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Microsatellite analysis of the GLC1B locus on chromosome 2 points to NCK2 as a new candidate gene for normal tension glaucoma

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ABSTRACT

Aims: The aim of this study was to investigate the association between normal tension glaucoma and the candidate disease locus glaucoma 1, open angle, B (GLC1B) on chromosome 2. There are many reports describing the results of association or linkage studies for primary open angle glaucoma (POAG), with GLC1B as one of the loci associated with normal or moderately elevated intraocular pressure. However, there are few reports about the association of genes or defined genomic regions with normal tension glaucoma, which is the leading type of glaucoma in Japan. The GLC1B locus is hypothesized to be a causative region for normal tension glaucoma.

Methods: Genomic DNA was extracted from whole blood of normal tension glaucoma ($n = 143$) and healthy controls ($n = 103$) of Japanese origin.

Results: Fifteen microsatellite markers within and/or near to the GLC1B locus were genotyped, and their association with normal tension glaucoma was analysed. Two markers D2S2264 and D2S176 had significant positive associations.

Conclusion: The D2S176 marker had the strongest significant association and it is located 24 kb from the nearest gene NCK2, which now becomes an important new candidate gene for future studies of its association with normal tension glaucoma.

The cause of glaucoma has been attributed largely to primary open angle glaucoma (POAG) associated with elevated IOP, but in Japan normal tension glaucoma (NTG) is the leading type of glaucoma. The Tajimi study group reported that the prevalence rate of NTG was 3.60% in Japan.[1] These studies suggested that more emphasis should be placed on the prevention, detection and treatment at an early stage of the disease in order to prevent irreversible blindness.

Elucidation of the genetic aetiology of glaucoma has been increasingly emphasised as an important step for a better understanding of the pathogenesis of this disease, and for ultimately improving the prevention strategies, diagnostic tools, and therapy in the new millennium.[2] A few reports describing the results of association or linkage studies for POAG[3] are available, and they have linked the disease to numerous chromosomal regions.[4–12] The application of linkage analysis to glaucoma with the exception of obvious Mendelian inheritance, has achieved only limited success thus far.

One of the candidate loci that has been identified for POAG is Glaucoma 1, open angle, B (GLC1B, MIM:606689) located on chromosome 2.[5] The identification of this locus was based on a linkage study of six Caucasian families in the UK and the GLC1B locus for adult-onset POAG was identified within a region of 11.2 cM flanked by markers at chromosome 2cen-q13. The POAG patients in these families had clinical characteristics of low to moderate IOP, disease onset in their late 40s, and a good response to medical therapy, indicating that the GLC1B locus might encode a POAG gene that is associated with normal or moderately elevated IOP. Interestingly, eight additional families, in which members had variable clinical presentations, did not show any linkage to this region. Another study on American families also excluded 2cen-q13.[13] These studies suggest that there is genetic heterogeneity for POAG and the possibility of a specific NTG gene.

The aim of our study was to determine the association between 15 selected microsatellite (MS) markers within or near to the GLC1B locus and NTG in Japanese subjects. The 15 MS markers are distributed across the GLC1B region with each neighbouring MS locus less than 100 kb apart and consequently in linkage disequilibrium (LD) with each other. We considered that the number and distribution of the MS markers were enough to identify disease-predisposing genomic variants in terms of LD. The MS markers chosen for this study are highly polymorphic and have a high degree of heterozygosity (on average, approximately 70%) and LD lengths in the 100–200 kb range.[14–19]

MATERIALS AND METHODS

Subjects for microsatellite typing

One hundred and forty-three patients with NTG for patients and 103 healthy individuals participated for controls in this study. The subjects were of Japanese origin from Yokohama City University, Yamanashi University, Gifu University, Kobe University, Yamaguchi University, Kumamoto University and Tokai University in Japan. The diagnosis of NTG was made according to the guidelines of Genetic Variation Project Team in the Japanese Society of Ophthalmology (declared in 2000), which defined NTG as glaucoma with an intraocular pressure (IOP) of less than 21 mm Hg by Goldmann applanation tonometry without medication, including during the 24 h diurnal

