the NBT/BCIP mixture at room temperature until color development.

Results

Effects of TCDD exposure on chick embryo heart and liver

We injected TCDD or corn oil vehicle control into white leghorn eggs prior to incubation. After incubation for 12 (E12) or 18 days (E18), chick embryos were dissected. Control embryos exhibited 20% mortality (4/20), whereas TCDD-exposed embryos exhibited 65% mortality (39/60) at E12. At E12, hearts from TCDD-exposed embryos were larger than control hearts, and histological examination revealed that most instances of enlargement occurred in left ventricle (Fig. 1A). To establish morphological change in the heart, we measured the wet weight of hearts. Heart wet weight relative to body wet weight increased when hearts were exposed to TCDD for 12 or 18 days (Fig. 1B). An increase in ANF, one of the hallmarks of the heart disease [31], was observed in hearts from E12 embryos (Fig. 2). Thus, morphological and functional alterations were evident in TCDD-exposed chick embryo hearts. Morphological abnormality was also observed in livers of TCDD-exposed chick embryos. TCDD exposure partially altered the color of the liver from pale pink to yellowish white (Fig. 3A). These changes in color were detected in both E12 and E18 livers (data not shown). However, no significant change in liver wet weight was detected when we compared the wet weights of TCDD-exposed and control livers, regardless of incubation periods (Fig. 3B). High-magnification images of TCDD-exposed liver sections showed that clearly extensive vesicle formation occurred in hepatocytes and that the abnormal vesicle formation was restricted to the periphery of TCDD-exposed liver, where color change was observed macroscopically (Figs. 4A–C). Accumulation of fatty vesicles was confirmed by Sudan IV staining (Fig. 4D). Staining of fatty vesicles was obvious in the region where abnormal vesicular formation was observed, suggesting that extensive vesicular fatty metamorphosis was induced in the periphery of TCDD-exposed liver.

Contribution of AhR-CYP1A pathway to TCDD signaling

Transcriptions of CYP1A family genes are initiated by binding of the complex to the DRE region [16,17]. To assess AhR expression at the protein level, an anti-AhR polyclonal antibody was generated. Affinity-purified anti-AhR antibody was specifically reacted with approximately 100-kDa peptide in both the heart and liver (Fig. 5A). In chick embryo, expression of AhR is detected from E2 to E10 in the heart and liver [23]. Consistent with the findings of Walker et al., Western blot analysis in the present study revealed constant AhR expression in the heart throughout embryogenesis. Expression of AhR was observed at approximately the same level in the liver as in the heart throughout embryogenesis (Fig. 5B).

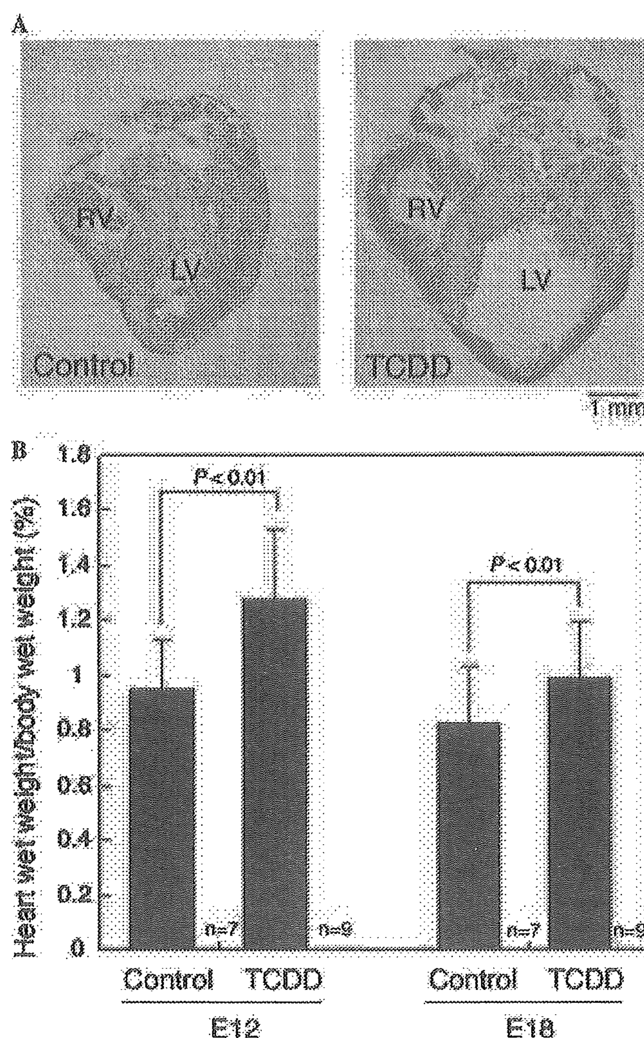

Fig. 1. (A) Representative images of frontal sections of chick embryo hearts 12 days after TCDD (1.0 pmol/g egg) or vehicle (corn oil for control) was injected into egg yolks. Sections are stained with hematoxylin and eosin. The TCDD-exposed heart (right panel) has an enlarged ventricular cavity compared to that of the control heart (left panel). RV, right ventricle; LV, left ventricle. (B) Heart wet weights after 12 (E12) and 18 days (E18) of incubation in the presence or absence (for control) of TCDD (1 pmol/g egg). Weights are relative to body wet weight. The *P* values refer to comparisons between TCDD-exposed and control hearts. The number of embryos in each group is specified.

To determine whether CYP1A4 and CYP1A5 gene expression occurred under our experimental condition, Northern blot analysis for these enzymes was done. In both analyses, an increase in the mRNAs was observed only in the liver when embryos were exposed to TCDD for 12 days, and no signals were observed in the heart

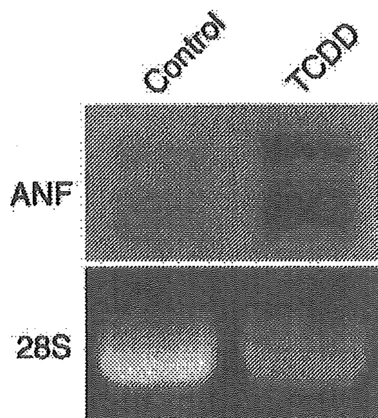

Fig. 2. Northern blot analysis of ANF expression levels in chicken embryo hearts. Approximately 20 μg total RNA was dissolved by agarose gel electrophoresis. Induction of ANF expression in the E12 heart was examined in TCDD-injected chick embryos. 28S, 28S ribosomal RNA. Control, vehicle (corn oil) was injected into egg yolks.

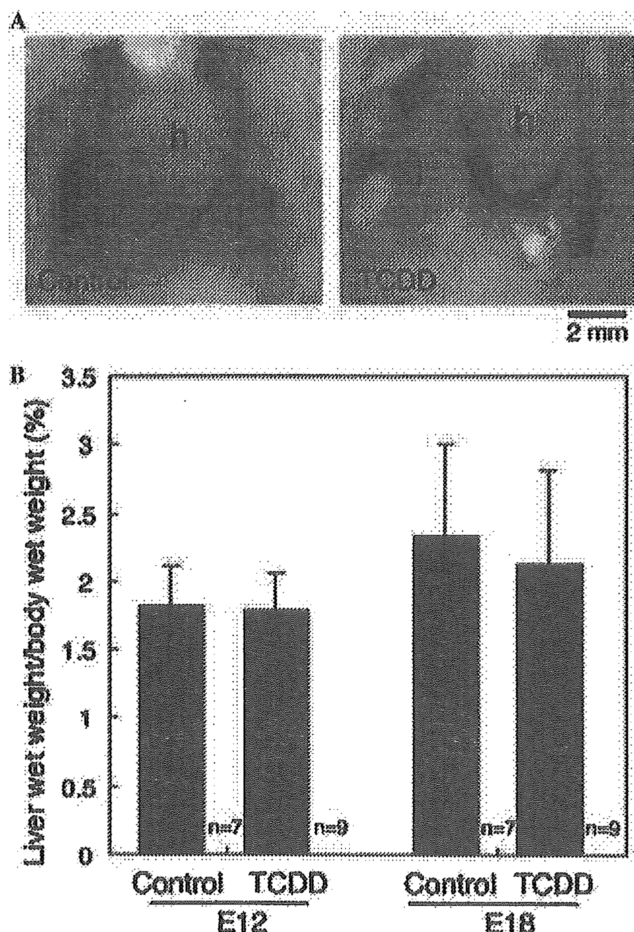

Fig. 3. (A) Representative image of abnormal liver in embryos collected after 12 days of incubation in the presence of TCDD (right panel). Control embryos (left panel) were exposed to vehicle only. An obvious change in color is seen in the TCDD-exposed liver. Arrowhead indicates the areas of color change: (h) heart, (l) liver. (B) Liver wet weights after 12 (E12) and 18 days (E18) of incubation in the presence or absence of TCDD. Weights are relative to body wet weight. The number of embryos in each group is specified.

