) may imply involvement of Id in the inflammation and angiogenesis typically observed in the synovial tissues of RA. If this is the case, Id can be an attractive target for the treatment not only of cancer, but also of RA, because expression of Id is usually weak in normal adult tissues, except for the proliferating cells (20).

In the present study, we examined whether the overexpression of Id alone can induce activation and angiogenesis in cultured human endothelial cells, and whether suppression of Id can inhibit activation and angiogenic processes of these cells induced by vascular endothelial growth factor (VEGF).

Materials and Methods

Monoclonal Abs

The mAbs used for flow cytometry were as follows: FITC-labeled anti-human α_v integrin (Beckman Coulter, Fullerton, CA), PE-labeled anti-human ICAM-1 (Beckman Coulter), PE-labeled anti-human β_1 integrin (DakoCytomation, Carpinteria, CA), CyChrome-labeled anti-human E-selectin (BD Biosciences, San Jose, CA), and unlabeled anti-human α_2 integrin (DakoCytomation). MsIgG1-RD1/MsIgG1-FITC (Beckman Coulter), IgG1-PE (Beckman Coulter), IgG1-CyChrome (BD Biosciences), and unlabeled IgG1 (DakoCytomation) were used as isotype controls.

Endothelial cell culture and transient transfection

Primary HUVECs were isolated as previously described (21) and cultured in MCDB151 medium (Sigma-Aldrich, St. Louis, MO) supplemented with

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; Id, inhibitor of DNA binding/differentiation; HLH, helix-loop-helix; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; bFGF, basic FGF; shRNA, small hairpin RNA; MMP, matrix metalloproteinase; RNAi, RNA interference.

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10% heat-denatured FBS (HyClone, Logan, UT), 500 μg/ml heparin (Sigma-Aldrich), and 2 ng/ml human acidic fibroblast growth factor (FGF; PeproTech, London, U.K.) in dishes treated with 0.1% gelatin (Wako Pure Chemical, Osaka, Japan). Cells were cultured at 37°C in an atmosphere of 5% CO_2, and routinely passaged by trypsinization when achieving confluence. HUVECs used in all experiments were of up to seven passages.

Transfection was performed using Effectene Transfection Reagent (Qiagen, Hilden, Germany). Overexpression of *ID* genes in HUVECs was achieved using pBLAST49-hId1a(c) and pBLAST49-hId3(c) plasmid vectors (InvivoGen, San Diego, CA). pBLAST49-mcs (InvivoGen) was used as a negative control.

To knockdown *ID1* and *ID3*, small hairpin RNAs (shRNAs) were designed. The sequences of *ID1*- and *ID3* shRNAs were as follows: TCCCAAAGAATCATGAAAGTCGCCAGTTCAAGAGACTGGCGACTTTCATGATTCTTTT and TCGGATCCAACCTCACAGCACCTCACTTCTTCAAGAGAGAAGTGAGGTGCTGTGAGGTTTTTTTTGGAAAAGCTTGG, respectively, with 3′ single-strand overhangs for ligation into RNA expression vectors (psiRNA-hH1 neo (InvivoGen) for Id1; pSilencer 2.0-U6 (Ambion, Austin, TX) for Id3) containing H1 or U6 RNA polymerase III promoter.

Transfection efficiency was examined using X-Gal staining assay (Gene Therapy Systems, San Diego, CA). LacZ expression vector was transfected into HUVECs under the same conditions in which Id3 transfectants and Id1/Id3 RNAi transfectants were generated. After incubation for 24 h, the cells were placed in the fixing buffer for 15 min at room temperature. Then, X-Gal staining solution was added to the dishes, and the cells were incubated for 10 h at 37°C. After incubation, stained and unstained cells were counted. The transfection efficiency was determined to be 62.6 ± 9.6%.

Endotoxin levels in the vector preparations were measured using endotoxin detection kit based on *Limulus* amebocyte lysate assay (Endospecy ES-50M; Seikagaku Corporation, Tokyo, Japan). Contamination of endotoxin was undetectable.

RT-PCR

Total RNA was isolated from the cells using RNeasy Mini kit (Qiagen), and was treated with DNase inhibitor (DNA-free (Ambion)). First-strand cDNAs were synthesized from RNA using ImProm-II reverse transcription system (Promega, Madison, WI). Quantitative RT-PCR was performed using a real-time RT-PCR machine (LightCycler), with LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) and gene-specific primer sets. The data were analyzed by fit-points method using the LightCycler analysis software. β-Actin or GAPDH were used to normalize total RNA levels. The assays were done in triplicate.

Induction of Id1 and Id3 in HUVECs after stimulation for 24 h by VEGF (20 ng/ml), TGFβ (0.5 ng/ml), TNF-α (20 ng/ml), or IL-1β (1 ng/ml) (all from PeproTech) was examined by conventional RT-PCR. The PCR products were electrophoresed, stained with SYBR Gold (Molecular Probes, Eugene, OR), and relative mRNA levels were calculated from the band intensity using β-actin as a reference.

Because the ordinary culture medium for HUVEC contained acidic FGF, the effect of basic FGF (bFGF; PeproTech) for the induction of Id1 and Id3 was examined under a different culture condition. HUVECs were cultured in serum-free medium (Human Endothelial-SFM; Invitrogen Life Technologies, Carlsbad, CA) supplemented with epidermal growth factor (10 ng/ml; Invitrogen Life Technologies) in accordance with the manufacturer's instruction, in the presence or absence of bFGF (10 ng/ml). After 72 h, Id1 and Id3 mRNA levels were quantitated as described above.

The primer pairs used for RT-PCR were the following: Id1, 5′-AGCCAGTCCGCCAAGAATCAT-3′(forward),5′-ACTCACTCCCCAGCATGAAG-3′ (reverse); Id3, 5′-CTCCACGCTCTGAAAAGACC-3′ (forward), 5′-ACTCAGATTAAGCCAGGTGGA-3′ (reverse); $p16^{INK4a}$, 5′-AGCCTTCGGCTGACTGGCTGG-3′ (forward), 5′-GCAGTTAAGGGGGCACGAGTG-3′ (reverse); ICAM-1, 5′-ACCTGGCAATGCCCAGACATCTGTGT-3′ (forward), 5′-GTACACGGTGAGGAAGGTTTTAGCTGTTG-3′ (reverse); E-selectin, 5′-AAAACTTCCATGAGGCCAAA-3′ (forward), 5′-GCATTCCTCTCTTCCAGAGC-3′ (reverse); matrix metalloproteinase (MMP)2, 5′-ATGACAGCTGCACCACTGAG-3′ (forward), 5′-TGATGTCATCCTGGGACAGA-3′ (reverse); and MMP9, 5′-GGCGCTCATGTACCCTATGT-3′ (forward), 5′-CCCTCAGTGAAGCGGTACAT-3′ (reverse).