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curve, open irido-corneal angle, glaucomatous optic disc appearance, and the corresponding glaucomatous visual field defect. The subjects had no systemic abnormalities. Glaucomatous optic disc change was diagnosed when the vertical cup/disc ratio of optic nerve head was more than or equal to 0.7, the rim width at superior portion (11–1 h) or inferior portion (5–7 h) was less than or equal to 0.1 of the disc diameter, the difference of the vertical cup/disc ratio was more than or equal to 0.2 between both eyes, or a nerve fibre layer defect was found. We have excluded individuals diagnosed at an age under 20 or over 60 years. Glaucomatous visual field defect was defined in a hemifield basis using reliable field data examined by the Humphrey static visual field analyser (Carl Zeiss-Meditec) C30-2 program according to the Anderson and Patella's criteria: the hemifield was judged abnormal when the pattern deviation probability plot showed a cluster of three or more non-edge contiguous points having sensitivity with a probability of less than 5% in the upper or lower hemifield and in one of these with a probability of less than 1%. The selection criteria were modified depending on the subjects' age in order to minimise the effects of damage to the retinal ganglion that occurs due to ageing; that is, the mean deviation measured by HFA C-30-2, (i) no requirement if the patient was diagnosed at the age under 50 years (ii) −10.00 dB or worse in at least one eye if the patient was diagnosed at the age between 50 and 55 (iii) −15.00 dB or worse in at least one eye if the patient was diagnosed at the age above 55.

During diagnosis, patients whose refraction values were myopic over −8.0 D of spherical equivalent and had changed due to cataract surgery, refractive surgery, etc were excluded from the study. In cases where a glaucomatous visual field defect was present in only one eye, the refraction value and glaucomatous visual field defect of the affected eye were adopted. Further, in cases where a glaucomatous visual field defect was present in both eyes, the refraction value and glaucomatous visual field defect of the more severely affected eye were adopted.

The control subjects were all healthy volunteers having regular medical check-ups. They are all under 50 years old. All personal identities associated with medical information and blood samples were carefully eliminated and replaced with anonymous identities in each recruiting institution.

This study was performed in accordance with the Declaration of Helsinki, and we obtained informed consent from all patients and healthy individuals whose DNA samples were used in the analyses. Our experimental procedures were conducted in accordance with the Declaration of Helsinki and approved by the relevant ethical committee in each participating university and centre.

DNA genotyping

DNA was extracted using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany) under standardised conditions to prevent any variation in DNA quality. This was followed by 0.8% agarose gel electrophoresis to check for DNA degradation and RNA contamination. Following measurement of the optical density to check for protein contamination, the DNA concentration was determined through three successive measurements using the PicoGreen fluorescence assay (Molecular Probes, Eugene, OR). PCR reactions were performed in a total volume of 12.5 μl containing PCR buffer, genomic DNA, 0.2 mM dinucleotide triphosphates (dNTPs), 0.5 μM primers, and 0.35 U Taq polymerase. The reaction mixture was subjected to 5 min at 94°C, then 30 cycles of 30 s for denaturing at 94°C, 30 s for annealing at 56°C, 1 min for extension at 72°C and then 10 min for final elongation at 72°C using a polymerase chain reaction (PCR) thermal cycler, GeneAmp System 9700 (Applied Biosystems, Foster City, CA). To determine the number of microsatellite repeats, PCR-amplified products were denatured for 2 min at 95°C, mixed with formamide and electrophoresed using an ABI3130 Genetic Analyzer (Applied Biosystems). The number of microsatellite repeats was estimated automatically using the GeneScan 672 software (Applied Biosystems) by the local Southern method with a size marker of GS500 TAMRA (Applied Biosystems).