(Fig. 6). No induction of CYP1A family genes in the heart was observed in E6, E8, and E10 TCDD-exposed chick embryos (data not shown). We further analyzed expression of the CYP1A gene by in situ hybridization. Signals for CYP1A4 were evident in TCDD-exposed liver, although the signal was confined to the normal liver tissue in which no vascular fatty metamorphosis was observed (Fig. 7). No significant difference was observed in the heart when the CYP1A gene was detected with antisense or sense probes (data not shown). Our data show clearly that AhR-mediated induction of CYP1A family gene expression occurred only in the liver after eggs were exposed to TCDD, although nearly the same amount of AhR protein was present in the heart.

Accumulation of TCDD in the liver was reported after in ovo injection [32–34]. This simply raises the question whether the different responses of the heart and liver to TCDD are caused by differences in TCDD concentration or by the nature of each cell type.

EROD assay

We prepared primary cultures of cardiac myocytes and hepatocytes from E12 embryos, and the same number of each type of cell was seeded into 96-well plates. EROD activity in wells was measured as an index of CYP1A family enzyme induction. Weak EROD activity induced by TCDD exposure was detected after 24 h of culture of cardiac myocytes, whereas EROD activity of TCDD-exposed hepatocytes was markedly increased (Fig. 8). Exposure to a high concentration (10 nM) of TCDD resulted in a decrease of EROD activity in hepatocytes. These data show that the different responses to TCDD of the heart and liver were due to the nature of each cell type.

Discussion

We found that morphological and functional alterations induced by TCDD were marked in the heart and liver of the chick embryo. The ventricular cavity of the heart was enlarged, and an increase in heart wet weight was observed. These alterations are implicated in the functional changes induced by TCDD exposure. Previous studies showed dilated cardiomyopathy with an increased heart weight induced by TCDD in E10 and E12 chick embryos [12,23]. In our experiment, induction of ANF, a hallmark of heart disease, was detected in the heart exposed to TCDD. This result supports the idea that the observed morphological alterations in heart were the result of functional defects induced by TCDD. We injected TCDD into egg yolks prior to incubation at a concentration of 1 pmol/g egg, a concentration approximately twice as high as that of LD_{50} for white leghorn chickens (*Gallus domesticus*) [7]. This might

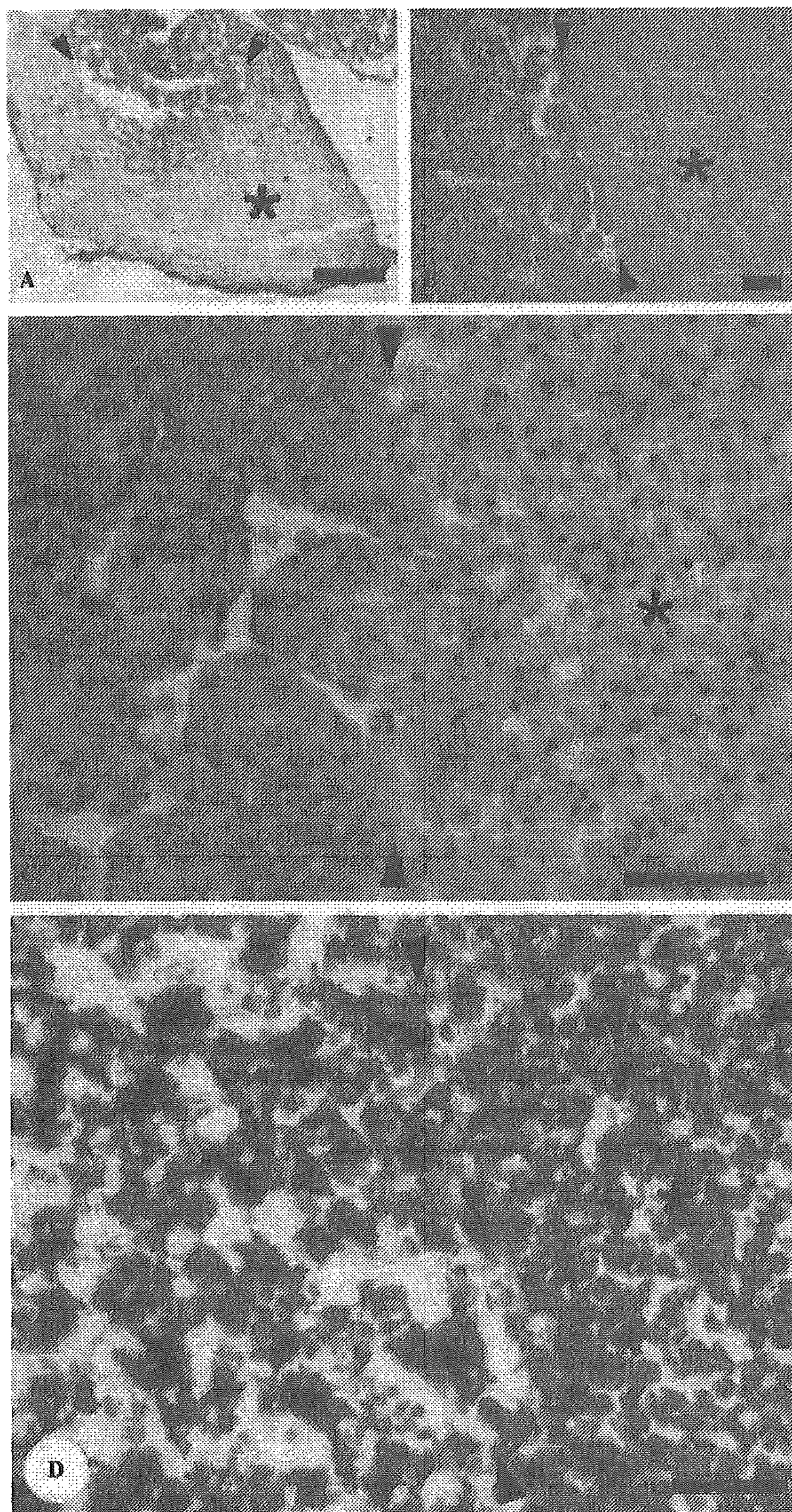

Fig. 4. Histological analysis of TCDD-exposed liver. TCDD (1 pmol/g egg) was injected into egg yolks prior to incubation. The liver dissected from chick embryos after 12 days of exposure to TCDD was fixed, sectioned, and stained with hematoxylin and eosin. Images of different magnifications from lower (A) to higher (C). Fatty vesicles are stained with Sudan IV (D). Arrowheads indicate boundaries between normal and abnormal liver tissue where color change is seen macroscopically (asterisk). Accumulation of fatty vesicles was observed in hepatocytes and was restricted to the abnormal liver. Bars indicated in (A)–(D) are 1 mm and 50 μm, respectively.