Cell proliferation assay

The proliferative activity of HUVECs was measured using the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan). Cells were cultured on gelatin-coated flat-bottom 96-well microtiter plate at 2×10^4 cells/well. After 24 h, 10 μl of WST-1 solution was added to each well, and the cells were incubated at 37°C in an atmosphere of 5% CO_2 for 4 h. The supernatant solutions were transferred to a new 96-well plate, and the absorbance of each well was measured at 480 nm. Experiments were performed in triplicate, and the proliferative activity was calculated as the mean ± SD of the triplicate wells divided by that of the controls.

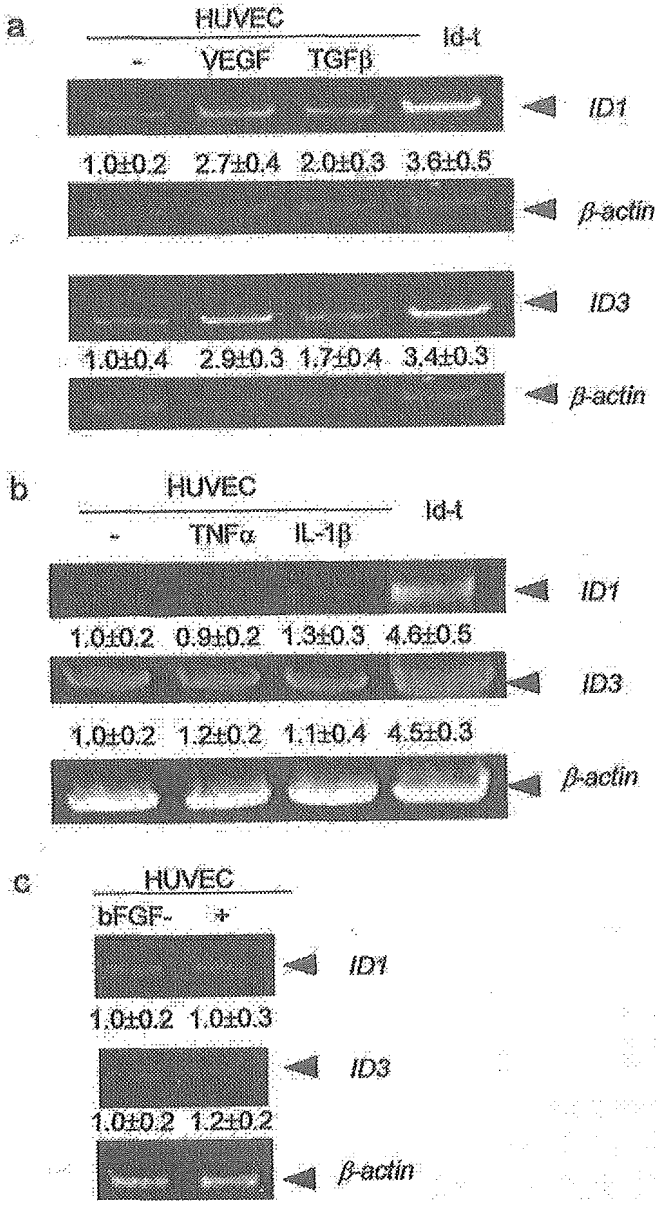

FIGURE 1. Expression of *ID1* and *ID3* mRNA is induced in HUVECs by VEGF or TGFβ. *a* and *b*, Id1 and Id3 mRNA levels were measured in HUVECs with or without stimulation with VEGF (20 ng/ml), TGFβ (0.5 ng/ml) (*a*), TNF-α (20 ng/ml), or IL-1β (1 ng/ml) (*b*) for 24 h. HUVECs transfected with *ID1* (Id1-t) or *ID3* (Id3-t) were used as the positive controls. *c*, In the case of bFGF, to exclude influence from acidic FGF contained in the ordinary culture medium, HUVECs were cultured in serum-free medium supplemented with epidermal growth factor for 72 h, in the presence or absence of bFGF (10 ng/ml). The densitometric intensity of each band was normalized to β-actin, and intensities relative to the control cells are shown under each panel.

Apoptosis induction

Apoptosis was induced in Id1-, Id3-, or mock-transfected HUVECs by serum deprivation (22). After the cells were cultured without FBS for 48 h, apoptotic cells were detected with Annexin V-FITC staining (Annexin V-FITC kit; Beckman Coulter). Freshly split HUVECs with or without treatment with agonistic anti-Fas/CD95 Ab (7C11; Beckman Coulter) were used as positive and negative controls, respectively.