Marker information

Microsatellite (MS) sequences were computationally detected from all the chromosomes. In this study, we utilised 15 MS markers within or near the GLC1B locus as shown in fig 1.

The PCR primer pairs of the 15 MS markers for the association test were designed in order to improve the efficiency of PCR (table S1, at http://bjo.bmj.com/content/vol92/issue9). The forward primer was labelled at the 5′ end with 6-FAM, NED, PED or VIC (ABI, Tokyo).

Statistical analysis

The measurements of the heights of individual peaks in the DNA were applied to association analysis. To calculate p values, we used χ^2 tests and made corrections using Bonferroni correction. The Hardy–Weinberg test for allele frequency distributions at the microsatellite loci was performed using a probability test for differentiation, as determined by GenePop 3.4. Other basic analyses were carried out using Microsoft Excel.

RESULTS

We genotyped 15 MS markers, and their locations are shown within the GLC1B locus in fig 1. The observed and expected frequencies of each genotype for the 15 markers in the case and control subjects were in Hardy–Weinberg equilibrium (data not shown). Two markers D2S2264 and D2S176 were significantly positive, as shown in tables 1, 2. In considering the LD range, the susceptibility genes for NTG were estimated to reside within a 100–150 kb region flanking the two positive markers D2S2264 and D2S176. The corrected p value for the 245 allele of D2S176 was ≤0.05. There was no significant association for the other 13 markers.

Figure 1 Locations of 15 microsatellite markers. To the left of the figure is 5′UTR. White boxes show the NCK2 gene (42 kb) and MAP4K4 gene (29 kb).

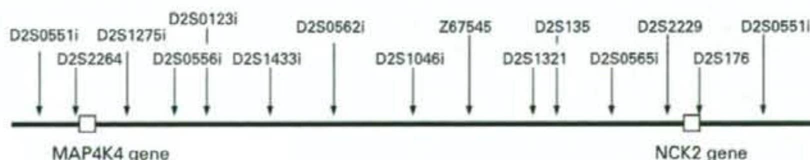

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Table 1 Statistical results for D2S2264

Allele	Patient	Negative	Positive	Negative	OR	×2	p*	Pc
236	33	249	29	173	0.79	0.74241	0.38889	3.888897
244	30	252	33	169	0.61	3.37535	0.066178	0.661784
245	1	281	0	202	–	0.71780	0.396868	3.968681
247	0	282	1	201	0.00	1.39893	0.236903	2.36903
248	0	282	1	201	0.00	1.39893	0.236903	2.36903
250	144	138	82	120	1.53	5.18313	0.022807	0.228072
252	18	264	15	187	0.85	0.20143	0.653568	6.535679
253	1	281	0	202	–	0.71780	0.396868	3.968681
254	54	228	41	161	0.93	0.09834	0.753832	7.538317
256	1	281	0	202	–	0.71780	0.396868	3.968681

*The corrected p value was corrected by the number of alleles.
OR, odds ratio.

Table 2 Statistical results for D2S176

Allele	Patient	Negative	Positive	Negative	OR	×2	p*	Pc
245	104	178	51	151	1.73	7.31510	0.006838	0.047864
247	6	276	7	195	0.61	0.80573	0.369385	2.585696
249	72	210	70	132	0.65	4.72355	0.029752	0.208266
251	73	209	49	153	1.09	0.16568	0.683979	4.787854
253	22	260	23	179	0.66	1.79341	0.180511	1.26358
255	4	278	2	200	1.44	0.17638	0.674504	4.72153
261	1	281	0	202	–	0.71780	0.396868	2.778077

*The corrected p value was corrected by the number of alleles.
OR, odds ratio.

DISCUSSION

Based on recent knowledge, the average length for an estimated LD between the disease-susceptible SNPs and the nearby MS alleles is 100 kb or longer.[14–19] In other words, if the disease-susceptible SNPs are located between two neighbouring MS markers at an interval of 200 kb or less, and the disease alleles of the two neighbouring MS markers are in LD, then the intervening disease-susceptible SNPs will also be in LD and associated with disease.