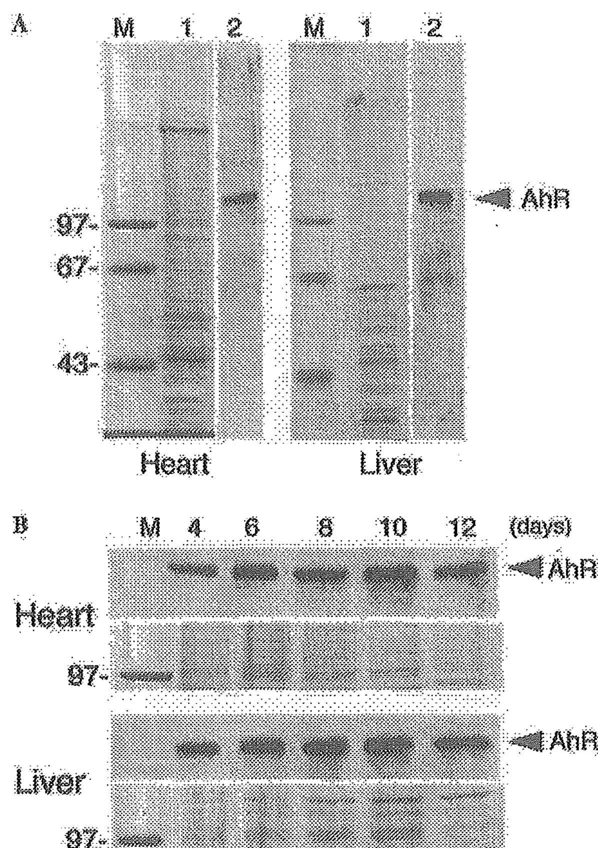

Fig. 5. (A) Western blot analysis of AhR with the purified anti-AhR antibody. Proteins (15 μg) from E12 embryo heart and liver were resolved by SDS–PAGE and stained with Coomassie brilliant blue (lane 1). The purified anti-AhR antibody reacted specifically with an approximately 100-kDa peptide both in heart and liver (lane 2). Lane M shows molecular weight markers, which are specified by numbers to the left of the panels. (B) Change in expression levels of AhR in heart and liver was examined by Western blot analysis. Heart (upper two panels) and liver (lower two panels) extracts (15 μg) from embryos at 4, 6, 8, 10, and 12 days were resolved by SDS–PAGE. Coomassie brilliant blue stained gels are shown on the lower panel of each pair. Western blots with the purified anti-AhR antibody are shown on upper panel of each pair. Levels of AhR expression are constant in the heart and liver throughout embryogenesis. Arrowheads indicate the expected molecular weight for AhR. Lane M shows molecular weight markers; numbers to the left of the panels indicate the 97-kDa marker peptide.

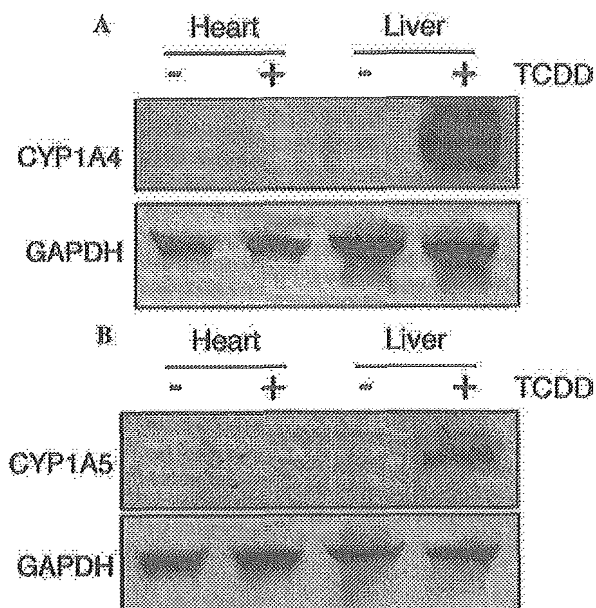

Fig. 6. Induction of CYP1A4 and CYP1A5 12 days after injection of TCDD or vehicle into egg yolks. Northern blots include mRNA of GAPDH as an internal standard. Induction of CYP1A4 (A) and CYP1A5 (B) by TCDD is evident only in the liver. Control (–), 1 pmol TCDD/g egg (+).

explain the high mortality (65% on E12) in our experiment. Even with this high concentration of TCDD, no significant change in wet weight of embryonic liver was observed. There are a number of studies that showed a statistically significant increase in heart weight [7,12,23]; however, inconsistent changes in liver weight were observed [7,32].

TCDD-induced toxicity is mediated through binding to AhR. Therefore, we examined expression of AhR at the protein level. Commercially available antibody raised against human AhR was less sensitive to chicken AhR (data not shown); therefore, expressed chicken AhR was used as antigen to obtain a specific antibody. Western blot analysis with the specific antibody showed that approximately the same levels of AhR were expressed in the heart and liver throughout embryogenesis. However, our results showed that a principal AhR response, induction of the CYP1A family genes, occurred in the liver but not in the heart in response to TCDD. Gannon et al. [20] reported that CYP1A4 was induced in chick embryo hearts when TCDD was injected into eggs. However, they injected TCDD at late stages of embryogenesis (E15 or E16) and used a 20-fold higher concentration of TCDD (1 nmol/egg) than we did in our experiments (1 pmol/g egg; approximately 0.05 nmol/egg) for a shorter exposure time (24 h). Walker et al. [23] reported that, under conditions similar to ours, AhR was expressed continuously in cardiac myocytes during cardiogenesis. They also reported that no induction of CYP1A family genes by TCDD was detectable at protein or mRNA levels except at the distal outflow tract of the embryonic heart. Our EROD assays confirmed that induction of CYP1A4 and CYP1A5 genes occurred only in the hepatocytes of E12 chick embryos, although weak activity indistinguishable from that in control hearts was observed. The same effects were observed when cells were exposed to TCDD for 48 h (data not shown), indicating that the CYP1A family genes were not inducible in cardiac myocytes in embryos or monocultures.

TCDD exposure also altered the function of the liver. A change in color of the liver surface from pale pink to yellowish white was observed. Histochemical analysis revealed a fatty vascular metamorphosis at the liver

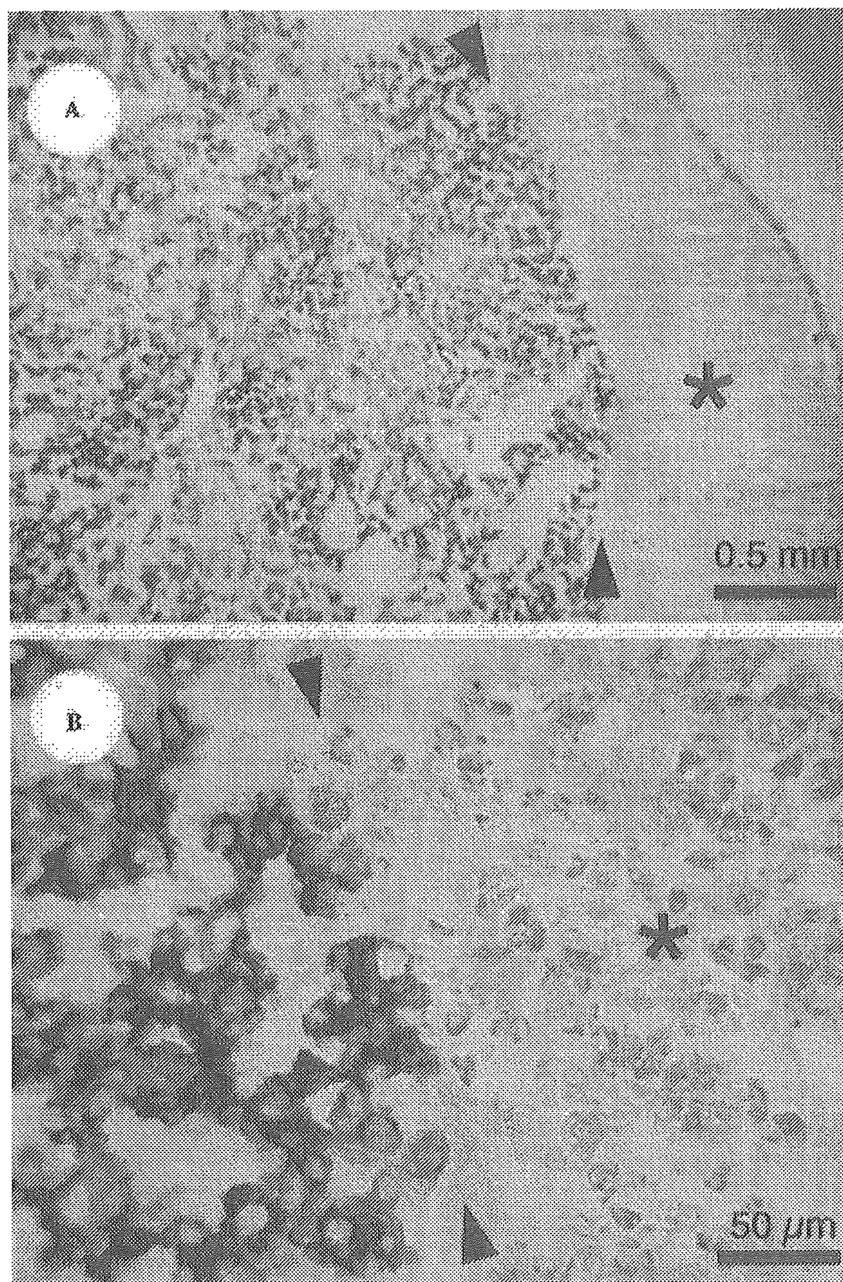

Fig. 7. In situ hybridization study of liver section with probes for CYP1A4. The liver dissected from chick embryos after 12 days of exposure to TCDD was fixed and sectioned, and the signals for CYP1A4 were detected with DIG-labeled antisense probes. Low (A) and high (B) magnification images show restricted expression of CYP1A4 in normal liver tissue but not in abnormal liver tissue (asterisk). Arrowheads indicate the boundary line between normal and abnormal liver tissue.