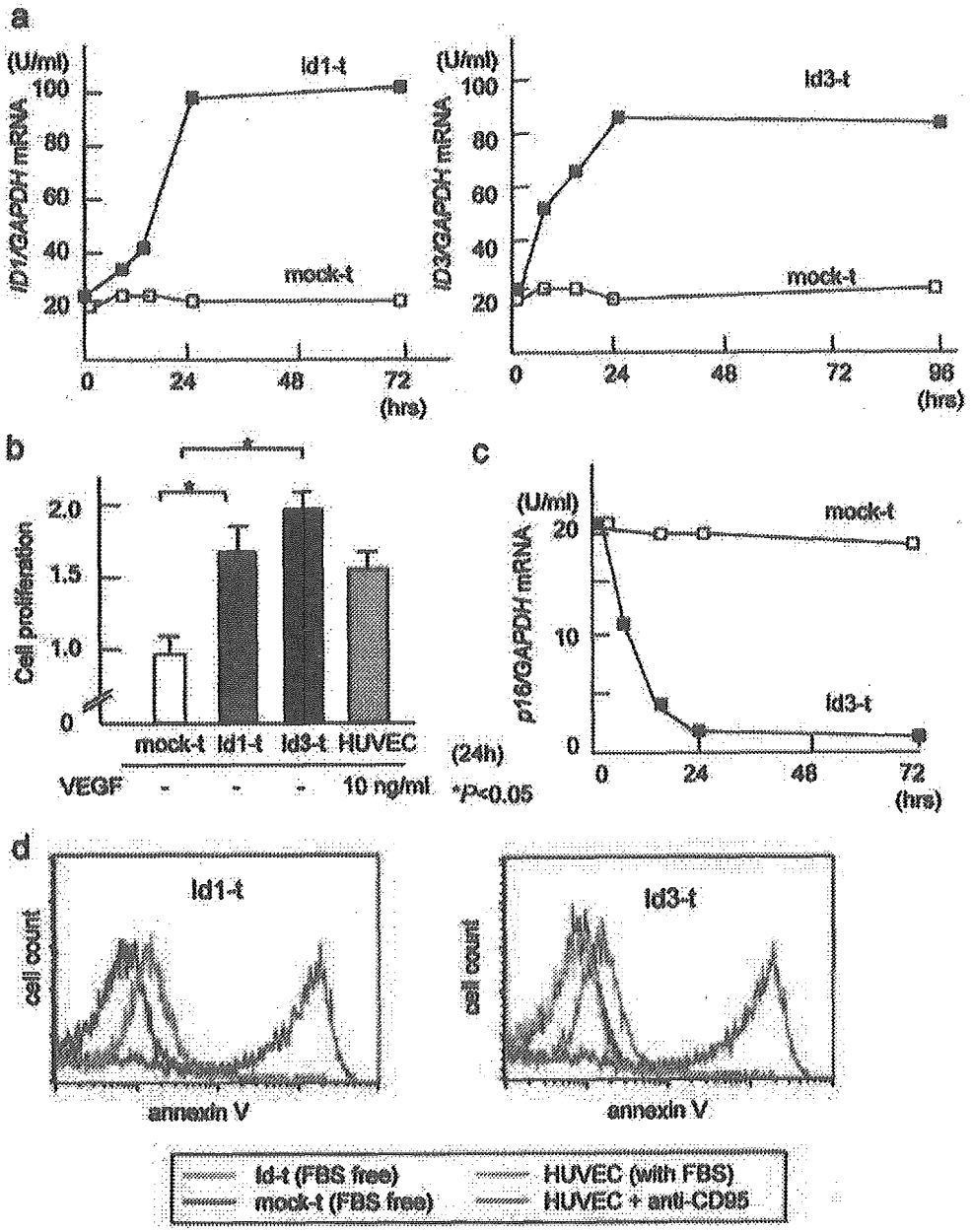

FIGURE 2. Overexpression of *ID1* or *ID3* induces proliferation of HUVECs. *a*, pBLAST49-hId1a(c) (Id1-t), pBLAST49-hId3(c) (Id3-t), or pBLAST49-mcs (mock-t) vector was transfected into HUVECs. Total RNA was isolated from each transfectant, and Id1 or Id3 mRNA level was sequentially quantitated using real-time RT-PCR. *b*, Proliferation of HUVECs at 24 h after transfection. Id1-t and Id3-t exhibited significantly higher proliferation in the absence of VEGF, compared with mock transfectants. Proliferation of Id transfectants was comparable with that of untransfected HUVECs stimulated with 10 ng/ml VEGF. Proliferation was measured using WST-1 assay. *c*, $p16^{INK4a}$ mRNA levels of *ID3* and mock transfectants were measured with real-time RT-PCR. $p16^{INK4a}$ mRNA expression was inversely correlated with Id3 mRNA shown in *a*. *d*, Apoptosis was induced in Id1, Id3, or mock transfectants by serum deprivation for 48 h, and the cells were stained with Annexin V-FITC. Freshly split HUVECs with or without treatment with agonistic anti-Fas/CD95 Ab (7C11) were used as positive and negative controls, respectively. Apoptosis induction was inhibited by overexpression of Id3, but not by Id1.

Flow cytometry

HUVECs were harvested by washing with 0.02% EDTA-PBS followed by treatment with trypsin-EDTA (InvivoGen), resuspended in PBS containing 0.1% BSA (Sigma-Aldrich) and 0.1% sodium azide, and incubated for 30 min with 50 ng/ml human γ-globulin (Sigma-Aldrich) at room temperature, to block for nonspecific binding.

For direct immunofluorescence staining, the cells were incubated with fluorescence-labeled mAbs or the isotype-matched controls for 30 min on ice, and then washed three times using a washing buffer consisting of PBS containing 0.2% BSA and 0.1% sodium azide. For indirect immunofluorescence staining, the cells were incubated with unlabeled primary mAbs or the isotype-matched Abs for 30 min on ice, washed with washing buffer, and then incubated with PE-conjugated goat anti-mouse IgG (Beckman Coulter), followed by another washing in washing buffer. Fluorescence intensity was analyzed in the EPICS XL (Beckman Coulter) or in the FACSCalibur (BD Biosciences).

Transmigration assay

Transmigration assays were performed using 8-μm-pore Transwell chambers (Corning, Corning, NY). HUVECs harvested by trypsinization were resuspended in MCDB151 medium containing 0.2% BSA, and seeded at 1×10^4 cells per well on the gelatin-coated upper chambers. Lower chambers were filled with 600 μl of the same medium supplemented with 10% FBS without or with either VEGF (20 ng/ml) or IL-1β (0.5 ng/ml). After incubation for 8 h at 37°C, 60 μl of 0.2% EDTA-PBS was added to the lower wells, and cells were harvested. The number of cells in the region corresponding to endothelial cells was counted in the EPICS, after acquiring cells in each sample for 50 s. The assays were done in triplicate.

Zymography

HUVECs were seeded on uncoated 100-mm dishes at 1×10^6 cells/well and were incubated with a 1:1 mixture of serum-free DMEM and F-12 Ham's medium (Sigma-Aldrich) for 12 h at 37°C. Then, supernatants were collected and, after removal of cells by centrifugation, were concentrated using the Centricon YM-10 concentrator (Millipore, Bedford, MA). Samples were then diluted with the same volume of sample buffer, and the MMPs were separated in SDS-PAGE using 10% polyacrylamide gels containing 0.1% gelatin. The gels were then incubated in 6.25% Triton X-100 (Wako Pure Chemical) for 1 h to remove SDS, and then placed for overnight in an incubation buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for development of enzyme activity. The gels were stained using Coomassie brilliant blue and destained in methanol/acetic acid. Gelatinase activity was detected as unstained bands.