We found an association between D2S176 on chromosome 2q12.2 and NTG in the Japanese. The association is not too strong, as it just reaches statistical significance (0.047864: corrected p value for D2S176). Therefore, it follows that this locus might contain an unknown candidate gene for NTG. The nearest gene to the D2S176 marker is the NCK2 gene (NM_003581),[20] which is about 24 kb from the marker and within the expected LD region of the D2S176 marker. This is the first report to point to the NCK2 gene as a disease-susceptibility gene for NTG. NCK2 gene encodes a member of the NCK family of adaptor proteins, and the adaptor protein which associates with tyrosine-phosphorylated growth factor receptors of their cellular substrates.[21]

Another weak, but significant, MS marker, D2S2264, is located on 2q11.2. This marker is within the MAP4K4 gene sequence (NM_145686) that codes a member of the serine/threonine protein kinase family.[22] There have been no previous reports suggesting any connection between NCK2 and/or MAP4K4 and NTG.

In conclusion, we performed an association analysis of normal tension glaucoma using a high-density set of polymorphic MS markers between cases and controls from the Japanese population. The MS markers were used in the association analysis to find statistically significant regions associated with potential susceptibility genes. Although the outcome of this study is insufficient to draw a definite conclusion about the true candidate gene for NTG, the study points to NCK2 as a disease candidate gene and further supports the GLC1B locus as an important genomic region that is associated with the genetic predisposition to glaucoma. The next step is to find susceptibility variants within the NCK2 and MAP4K4 genes by SNP analysis. The future analysis of these genes is expected to open the door to a better understanding of the genetic predisposition to NTG.

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Patient consent: Obtained.

REFERENCES

1. **Iwase A,** Suzuki Y, Araie M, *et al.* The prevalence of primary open-angle glaucoma in Japanese: the Tajimi Study. *Opthalmology* 2004;**111**:1641–8.
2. **Hyman L,** Klein B, Nemesure B, *et al.* Ophthalmic genetics: at the dawn of discovery. *Arch Opthalmol* 2007;**125**:9–10.
3. **Fan BJ,** Wang DY, Lam DS, *et al.* Gene mapping for primary open angle glaucoma. *Clin Biochem* 2006;**39**:249–58.
4. **Sheffield VC,** Stone EM, Alward WL, *et al.* Genetic linkage of familial open angle glaucoma to chromosome 1q21–q31. *Nat Genet* 1993;**4**:47–50.
5. **Stoilova D,** Child A, Trifan OC, *et al.* Localization of a locus (GLC1B) for adult-onset primary open-angle glaucoma (POAG). *Genomics* 2003;**112**:600–9.
6. **Wirtz MK,** Samples JR, Kramer PL, *et al.* Mapping a gene for adult-onset primary open-angle glaucoma to chromosome 3q. *Am J Hum Genet* 1997;**60**:296–304.
7. **Wirtz MK,** Samples JR, Ruse K, *et al.* GLC1F, a new primary open-angle glaucoma locus, maps to 7q35–q36. *Am J Hum Genet* 1997;**60**:296–304.
8. **Monemi S,** Spaeth G, Dasilva A, *et al.* Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22.1. *Hum Mol Genet* 2005;**14**:725–33.
9. **Trifan OC,** Traboulsi EI, Stoilova D, *et al.* A third locus (GLC1D) for adult-onset primary open-angle glaucoma maps to the 8q23 region. *Am J Ophthalmol* 1998;**126**:17–28.