periphery, where the color change was observed. Similarly, alteration in hepatocytes with transient microvesicular formation was observed in the liver of AhR null mice. And the vesicular formation was due to a metabolic deficit in hepatocyte function [35]. Studies of TCDD toxicity in the liver have been done with 7- to 9-week-old immature chicks. And it is reported that lipoprotein lipase and glucose transporting activities were decreased by TCDD, which resulted in reduced fatty acid synthesis, wasting syndrome, profound loss of adipose tissue and lean body mass, and increased liver wet weight [36,37]. We consistently observed an accumulation of fatty vesicles in the liver. In situ hybridization analysis showed that the CYP1A gene was not expressed in the liver periphery of TCDD-exposed embryos. This novel finding suggests alteration of hepatocytes induced by TCDD toward a loss of function for metabolizing xenobiotics. The CYP1A gene can produce reactive oxygen species during its catalytic cycle, and CYP1A-based production of H_2O_2 suppresses expression of the CYP1A gene itself in HepG2 cells [38]. Decrease of EROD activity in hepatocytes as shown in Fig. 8 may due to the same suppressive effects of

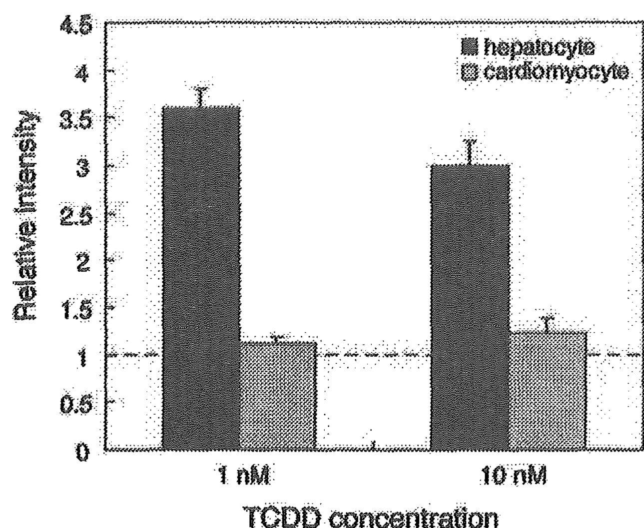

Fig. 8. Induction of EROD activity in hepatocytes and cardiac myocytes from chick embryos after 12 days of exposure to TCDD. Average EROD intensity relative to control intensity is given for two TCDD concentrations. A relative intensity of 1.0 means there is no TCDD effect. Error bars represent standard deviations.

the reactive oxygen. Thus, absence of CYP1A gene expression in the liver periphery of TCDD-exposed embryos can be explained by a similar autoregulation mechanism.

Possible mechanisms for TCDD-induced toxicity have been postulated. Toxicity might result from transcriptional activation or repression of genes by binding the AhR–Arnt complex to the regulatory sequence upstream of the genes. TCDD toxicity might also result from the regulatory alteration of unrelated genes by sequestering bHLH-PAS proteins [35]. Transcriptional activation of toxicity-implicated genes by TCDD has been postulated because immunohistochemical analysis revealed that AhR and Arnt are present in the heart [23].

We found that expression of the CYP1A family genes was induced in the liver but not in the heart. Our findings suggest that the signaling cascade through AhR in response to TCDD is different in the heart from that in the liver. Paracrine factors, for example, may be implicated in the pathway leading to heart defects. Defects in the liver or other organs cause an increased circulatory load. The increased load induces expression of paracrine factors such as endothelin that can induce cardiac hypertrophy [39]. Further analysis is needed to elucidate the mechanism underlying the heart defects induced by TCDD. The physiological function of AhR in the heart should also be determined.

Acknowledgments

We thank Professor N. Miyairi (College of Pharmacy, Nihon University) for technical assistance. This work was supported in part by a Special Assistance Grant for Promoting the Advancement of Education and Research Program.

References

[1] A. Poland, J.C. Knutson, Annu. Rev. Pharmacol. Toxicol. 22 (1982) 517–554.
[2] M.I. Luster, R.E. Faith, G. Clark, Ann. N.Y. Acad. Sci. 320 (1979) 473–486.
[3] M.W. Hornung, J.M. Spitsbergen, R.E. Peterson, Toxicol. Sci. 47 (1999) 40–51.
[4] T.R. Henry, J.M. Spitsbergen, M.W. Hornung, C.C. Abnet, R.E. Peterson, Toxicol. Appl. Pharmacol. 142 (1997) 56–68.
[5] C.L. Summer, J.P. Giesy, S.J. Bursian, J.A. Render, T.J. Kubiak, P.D. Jones, D.A. Verbrugge, R.J. Aulerich, J. Toxicol. Environ. Health 49 (1996) 409–438.
[6] C.L. Summer, J.P. Giesy, S.J. Bursian, J.A. Render, T.J. Kubiak, P.D. Jones, D.A. Verbrugge, R.J. Aulerich, J. Toxicol. Environ. Health 49 (1996) 389–407.
[7] D.C. Powell, R.J. Aulerich, J.C. Meadows, D.E. Tillitt, J.P. Giesy, K.L. Stromberg, S.J. Bursian, Arch. Environ. Contam. Toxicol. 31 (1996) 404–409.
[8] A.B. Rifkind, S. Sassa, J. Reyes, H. Muschick, Toxicol. Appl. Pharmacol. 78 (1985) 268–279.
[9] H. Huuskonen, M. Unkila, R. Pohjanvirta, J. Tuomisto, Toxicol. Appl. Pharmacol. 124 (1994) 174–180.
[10] P.M. Allred, J.R. Strange, Arch. Environ. Contam. Toxicol. 6 (1977) 483–489.
[11] T. Sawyer, D. Jones, K. Rosanoff, G. Mason, J. Piskorska-Pliszczynska, S. Safe, Toxicology 39 (1986) 197–206.
[12] M.K. Walker, T.F. Catron, Toxicol. Appl. Pharmacol. 167 (2000) 210–221.
[13] L. Canga, L. Paroli, T.J. Blanck, R.B. Silver, A.B. Rifkind, Mol. Pharmacol. 44 (1993) 1142–1151.
[14] S.E. Heid, M.K. Walker, H.I. Swanson, Toxicol. Sci. 61 (2001) 187–196.
[15] P.H. Sugden, A. Clerk, J. Mol. Med. 76 (1998) 725–746.
[16] O. Hankinson, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 307–340.
[17] J.P. Whitlock Jr., Annu. Rev. Pharmacol. Toxicol. 39 (1999) 103–125.
[18] D.W. Nebert, A. Puga, V. Vasiliou, Ann. N.Y. Acad. Sci. 685 (1993) 624–640.
[19] D. Gilday, M. Gannon, K. Yutzey, D. Bader, A.B. Rifkind, J. Biol. Chem. 271 (1996) 33054–33059.
[20] M. Gannon, D. Gilday, A.B. Rifkind, Toxicol. Appl. Pharmacol. 164 (2000) 24–37.
[21] S.S. Mahajan, A.B. Rifkind, Toxicol. Appl. Pharmacol. 155 (1999) 96–106.
[22] P.R. Sinclair, N. Gorman, H.S. Walton, J.F. Sinclair, C.A. Lee, A.B. Rifkind, Drug. Metab. Dispos. 25 (1997) 779–783.
[23] M.K. Walker, R.S. Pollenz, S.M. Smith, Toxicol. Appl. Pharmacol. 143 (1997) 407–419.
[24] G.L. Wang, B.H. Jiang, E.A. Rue, G.L. Semenza, Proc. Natl. Acad. Sci. USA 92 (1995) 5510–5514.
[25] A.G. Goodridge, J. Biol. Chem. 248 (1973) 1924–1931.
[26] U.K. Laemmli, Nature 227 (1970) 680–685.
[27] J. Sambrook, D. Russell, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 2001.
[28] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[29] D. Henrique, J. Adam, A. Myat, A. Chitnis, J. Lewis, D. Ish-Horowicz, Nature 375 (1995) 787–790.
[30] K. Takebayashi-Suzuki, M. Yanagisawa, R.G. Gourdie, N. Kanzawa, T. Mikawa, Development 127 (2000) 3523–3532.