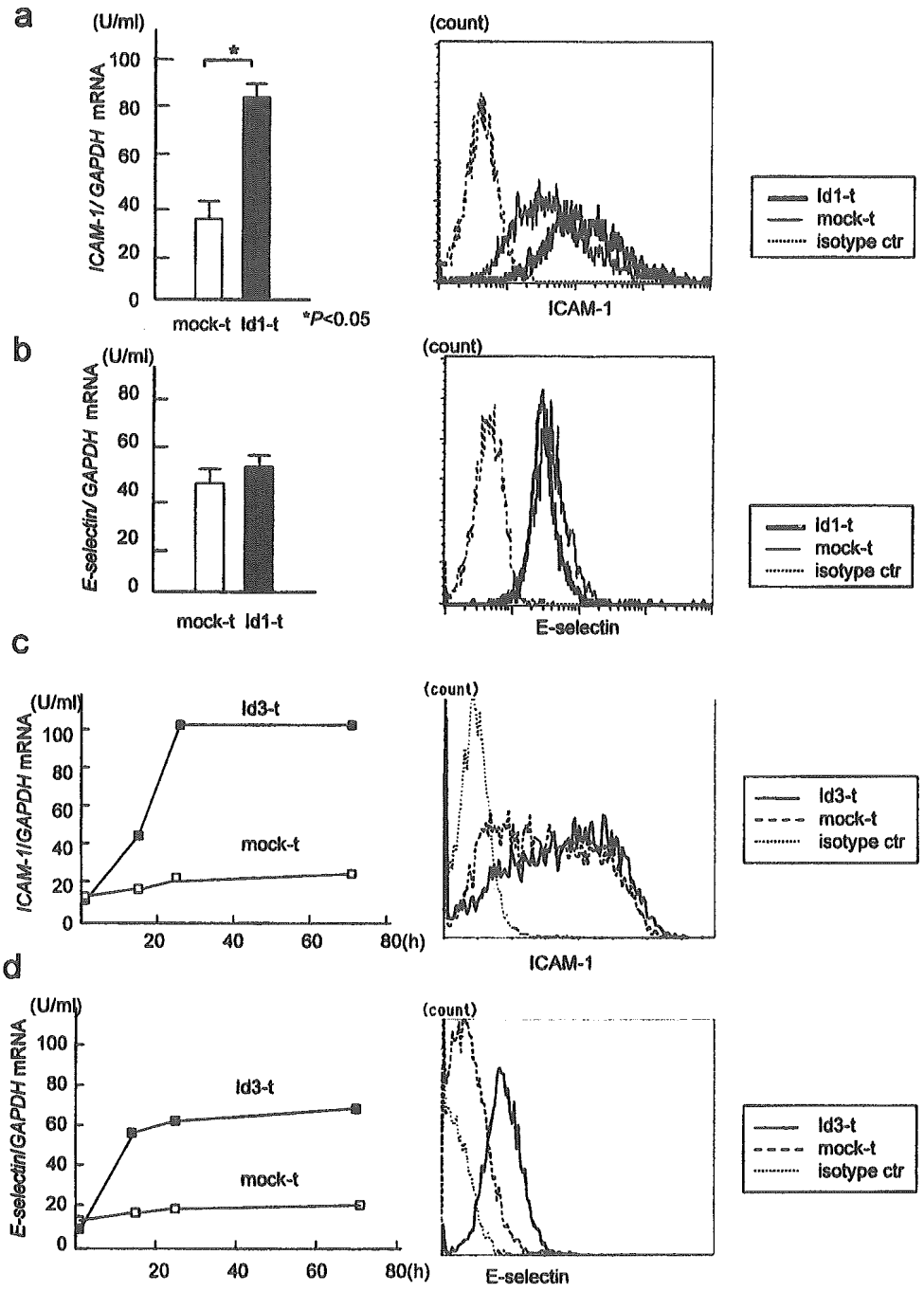

FIGURE 3. Overexpression of Id1 or Id3 induces activation of HUVECs. *a* and *b*, mRNA levels and surface expression of ICAM-1 (*a*) and E-selectin (*b*) in Id1 transfectants at 24 h after transfection. *c* and *d*, Kinetics of ICAM-1 (*c*) and E-selectin (*d*) mRNA levels in Id3 transfectants (*left*) and surface expression at 24 h. Significant up-regulation of ICAM-1 was observed in both transfectants, whereas expression of E-selectin was up-regulated only in Id3 transfectants.

In vitro tube formation

In vitro tube formation assay was performed using the Matrigel basement membrane matrix (BD Biosciences). Matrigel, kept on ice, was placed at 1 ml/well in six-well culture plates. The plates were then incubated at 37°C for 30 min to allow Matrigel to solidify. HUVECs were seeded at 5×10^4 cells per well on the top of the solidified Matrigel in the presence or absence of VEGF, and the plate was incubated at 37°C for 24 h. Tube formation on Matrigel was observed and analyzed under the microscope. The degree of angiogenesis was measured by multiplying the number of branch points by the total number of branches (23).

Statistics

Statistical significance was analyzed with Student's unpaired *t* test using StatView for Windows, version 5.0 (SAS Institute, Cary, NC).

Results

Induction of Id expression by VEGF and TGFβ in HUVECs

To gain insight into the significance of Id in relation to physiological endothelial cell activation and angiogenesis, we examined whether mRNA expression of Id1 and Id3 could be induced by known stimulators of endothelial cells such as VEGF, TGFβ, TNF-α, IL-1β, and bFGF. Significant up-regulation was observed for Id1 and Id3 mRNA when stimulated with VEGF (20 ng/ml) or TGFβ (0.5 ng/ml) (Fig. 1*a*). In contrast, expression of Id was not induced by IL-1β, TNF-α, or bFGF (Fig. 1, *b* and *c*).