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10. **Wiggs JL,** Lynch S, Ynagi G, *et al.* A genomewide scan identifies novel early-onset primary open-angle glaucoma loci on 9q22 and 20p12. *Am J Hum Genet* 2004;**74**:1314–20.
11. **Sarfarazi M,** Child A, Stoilova D, *et al.* Localization of the fourth locus (GLC1E) for adult-onset primary open-angle glaucoma to the 10p15–p14 region. *Am J Hum Genet* 1998;**62**:641–52.
12. **Allingham RR,** Wiggs JL, Hauser ER, *et al.* Early adult-onset POAG linked to 15q11–13 using ordered subset analysis. *Invest Ophthalmol Vis Sci* 2005;**46**:2002–5.
13. **Allingham RR,** Wiggs JL, Damji KF, *et al.* Adult-onset primary open angle glaucoma does not localize to chromosome 2cen-q13 in North American families. *Hum Hered* 1998;**48**:251–5.
14. **Oka A,** Tamiya G, Tomizawa M, *et al.* Association analysis using refined microsatellite markers localize a susceptible locus for psoriasis vulgaris within a 111-kb segment telomeric of the HLA-C gene. *Hum Mol Genet* 1999;**8**:2165–70.
15. **Ota M,** Mizuki N, Katsuyama Y, *et al.* The critical region for Behçet disease in the human major histocompatibility complex is reduced to a 46-kb segment centromeric of HLA-B by association analysis using refined microsatellite mapping. *Am J Hum Genet* 1999;**64**:1406–10.
16. **Keicho N,** Ohashi J, Tamiya G, *et al.* Fine localization of a major disease-susceptibility locus for diffuse panbronchiolitis. *Am J Hum Gent* 2000;**66**:501–7.
17. **Mizuki N,** Ota M, Yabuki K, *et al.* Localization of the pathogenic gene of Behçet's diseases by microsatellite analysis of three different populations. *Invest Ophthalmol Vis Sci* 2000;**41**:3702–8.
18. **Zhang Y,** Leaves NI, Anderson GG, *et al.* Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. *Nat Genet* 2003;**34**:181–6.
19. **Ota M,** Katsuyama Y, Kimura A, *et al.* A second susceptibility gene for developing rheumatoid arthritis in the human MHC is localized within a 70-kb interval telomeric of the TNF genes in the HLA class III region. *Genomics* 2001;**71**:263–70.
20. **Liu J,** Li M, Ran X, *et al.* Structual insight into the binding diversity between the human NCK2 SH3 domains and proline rich proteins. *Biochemistry* 2006;**45**:7171–84.
21. **Rivera GM,** Antoku S, Gelkop S, *et al.* Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. *Proc Natl Acad Sci USA* 2006;**103**:9536–41.
22. **Machida N,** Umikawa M, Takei K, *et al.* Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. *J Biol Chem* 2004;**279**:15711–14.

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Association of the toll-like receptor 4 gene polymorphisms with Behçet's disease

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Table 1 Intima-media thickness values at different carotid artery districts (mm; mean (SD)) in patients with RA subdivided according to the presence of absence of serum anti-CCP antibodies

	Normal controls n = 75	Total patients with RA n = 81	Patients with RA anti-CCP– n = 29	Patients with RA anti-CCP+ n = 52	Anti-CCP– versus anti-CCP+P
Age (mean (SD))	61 (13)	63 (10)	62 (10)	63 (11)	0.54
Sex (males %)	29.3	28.4	13.7	36.5	0.02 (ξ^2)
Disease duration (years)	–	11 (9)	10 (7)	13 (10)	0.41
Common carotid	0.81 (0.24)	0.84 (0.22)†	0.82 (0.18)	0.85 (0.24)	0.44
Carotid bifurcation	0.89 (0.24)	1.02 (0.25)†	1.05 (0.26)	1.01 (0.24)	0.52
Internal carotid	0.74 (0.23)	0.76 (0.21)†	0.70 (0.16)	0.80 (0.23)	0.03
Carotid artery*	0.86 (0.25)	0.87 (0.19)†	0.85 (0.16)	0.89 (0.20)	0.47

*Values of carotid artery are the average of common carotid, carotid bifurcation and internal carotid intima-media thickness values.
†$p<0.05$ versus normal controls.
RA, rheumatoid arthritis; anti-CCP, anti-cyclic citrullinated peptides.

patients with RA without overt CVD was analysed by ultrasound, as described.[6] Seventy-five age- and sex-matched healthy subjects with a similar distribution of risk factors (smoking, high body mass index, hypercholesterolaemia, hypertension, diabetes mellitus and CVD family history) formed the control group. Evaluation of anti-CCP was performed in all patients by an enzyme-linked immunosorbent assay (Diastat, Axis-Shield Diagnostics, Dundee, UK). The study was approved by the local ethical committee.