[31] J.J. Mercadier, J.L. Samuel, J.B. Michel, M.A. Zongazo, D. de la Bastie, A.M. Lompre, C. Wisnewsky, L. Rappaport, B. Levy, K. Schwartz, Am. J. Physiol. 257 (1989) H979–H987.
[32] J.T. Sanderson, G.D. Bellward, Toxicol. Appl. Pharmacol. 132 (1995) 131–145.
[33] D.M. Janz, G.D. Bellward, Toxicol. Appl. Pharmacol. 139 (1996) 292–300.
[34] A.L. Blankenship, K. Hilscherova, M. Nie, K.K. Coady, S.A. Villalobos, K. Kannan, D.C. Powell, S.J. Bursian, J.P. Giesy, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 136 (2003) 47–62.
[35] J.V. Schmidt, G.H. Su, J.K. Reddy, M.C. Simon, C.A. Bradfield, Proc. Natl. Acad. Sci. USA 93 (1996) 6731–6736.
[36] F. El-Sabeawy, E. Enan, B. Lasley, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 129 (2001) 317–327.
[37] B. Stanton, S. Watkins, J.B. German, B. Lasley, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 129 (2001) 137–150.
[38] Y. Morel, N. Mermod, R. Barouki, Mol. Cell. Biol. 19 (1999) 6825–6832.
[39] H. Ito, Y. Hirata, M. Hiroe, M. Tsujino, S. Adachi, T. Takamoto, M. Nitta, K. Taniguchi, F. Marumo, Circ. Res. 69 (1991) 209–215.

Human Molecular Genetics, 2004, Vol. 13, No. 23 2907–2917
doi:10.1093/hmg/ddh318
Advance Access published on September 30, 2004

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

Yuki Hitomi[1], Naoyuki Tsuchiya[1,*], Aya Kawasaki[1], Jun Ohashi[1], Takeshi Suzuki[2], Chieko Kyogoku[1,3], Toru Fukazawa[4], Sasitorn Bejrachandra[5], Usanee Siriboonrit[5], Dasnayanee Chandanayingyong[5], Puan Suthipinittharm[6], Betty P. Tsao[7], Hiroshi Hashimoto[4], Zen-ichiro Honda[2] and Katsushi Tokunaga[1]

[1]Department of Human Genetics and [2]Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan, [3]Department of Medicine, University of Minnesota School of Medicine, Minneapolis, MN 55455, USA, [4]Department of Rheumatology and Internal Medicine, Juntendo University, Tokyo, Japan, [5]Department of Transfusion Medicine and [6]Department of Dermatology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand and [7]Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90095-1670, USA

Received June 15, 2004; Revised September 17, 2004; Accepted September 24, 2004

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

*To whom correspondence should be addressed. Tel: +81 358413693; Fax: +81 358028619; Email: tsuchiya-tky@umin.ac.jp

Human Molecular Genetics, Vol. 13, No. 23 © Oxford University Press 2004; all rights reserved

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,

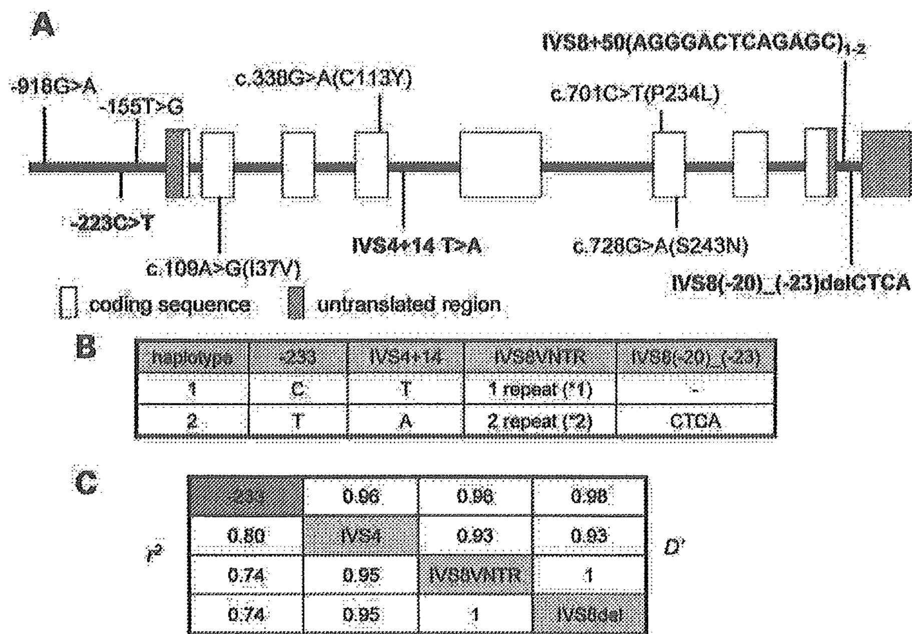

Figure 1. Human *CD72* gene structure, detected variations and haplotypes. (**A**) Exon–intron structure of *CD72* gene. Detected variations are shown in accordance with the nomenclature proposed by den Dunnen and Antonarakis (36). Variations shown in bold are polymorphisms, and others are rare variations. 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

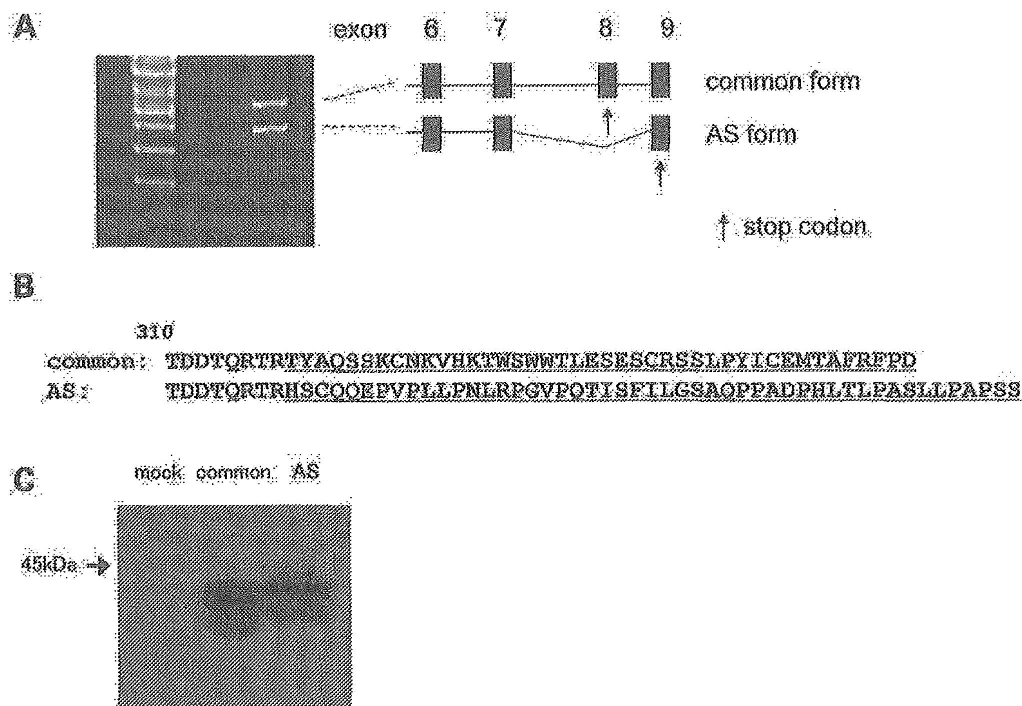

Figure 2. A novel alternative splicing isoform of *CD72*. (A) Two *CD72* fragments detected by RT–PCR. Sequence analysis revealed that the shorter fragment was the product of exon 8 skipping [alternatively spliced (AS) isoform]. In the AS isoform, the stop codon which normally appears in exon 8 is skipped, 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

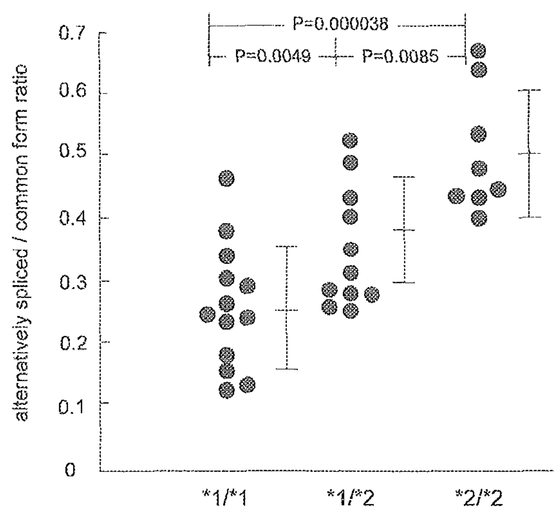

Figure 3. Association of relative proportion of AS isoform with *CD72* genotype. RNA purified from PBMCs of 32 healthy individuals (13 with *CD72*-*1/*1, 11 with *1/*2, 8 with *2/*2 genotype) was semi-quantitatively examined by RT–PCR. Mean ± SD of each genotype group is shown. Statistical difference 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.