Induction of proliferation of HUVECs by overexpression of Id

Next, we asked whether overexpression of Id alone could induce activation and proliferation of HUVECs. To address this question,

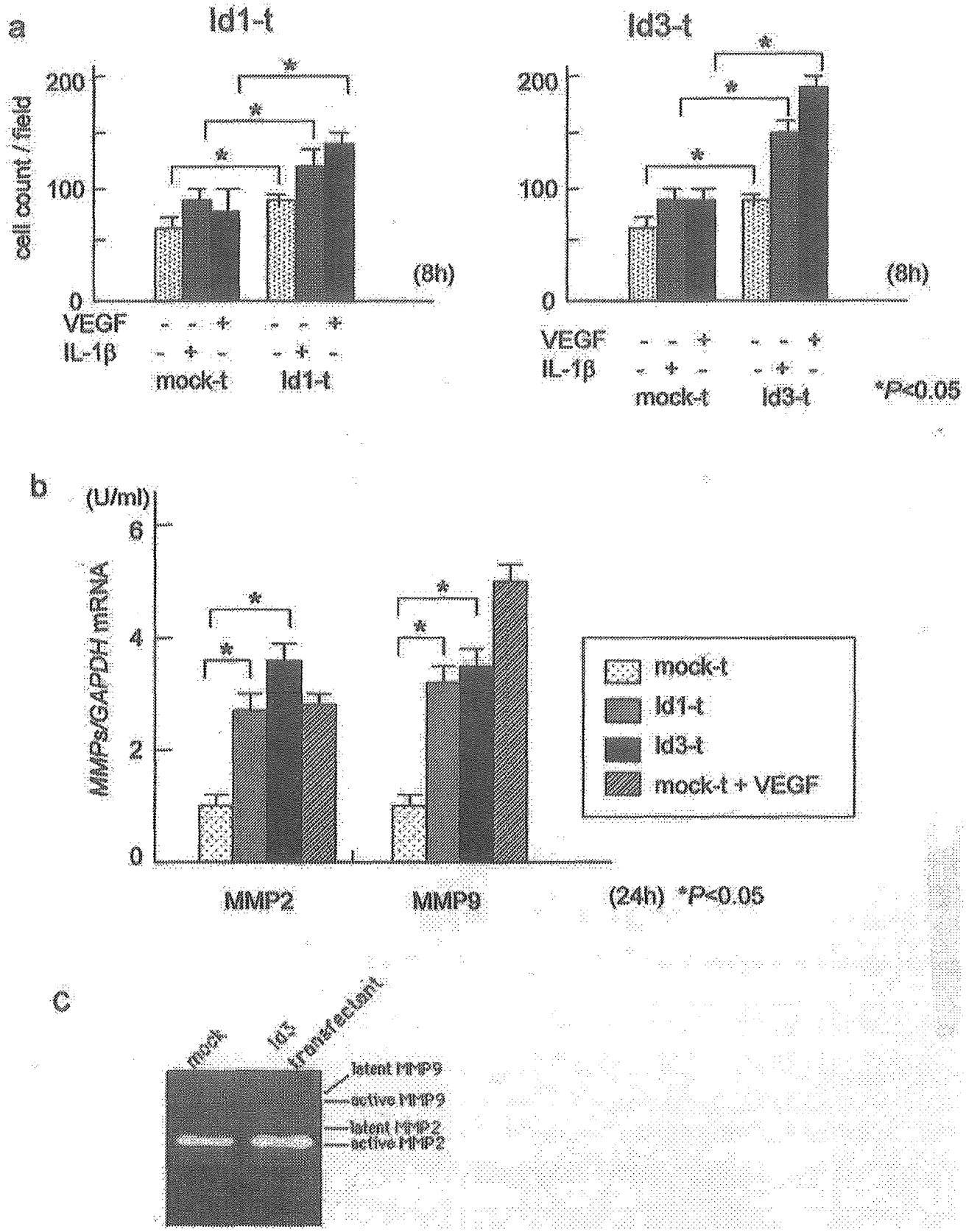

FIGURE 4. Overexpression of Id1 or Id3 induces angiogenic properties in HUVECs. *a*, Transmigration assay. Id1-t and Id3-t demonstrated significant increase in the transmigration activity, especially when the cells were stimulated with IL-1β (0.5 ng/ml) or VEGF (20 ng/ml). *b*, mRNA levels of MMP2 and MMP9. MMP2 and MMP9 expression was significantly increased in Id1-t and Id3-t. *c*, Zymography. Up-regulation of MMP2 and MMP9 activity was confirmed at the protein level.

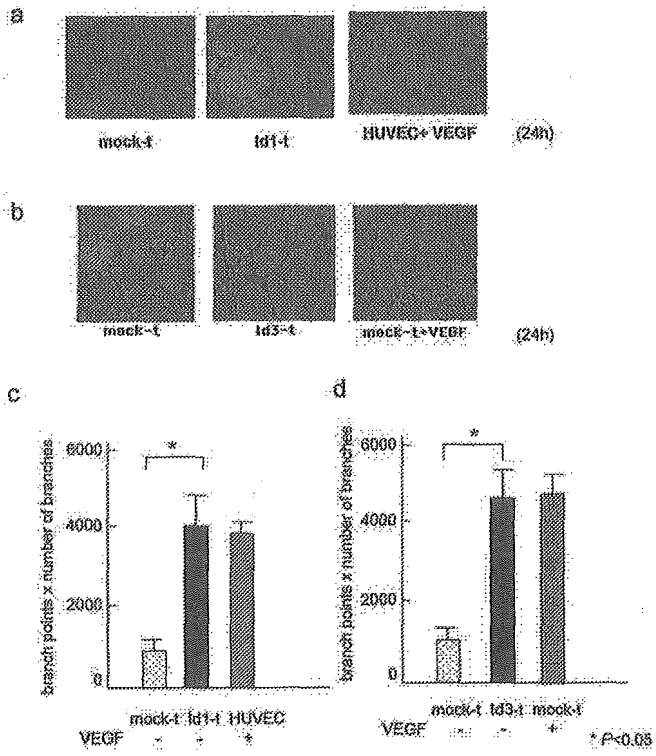

FIGURE 5. Enhanced tube formation by overexpression of Id1 or Id3 in the Matrigel. *a* and *b*, Id1-t (*a*) and Id3-t (*b*) exhibited enhanced tube formation in the Matrigel in the absence of VEGF (original magnification, ×100). *c* and *d*, The degree of tube formation in the transfectants at the basal level was comparable with mock transfectants cultured in the presence of VEGF (10 ng/ml).

transient overexpression of Id1 or Id3 was induced in HUVECs by transfection (Fig. 2*a*). Proliferation was significantly enhanced in both transfectants as compared with mock transfectant, to levels similar to the proliferative activity of untransfected HUVECs when stimulated with VEGF (Fig. 2*b*).

Id has been shown to regulate the cell cycle via transcriptional regulation of $p16^{INK4a}$ (16). In Id transfectants, the $p16^{INK4a}$ expression was found to be inversely correlated with expression level of *ID* mRNA (Fig. 2, *a* and *c*).

Id has also been shown to regulate apoptosis either positively or negatively, depending on the cell types (24, 25). The effect of Id overexpression on apoptosis was examined by serum starvation-induced apoptosis model. After the cells were cultured for 48 h without FBS, Id1 and mock transfectants exhibited comparable enhancement of annexin V binding compared with freshly split HUVEC. In contrast, annexin V binding was significantly inhibited in Id3 transfectants, suggesting that overexpression of Id3, but not Id1, inhibits HUVEC apoptosis induced by serum starvation (Fig. 2*d*).

Induction of activation markers in HUVECs by overexpression of Id

To examine whether Id overexpression alone can induce activation of HUVECs, we measured the expression levels of ICAM-1 (CD54) and E-selectin (CD62E) after transfection of Id1 or Id3 in the absence of VEGF. mRNA levels of ICAM-1 was significantly increased both in Id1 and Id3 transfectants (Fig. 3, *a* and *c*). Correspondingly, the cell surface expression of ICAM-1 was significantly up-regulated in Id1 transfectants (Fig. 3*a*) and, to a lesser extent, in Id3 transfectants (*c*). In contrast, mRNA level and surface expression of E-selectin was clearly up-regulated in Id3 transfectants (Fig. 3*d*) but not in Id1 transfectants (*b*). These results suggested that both Id1 and Id3 can induce activation of HUVEC, but their downstream pathways may not be identical.

Induction of angiogenic processes by overexpression of Id

We next examined whether overexpression of Id in HUVECs can induce angiogenesis. Both Id1 and Id3 transfectants exhibited significant increase in the transmigration activity, especially when the cells were cultured with IL-1β (0.5 ng/ml) or VEGF (20 ng/ml) (Fig. 4*a*).

MMP2 and MMP9 are MMPs relevant to angiogenic processes. Basal expression levels of MMP2 and MMP9 mRNA were significantly increased both in Id1 and Id3 transfectants, and were comparable to those of VEGF-stimulated mock transfectants (Fig. 4*b*). Zymography confirmed up-regulation of MMP2 and MMP9 enzymatic activities (Fig. 4*c*). In the transfectants, not only the total MMP2 and MMP9 expression levels, but also expression levels of the active forms of MMP2 and MMP9 were increased compared with mock transfectants. Furthermore, tube formation in the Matrigel assay was markedly enhanced both in Id1 and Id3 transfectants even in the absence of VEGF (Fig. 5). Taken together, these observations indicate that overexpression of Id alone can induce angiogenic processes in HUVECs.

Inhibition of VEGF-induced proliferation and activation of HUVECs by ID1 *and* ID3 *shRNA*

In the next sets of experiments, we addressed the question whether Id proteins are essential for proliferation, activation, and angiogenic processes of HUVECs induced by VEGF. To inhibit expression of Id1 and Id3, we used RNA interference (RNAi). HUVECs were doubly transfected with RNA expression vectors containing *ID1* and *ID3* shRNA at 0.125, 0.5, or 1 ng/ml. A dose-dependent inhibition of VEGF-induced Id3 (Fig. 6*a*) and Id1 (data not shown) expression was observed. Subsequent studies were conducted using 1 ng/ml *ID1* and *ID3* shRNA transfectants.

Id1/Id3 double-knockdown transfectants exhibited significantly decreased proliferation when stimulated with VEGF (Fig. 6*b*). With respect to adhesion molecules, mock transfectants showed modest up-regulation of surface expression of ICAM-1 (Fig. 6*c*), E-selectin (*d*), and substantial up-regulation of α_v integrin (*e*), when stimulated with VEGF. In *ID1* and *ID3* shRNA transfectants, up-regulation of these molecules were completely inhibited (Fig. 6, *c–e*). When HUVECs were treated with either *ID1* or *ID3* shRNA, VEGF-induced ICAM-1 expression was inhibited, but induction of E-selectin and α_v integrin were not (data not shown).

We next tested whether Id1/Id3 knockdown also inhibits HUVEC activation by IL-1β and TNF-α. IL-1β modestly up-regulated ICAM-1 expression, which was not inhibited by knockdown of Id1 and Id3 (Fig. 6*f*). In contrast, TNF-α-induced up-regulation of E-selectin was inhibited by knockdown of Id1 and Id3 (Fig. 6*g*).

Inhibition of VEGF-induced angiogenesis by ID1 *and* ID3 *shRNA*

We next examined whether Id1/Id3 double knockdown can inhibit the VEGF-induced angiogenic processes of HUVECs. VEGF-induced transmigration activities were abolished in *ID1* and *ID3* shRNA transfectants (Fig. 7*a*). mRNA expression of MMP2, but not MMP9, was significantly decreased by Id1/Id3 knockdown, when HUVECs were cultured in the presence of VEGF (20 ng/ml) (Fig. 7*b*). VEGF-induced tube formation in Matrigel was markedly decreased in *ID1* and *ID3* shRNA transfected cells (Fig. 7, *c* and *d*). Such inhibition of the angiogenic processes was accompanied by a down-regulation of α_2 and β_1 integrins in *ID1* and *ID3* shRNA transfectants (Fig. 6, *h* and *i*). Thus, these results indicate