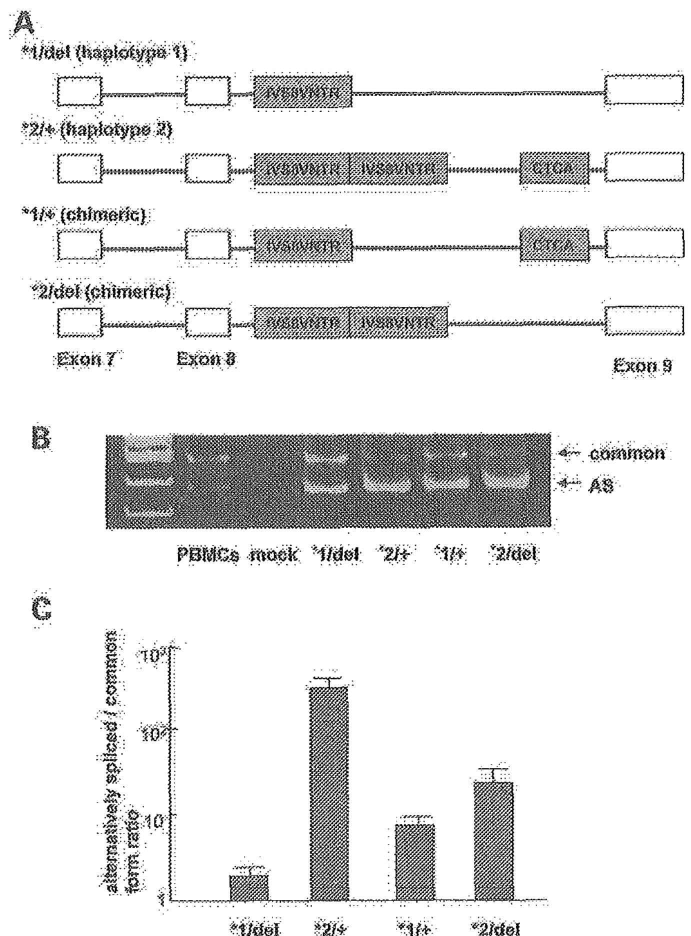

Figure 4. *CD72* minigene assay. (**A**) Four types of *CD72* minigene constructs. *1/del contains one repeat of 13-nucleotide repeat polymorphisms (IVS8VNTR) and CTCA deletion at −20 to −23 bp upstream from the splice acceptor site of intron 8, and corresponds to haplotype 1 (Fig. 1B). *2/+ contains 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′-GGCAGGAGAGCCAAAAACAA-3′ and 5′-ACCCCATTCTACCATGGGAA-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′-GCAGAGCTGCTCAGGACCAT-3′ and 5′-ACCCCATTCTACCATGGGAA-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.

ACKNOWLEDGEMENTS

We would like to thank Dr Takeshi Tsubata (Department of Immunology, Tokyo Medical and Dental University) and Dr Daisuke Sakurai (Department of Immunology, Dokkyo Medical University) for valuable suggestions. This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (C) 'Medical Genome Science', Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture of Japan, Health and Labor Sciences Research Grant from the Ministry of Health Labor and Welfare of Japan, and by Takeda Science Foundation.

REFERENCES

1. Tsao, B.P. (2003) The genetics of human systemic lupus erythematosus. *Trends Immunol.*, **24**, 595–602.
2. Prokunina, L. and Alarcon-Riquelme, M. (2004) The genetic basis of systemic lupus erythematosus—knowledge of today and thoughts for tomorrow. *Hum. Mol. Genet.*, **13**, R143–R148.
3. Kelly, J.A., Moser, K.L. and Harley, J.B. (2002) The genetics of systemic lupus erythematosus: putting the pieces together. *Genes Immun.*, **3** (Suppl. 1), S71–S85.
4. Tsuchiya, N., Ohashi, J. and Tokunaga, K. (2002) Variations in immune response genes and their associations with multifactorial immune disorders. *Immunol. Rev.*, **190**, 169–181.
5. Wakeland, E.K., Liu, K., Graham, R.R. and Behrens, T.W. (2001) Delineating the genetic basis of systemic lupus erythematosus. *Immunity*, **15**, 397–408.
6. Hatta, Y., Tsuchiya, N., Matsushita, M., Shiota, M., Hagiwara, K. and Tokunaga, K. (1999) Identification of the gene variations in human *CD22*. *Immunogenetics*, **49**, 280–286.
7. Matsushita, M., Tsuchiya, N., Oka, T., Yamane, A. and Tokunaga, K. (1999) New variations of human SHP-1. *Immunogenetics*, **49**, 577–579.
8. Kawasaki, A., Tsuchiya, N., Fukazawa, T., Hashimoto, H. and Tokunaga, K. (2001) Presence of four major haplotypes in human *BCMA* gene: lack of association with systemic lupus erythematosus and rheumatoid arthritis. *Genes Immun.*, **2**, 276–279.
9. Kawasaki, A., Tsuchiya, N., Fukazawa, T., Hashimoto, H. and Tokunaga, K. (2002) Analysis on the association of human *BLYS* (*BAFF*, TNFSF13B) polymorphisms with systemic lupus erythematosus and rheumatoid arthritis. Genes Immun., 3, 424–429.
10. Kuroki, K., Tsuchiya, N., Tsao, B.P., Grossman, J.M., Fukazawa, T., Hagiwara, K., Kano, H., Takazoe, M., Iwata, T., Hashimoto, H. and Tokunaga, K. (2002) Polymorphisms of human *CD19* gene: possible association with susceptibility to systemic lupus erythematosus in Japanese. *Genes Immun.*, **3** (Suppl. 1), S21–S30.