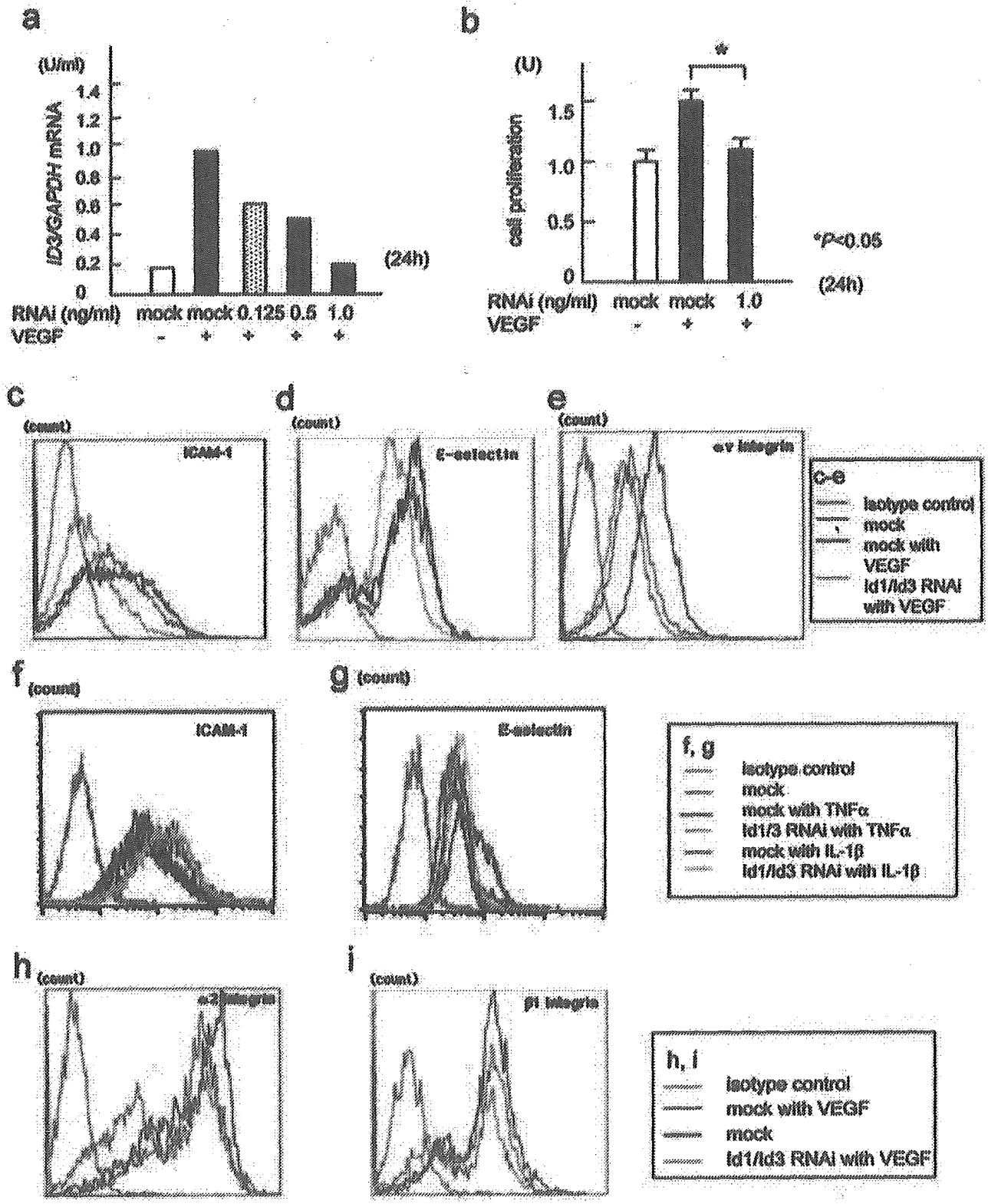

FIGURE 6. Silencing of Id1 and Id3 inhibits VEGF-induced proliferation and activation of HUVECs. *a*, HUVECs were transfected with *ID1* and *ID3* shRNA expression vectors at various concentrations or pSilencer alone (mock). After HUVECs were cultured for 24 h with or without VEGF (20 ng/ml), mRNA levels of *ID3* and *GAPDH* were quantitated. Dose-dependent suppression of *ID3* induction was observed. *ID1* induction was also inhibited (data not shown). *b*, Reduced VEGF-induced proliferation of double-knockdown transfectants. Proliferation was analyzed using WST-1 at 24 h. VEGF-induced proliferation was significantly inhibited in Id1/Id3 double-knockdown transfectants. *c–e*, Inhibition of VEGF-induced activation of HUVECs by Id1/Id3 double knockdown. Surface expression of ICAM-1 (*c*), E-selectin (*d*), and α_v integrin (*e*) was measured in mock transfectants with or without stimulation with VEGF (20 ng/ml), and *ID1* and *ID3* shRNA double transfectants stimulated with VEGF. Id1/Id3 double knockdown resulted in inhibition of VEGF-induced expression of ICAM-1, E-selectin, and α_v integrin. *f*, ICAM-1 expression was modestly up-regulated at 24 h after IL-1β treatment (1 ng/ml) in mock-t, which was not inhibited by knockdown of Id1/Id3. *g*, E-selectin expression was up-regulated at 5 h after TNF-α treatment (20 ng/ml) in mock-t, which was inhibited by silencing of Id1 and Id3. *h* and *i*, Surface expression of α_2 (*h*) and β_1 (*i*) integrins in *ID1*, *ID3* shRNA transfectants stimulated with VEGF (20 ng/ml). Both integrins were down-regulated.

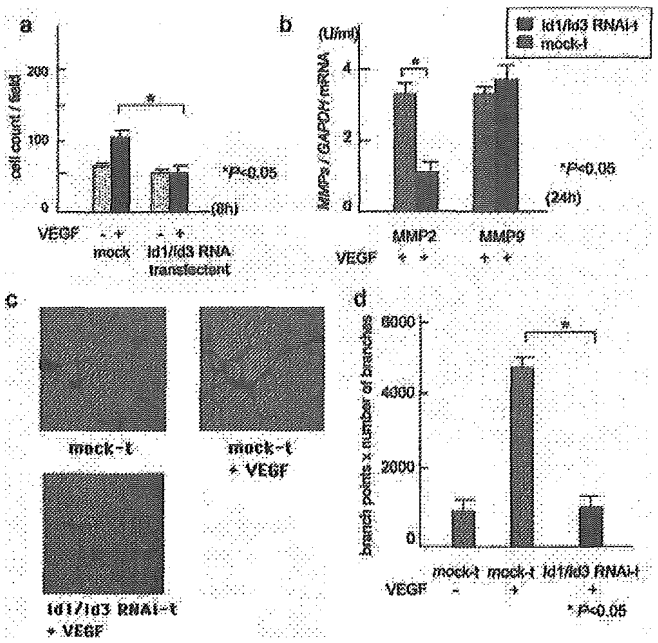

FIGURE 7. Silencing of Id1 and Id3 inhibits VEGF-induced angiogenic processes of HUVECs. *a*, Transmigration activity was significantly decreased in *ID1* and *ID3* shRNA transfectant after stimulation with VEGF (20 ng/ml) for 8 h. *b*, MMP mRNA expression in mock or *ID1* and *ID3* shRNA transfectants cultured for 8 h in the presence of VEGF (20 ng/ml). MMP2 mRNA expression was decreased in the double-knockdown cells, whereas MMP9 mRNA was not inhibited. *c*, Tube formation assay. Untransfected, mock-transfected, and *ID1*, *ID3* shRNA double-transfected HUVECs were cultured in the presence of VEGF (20 ng/ml) for 24 h. Marked inhibition of tube formation was observed by Id1/Id3 double knockdown. Original magnification, ×100. *d*, Quantitation of tube formation.

that the expression of Id plays a crucial role in the VEGF-induced angiogenic processes in HUVECs.

Discussion

In the present study, we demonstrated that overexpression of Id alone can induce proliferation, activation, and angiogenic processes of HUVECs in the absence of VEGF, to levels similar to that of VEGF-stimulated untransfected HUVECs. Moreover, knockdown of *ID1* and *ID3* in HUVECs almost completely abolished the VEGF-induced proliferation, activation, and angiogenic processes. These findings indicate a crucial role for Id in some of the VEGF signaling pathways in HUVECs.

Using $Id1^{+/-}Id3^{-/-}$ mice, it has been shown that Id expression is required to support angiogenesis in tumors (19), and that recruitment of $VEGFR1^{+}$ myeloid cells and $VEGFR2^{+}$ circulating endothelial precursor cells expressing Id is necessary for tumor growth (26). The new information presented in this study, including the requirement of Id also in human endothelial cells, up-regulation of ICAM-1 and E-selectin by forced expression of Id, and inhibition of endothelial cell activation and angiogenesis by double knockdown of Id1 and Id3, further emphasizes the crucial role of Id in endothelial cell activation and angiogenesis.

Previous reports demonstrated that Id controls the cell cycle in several ways (15), for example, by binding to Rb protein and blocking its tumor suppressor function (6, 12), or by inhibiting the binding of Ets1 and Ets2 transcription factors to $p16^{INK4a}$ promoter and repressing its expression (16). Our data suggested that at least the latter mechanism is operative in HUVECs. Id family proteins have been generally shown to promote apoptosis in a variety of conditions (24). However, in some settings, Id was shown to have antiapoptotic activity (25). Our present data suggested that overexpression of Id3, but not Id1, can protect HUVEC from apoptosis induced by serum starvation. In HUVECs, it was previously shown that VEGF prevents apoptosis induced by serum starvation (22). These results suggest that Id3 may mediate antiapoptotic effect induced by VEGF in HUVECs, and such an effect may also partly account for enhanced proliferation of Id3 transfectants.

As for ICAM-1 and E-selectin induction, it was recently demonstrated that VEGF induces these adhesion molecules in HUVECs through NF-κB (27), and that Id1 activates NF-κB transcription in prostate cancer cells (25). Our present data provide evidence that Id is involved in the signaling pathway that connects VEGF receptors and NF-κB activation in HUVECs. In our system, we have not distinguished contribution of each VEGFR for the induction of Id, which should be investigated in future studies. Although gene silencing of Id1 and Id3 efficiently inhibited ICAM-1 and E-selectin induction by VEGF and TNF-α, up-regulation of ICAM-1 by IL-1β was not inhibited. Taken together with the lack of *ID1* and *ID3* mRNA induction after stimulation with IL-1β, our results suggest that Id may not be involved in the IL-1β pathway of HUVEC activation. In the case of TNF-α stimulation, because mRNA of *ID* was not up-regulated, the presence of basal level of Id may be necessary for the activation of HUVEC by TNF-α. The signaling pathways of VEGF, TNF-α, and IL-1β in endothelial cells have not yet been fully delineated (28), and further studies are necessary to address the relationships of these pathways and Id.

When HUVECs were treated with VEGF or TGFβ, expression of Id was induced. Expression of Id3 has been shown to be induced by the Ras-ERK pathway in thymocytes (17), and through type I receptor of the TGFβ via Smad1/5 signaling in HUVECs (18), whereas Id1 expression was associated with Raf/MEK1/2 activation in a human prostatic cancer cell line (29) and Smad1/5 signaling in HUVECs (18). Induction of Id in HUVECs by VEGF is reasonable, because MAPK cascade exists in the downstream of VEGF signaling pathways (30). In contrast, stimulation with IL-1β, TNF-α, or bFGF did not induce Id1 or Id3 in HUVECs, suggesting that Id may not be a ubiquitous mediator of inflammation, but more specifically associated with signaling pathways leading to angiogenesis.

For the knockdown experiments, we used double knockdown of Id1 and Id3, because knockdown of either one of them resulted in only partial inhibition of VEGF-induced activation or angiogenesis. Such results were expected, because it has been demonstrated that Id1 and Id3 have similar promoter region sequences, and exert overlapping biochemical functions; thus either one might be able to compensate the other, at least partially. In contrast, our results indicated that knockdown of both Id1 and Id3 was sufficient to abolish most of the angiogenic processes induced by VEGF in HUVECs, and Id2 and Id4 cannot compensate for the lack of Id1 and Id3. *ID1* and *ID3* shRNA sequences were designed from the 5′ region specific to each gene. Because RNAi can block gene expression only when the shRNA sequence is highly matched with the target gene (31), Id2 and Id4 expression were not considered to be suppressed.

Although Id3 overexpression up-regulated MMP9, knockdown of Id did not inhibit VEGF-induced up-regulation of MMP9. This suggests the presence of other pathways that lead to MMP9 up-regulation. Nevertheless, Id knockdown resulted in marked inhibition of tube formation in Matrigel. Such discrepancy may be explained by reduced expression of α_2 and β_1 integrins, whose

ligands are collagen and laminin, the main components of basement membranes and also of Matrigel (32, 33). Thus, Id seems to be involved in multiple pathways leading to activation of endothelial cells and angiogenesis. Although the functions of Id1 and Id3 are substantially overlapping (11, 14), it is intriguing to delineate the pathways from each Id protein to a multitude of endothelial cell activation and angiogenic processes. Indeed, our observations indicated some notable differences between the effects of Id1 and Id3 overexpression; for example, Id1 overexpression failed to up-regulate E-selectin and to protect HUVEC from apoptosis induced by serum starvation. It has recently been reported that Id1 down-regulates expression of thrombospondin-1, a suppressor of angiogenesis (34). It is also of interest to examine whether all of the angiogenic processes associated with Id can be explained by down-regulation of thrombospondin-1. To gain further insights, DNA microarray analysis is underway.

In conclusion, we demonstrated that Id plays an indispensable role for the VEGF-induced activation and angiogenic processes of HUVECs. Based on the inhibition of endothelial cell activation and angiogenesis with RNAi, as well as the low expression of Id in most of the normal adult tissues, Id should be considered an attractive target for the development of new therapeutic approaches for disorders associated with excessive angiogenesis.

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