11. Kyogoku, C., Dijstelbloem, H.M., Tsuchiya, N., Hatta, Y., Kato, H., Yamaguchi, A., Fukazawa, T., Jansen, M.D., Hashimoto, H., van de Winkel, J.G.J. *et al.* (2002) Association of Fcγ receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of *FCGR2B* to the genetic susceptibility to SLE. *Arthritis Rheum.*, **46**, 1242–1254.
12. Siriboonrit, U., Tsuchiya, N., Sirikong, M., Kyogoku, C., Bejrachandra, S., Suthipinittharm, P., Luangtrakool, K., Srinak, D., Trongpradit, R., Fujiwara, K. *et al.* (2003) Association of Fcγ receptor 2B and 3B polymorphisms with susceptibility to systemic lupus erythematosus in Thais. *Tissue Antigens*, **61**, 374–383.
13. Chu, Z.T., Tsuchiya, N., Kyogoku, C., Ohashi, J., Qian, Y.P., Xu, S.B., Mao, C.Z., Chu, J.Y. and Tokunaga, K. (2004) Association of Fcγ receptor 2B polymorphism with susceptibility to systemic lupus erythematosus in Chinese: a common susceptibility gene in the Asian populations. *Tissue Antigens*, **63**, 21–27.
14. Kyogoku, C., Tsuchiya, N., Wu, H., Tsao, B.P. and Tokunaga, K. (2004) Association of Fcγ receptor IIA, but not of IIB and IIIA, polymorphisms with systemic lupus erythematosus: a family-based association study in Caucasians. *Arthritis Rheum.*, **50**, 671–673.
15. Li, X., Wu, J., Carter, R.H., Edberg, J.C., Su, K., Cooper, G.S. and Kimberly, R.P. (2003) A novel polymorphism in the Fcγ receptor IIB (CD32B) transmembrane region alters receptor signaling. *Arthritis Rheum.*, **48**, 3242–3252.
16. Magnusson, V., Zunec, R., Odeberg, J., Sturfelt, G., Truedsson, L., Gunnarsson, I. and Alarcon-Riquelme, M.E. (2004) Polymorphisms of the Fcγ receptor type IIB gene are not associated with systemic lupus erythematosus in the Swedish population. *Arthritis Rheum.*, **50**, 1348–1350.
17. Su, K., Wu, J., Edberg, J.C., Li, X., Ferguson, P., Cooper, G.S., Langefeld, C.D. and Kimberly, R.P. (2004) A promoter haplotype of the immunoreceptor tyrosine-based inhibitory motif-bearing FcγRIIb alters receptor expression and associates with autoimmunity. I. Regulatory *FCGR2B* polymorphisms and their association with systemic lupus erythematosus. *J. Immunol.*, **172**, 7186–7191.
18. Su, K., Li, X, Edberg, J.C., Wu, J., Ferguson, P. and Kimberly, R.P. (2004) A promoter haplotype of the immunoreceptor tyrosine-based inhibitory motif-bearing FcγRIIb alters receptor expression and associates with autoimmunity. II. Differential binding of GATA4 and Yin-Yang 1 transcription factors and correlated receptor expression and function. *J. Immunol.*, **172**, 7192–7199.
19. Nakayama, E., Von Hoegen, I. and Parnes, J.R. (1989) Sequence of the Lyb-2 B-cell differentiation antigen defines a gene superfamily of receptors with inverted membrane orientation. *Proc. Natl Acad. Sci. USA*, **86**, 1352–1356.
20. Von Hoegen, I., Nakayama, E. and Parnes, J.R. (1990) Identification of a human protein homologous to the mouse Lyb-2 B-cell differentiation antigen and sequence of the corresponding cDNA. *J. Immunol.*, **144**, 4870–4877.
21. Sato, H. and Boyes, E.A. (1976) A new alloantigen expressed selectively on B cells: the Lyb-2 system. *Immunogenetics*, **3**, 525–531.
22. Beavil, A.J., Edmeades, R.L., Gould, H.J. and Sutton, B.J. (1992) α-Helical coiled-coil stalks in the low-affinity receptor for IgE (FcεRα/CD23) and related C-type lectins. *Proc. Natl Acad. Sci. USA*, **89**, 753–757.
23. Robinson, W.H., Landolfi, M.M., Schafer, H. and Parnes, J.R. (1993) Biochemical identity of the mouse Ly-19.2 and Ly-32.2 alloantigens with the B cell differentiated antigen Lyb-2/CD72. *J. Immunol.*, **151**, 4764–4772.
24. Adachi, T., Wakabayashi, C., Nakayama, T., Yakura, H. and Tsubata, T. (2000) CD72 negatively regulates signaling through the antigen receptor of B cells. *J. Immunol.*, **164**, 1223–1229.
25. Adachi, T., Flaswinkel, H., Yakura, H., Reth, M. and Tsubata, T. (1998) The B cell surface protein CD72 recruits the tyrosine phosphatase SHP-1 upon tyrosine phosphorylation. *J. Immunol.*, **160**, 4662–4665.
26. Wu, Y., Nadler, M.J., Brennan, L.A., Gish, G.D., Timms, J.F., Fusaki, N., Jongstra-Bilen, J., Tada, N., Pawson, T., Wither, J. *et al.* (1998) The B-cell transmembrane protein CD72 binds to and is an *in vivo* substrate of the protein tyrosine phosphatase SHP-1. *Curr. Biol.*, **8**, 1009–1017.
27. Pan, C., Baumgarth, N. and Parnes, J.R. (1999) CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity*, **11**, 495–506.
28. Kumanogoh, A., Watanabe, C., Lee, I., Wang, X., Shi, W., Araki, H., Hirata, H., Iwahori, K., Uchida, J., Yasui, T. *et al.* (2000) Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity*, **13**, 621–631.
29. Ishida, I., Kumanogoh, A., Suzuki, K., Akahani, S., Noda, K. and Kikutani, H. (2003) Involvement of CD100, a lymphocyte semaphorin, in the activation of the human immune system via CD72: implications for the regulation of immune and inflammatory responses. *Int. Immunol.*, **15**, 1027–1034.
30. Kikutani, H. and Kumanogoh, A. (2003) Semaphorins in interactions between T cells and antigen-presenting cells. *Nat. Rev. Immunol.*, **3**, 159–167.
31. Wu, H.J., Venkataraman, C., Estus, S., Dong, C., Davis, R.J., Flavell, R.A. and Bondada, S. (2001) Positive signaling through CD72 induces mitogen-activated protein kinase activation and synergizes with B cell receptor signals to induce X-linked immunodeficiency B cell proliferation. *J. Immunol.*, **167**, 1263–1273.
32. Ogimoto, M., Ichinowatari, G., Watanabe, N., Tada, N., Mizuno, K. and Yakura, H. (2004) Impairment of B cell receptor-mediated Ca^{2+} influx, activation of mitogen-activated protein kinases and growth inhibition in CD72-deficient BAL-17 cells. *Int. Immunol.*, **16**, 971–982.
33. Fusaki, N., Tomita, S., Wu, Y., Okamoto, N., Goisuka, R., Kitamura, D. and Hozumi, N. (2000) BLNK is associated with the CD72/SHP-1/Grb2 complex in the WEHI231 cell line after membrane IgM cross-linking. *Eur. J. Immunol*, **30**, 1326–1330.
34. Qu, W.M., Miyazaki, T., Terada, M., Lu, L.M., Nishihara, M., Yamada, A., Mori, S., Nakamura, Y., Ogasawara, H., Yazawa, C. *et al.* (2000) Genetic dissection of vasculitis in MRL/lpr lupus mice: a novel susceptibility locus involving the *CD72*^{c} allele. *Eur. J. Immunol.*, **30**, 2027–2037.
35. James, J.A., Kaufman, K.M., Gross, T.F., Heinlin, L., Brown, E.J. and Harley, J.B. (2002) CD72c is required for SLE in an induced murine model [abstract]. *Arthritis Rheum.*, **46** (suppl.), S559.
36. den Dunnen, J.T. and Antonarakis, S.E. (2000) Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum. Mutat.*, **15**, 7–12.
37. Attanasio, C., David, A. and Neerman-Arbez, M. (2003) Outcome of donor splice site mutations accounting for congenital afibrinogenemia reflects order of intron removal in the fibrinogen alpha gene (FGA). *Blood*, **101**, 1851–1856.
38. Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C.J., Hofmann, K. and Bairoch, A. (2002) The PROSITE database, its status in 2002. *Nucleic Acids Res.*, **30**, 235–238.
39. Shen, F.W., Spanodis, M. and Boyse, E.A. (1977) Multiple alleles of the *Lyb-2* locus. *Immunogenetics*, **5**, 481–484.
40. Tung, J.F., Shen, F.W., LaRegina, V. and Boyse, E.A. (1986) Antigenic complexity and protein-structural polymorphism in the Lyb-2 system. *Immunogenetics*, **23**, 208–210.
41. Robinson, W.H., Ying, H., Miceli, M.C. and Parnes, J.R. (1992) Extensive polymorphism in the extracellular domain of the mouse B cell differentiation antigen Lyb-2/CD72. *J. Immunol.*, **149**, 880–886.
42. Ying, H., Nakayama, E., Robinson, W.H. and Parnes, J.R. (1995) Structure of the mouse *CD72 (Lyb-2)* gene and its alternatively spliced transcripts. *J. Immunol.*, **154**, 2743–2752.
43. Rojas, A., Xu, F. and Thomas, J.W. (2003) Structure and function of CD72 in the non-obese diabetic (NOD) mouse. *Autoimmunity*, **36**, 233–239.
44. Kyogoku, C., Kono, H., Tsuchiya, N., Suzuki, T., Yamamoto, K., Tokunaga, K. and Honda, Z. (2003) SLE-associated polymorphism of FcγRIIB Ile232Thr affects localization at lipid rafts and attenuation of BCR signaling [abstract]. *Arthritis Rheum.*, **48** (suppl.), S647.
45. Ono, M., Okada, H., Bolland, S., Yanagi, S., Kurosaki, T. and Ravetch, J.V. (1997) Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell*, **90**, 293–301.
46. Pagani, F. and Baralle, F.E. (2004) Genomic variants in exons and introns: identifying the splicing spoilers. *Nat. Rev. Genet.*, **5**, 389–396.
47. Mikolajczak, S.A., Ma, B.Y., Yoshida, T., Yoshida, R., Kelvin, D.J. and Ochi, A. (2004) The modulation of CD40 ligand signaling by transmembrane CD28 splice variant in human T cells. *J. Exp. Med.*, **199**, 1025–1031.

48. Ueda, H., Howson, J.M.M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., Rainbow, D.B., Hunter, K.M.D., Smith, A.N., Genova, G.D. *et al.* (2003) Association of the T-cell regulatory gene *CTLA4* with susceptibility to autoimmune disease. *Nature*, **423**, 506–511.
49. Vijayakrishnan, L., Slavik, J.M., Illes, Z., Greenwald, R.J., Rainbow, D., Greve, B., Peterson, L.B., Hafler, D.A., Freeman, G.J., Sharpe, A.H. *et al.* (2004) An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity*, **20**, 563–575.
50. Tone, M., Tone, Y., Fairchild, P.J., Wykes, M. and Waldmann, H. (2001) Regulation of CD40 function by its isoforms generated through alternative splicing. *Proc. Natl Acad. Sci. USA*, **98**, 1751–1756.
51. Stamm, S. (2002) Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome. *Hum. Mol. Genet.*, **11**, 2409–2416.
52. Modrek, B., Resch, A., Grasso, C. and Lee, C. (2001) Genome-wide detection of alternative splicing in expressed sequences of human genes. *Nucleic Acids Res.*, **29**, 2850–2859.
53. Johnson, J.M., Castle, J., Garrett-Engele, P., Kan, Z., Loerch, P.M., Armour, C.D., Santos, R., Schadt, E.E., Stoughton, R. and Shoemaker, D.D. (2003) Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science*, **302**, 2141–2144.
54. Tan, E.M., Cohen, A.S., Fries, J.F., Masi, A.T., McShane, D.J., Rothfield, N.F., Schaller, J.G., Talal, N. and Winchester, R.J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.*, **25**, 1271–1277.
55. Sasieni, P.D. (1997) From genotypes to genes: doubling the sample size. *Biometrics*, **53**, 1253–1261.
56. Terwilliger, J. and Ott, J. (1994) *Handbook for Human Genetic Linkage.* Johns Hopkins University Press, Baltimore, pp. 188–193.
57. Lau, J., Ioannidis, J.P.A. and Schmid, C.H. (1997) Quantitative synthesis in systematic reviews. *Ann. Intern. Med.*, **127**, 820–826.

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

Daisuke Sakurai,*¶ Naoyuki Tsuchiya,[2]* Akihiro Yamaguchi,† Yurai Okaji,‡ Nelson H. Tsuno,§ Tetsuji Kobata,¶ Koki Takahashi,§ and Katsushi Tokunaga*

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

Departments of *Human Genetics, †Allergy and Rheumatology, ‡Surgical Oncology, and §Transfusion Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and ¶Division of Immunology, Institute for Medical Science, Dokkyo University School of Medicine, Tochigi, Japan

Received for publication March 16, 2004. Accepted for publication August 26, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (C) "Medical Genome Science," and Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture of Japan, and by Health and Labour Sciences Research Grant for Research on Allergic Disease and Immunology from the Ministry of Health Labour and Welfare of Japan.

[2] Address correspondence and reprint requests to Dr. Naoyuki Tsuchiya, Department of Human Genetics, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113-0033. E-mail address: tsuchiya-tky@umin.ac.jp

[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.

Copyright © 2004 by The American Association of Immunologists, Inc.

0022-1767/04/$02.00

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: TCCCAAAGAATCATGAAAGTCGCCAGTTCAAGAGACTGGCGA CTTTCATGATTCTTTT and TCGGATCCAACCTCACAGCACCTC ACTTCTTCAAGAGAGAAGTGAGGTGCTGTGAGGTTTTTTTGG AAAAGCTTGG, 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′-AGC CAGTCCGCCAAGAATCAT-3′(forward),5′-ACTCACTCCCCAGCA TGAAG-3′ (reverse); Id3, 5′-CTCCACGCTCTGAAAAGACC-3′ (forward), 5′-ACTCAGATTAAGCCAGGTGGA-3′ (reverse); $p16^{INK4a}$, 5′-AGCCTTCGGCTGACTGGCTGG-3′ (forward), 5′-GCAGTTAAG GGGGCACGAGTG-3′ (reverse); ICAM-1, 5′-ACCTGGCAATGCCC AGACATCTGTGT-3′ (forward), 5′-GTACACGGTGAGGAAGGTTT TAGCTGTTG-3′ (reverse); E-selectin, 5′-AAAACTTCCATGAGGCC AAA-3′ (forward), 5′-GCATTCCTCTCTTCCAGAGC-3′ (reverse); matrix metalloproteinase (MMP)2, 5′-ATGACAGCTGCACCACT GAG-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.

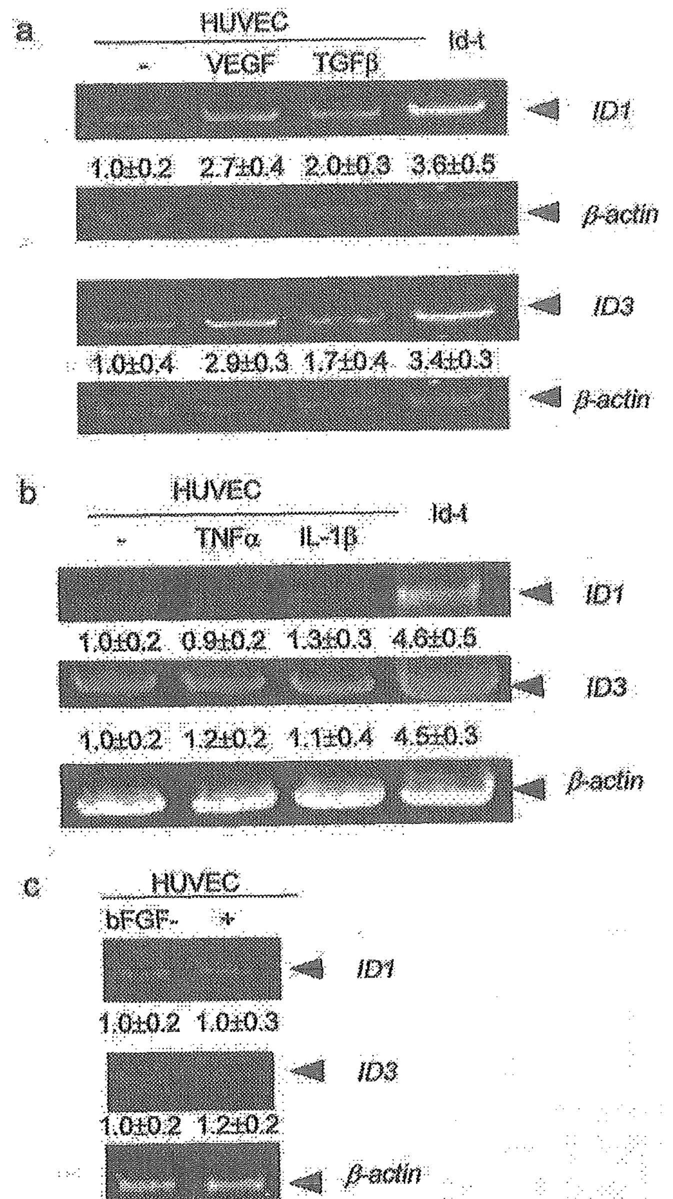

FIGURE 1. Expression of *ID1* and *ID3* mRNA is induced in HUVECs by VEGF or TGFβ. *a* and *b*, Id1 and Id3 mRNA levels were measured in HUVECs with or without stimulation with VEGF (20 ng/ml), TGFβ (0.5 ng/ml) (*a*), TNF-α (20 ng/ml), or IL-1β (1 ng/ml) (*b*) for 24 h. HUVECs transfected with *ID1* (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.