IMT values were higher in the patients than in controls at all artery domains examined (common, bifurcation and internal carotid) (table 1). Patients with RA with detectable circulating anti-CCP had higher IMT at internal carotid arterial wall than patients without evidence of these antibodies. The fact that we found differences only at the internal carotid may be due to a low number of enrolled patients, but it may also be explained by the observation that atherosclerosis primarily involves the upper carotid tract (internal carotid and bifurcation).[7]

The patients who were anti-CCP positive did not differ from the other patients for age, disease duration, traditional risk factors and treatment (data not shown), but included a higher number of males. This finding agrees with the demonstration that male patients with RA are more likely to be seropositive for, and have higher titres of anti-CCP compared with female patients.[8] Although this may represent a confounding factor that might explain the higher internal carotid IMT found in the patients who were anti-CCP positive, a multivariate analysis showed that only age, smoking and anti-CCP, but not sex or other traditional risk factors, were predictors of internal carotid thickening in our series.

The role of age and smoking as predictors of atherosclerosis in RA has been described in several studies.[1 2 9 10] However, to our knowledge, this is the first report showing an association between anti-CCP and subclinical atherosclerosis in patients with RA. The finding that smoking may trigger immunity to citrullinated proteins in genetically predisposed subjects with RA [10] may represent a fascinating pathogenic link between smoking, anti-CCP and atherosclerosis acceleration in RA. Further studies with higher number of patients are ongoing to verify the benefit of anti-CCP determination in identifying patients with RA at high risk for CVD.

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REFERENCES

1. **Shoenfeld Y,** Gerli R, Doria A, Matsuura E, Matucci Cerinic M, Ronda N, *et al.* Accelerated atherosclerosis in autoimmune rheumatic diseases. *Circulation* 2005;**112**:3337–47.
2. **Kaplan M.** Cardiovascular disease in rheumatoid arthritis. *Curr Opin Rheumatol* 2006;**18**:289–97.
3. **Turesson C,** McClelland RL, Christianson TJH, Matteson EL. Severe extra-articular disease manifestations are associated with an increased risk of first ever cardiovascular events in patients with rheumatoid arthritis. *Ann Rheum Dis* 2007;**66**:70–75.
4. **Turesson C,** Jacobsson LTH, Sturfelt G, Matteson EL, Mathsson L, Rönnelid J. Rheumatoid factor and antibodies to cyclic citrullinated peptides are associated with severe extra-articular manifestations in rheumatoid arthritis. *Ann Rheum Dis* 2007;**66**:59–64.
5. **van Venrooij WJ,** Zendman AJW, Pruijn GJM. Autoantibodies to citrullinated antigens in (early) rheumatoid arthritis. *Autoimmun Rev* 2006;**6**:37–41.
6. **Gerli R,** Schillaci G, Giordano A, Bartoloni Bocci E, Bistoni O,Vaudo G, *et al.* CD4+CD28- T lymphocytes contribute to early atherosclerotic damage in rheumatoid arthritis patients. *Circulation* 2004;**109**:2744–8.
7. **Rubba P,** Panico S, Bond MG, Covetti G, Celentano E, Iannuzzi A, *et al.* Site-specific atherosclerotic plaques in the carotid arteries of middle-aged women from southern Italy: associations with traditional risk factors and oxidation markers. *Stroke* 2001;**32**:1953–9.
8. **Jawaheer D,** Lum RF, Gregersen PK, Criswell LA. Influence of male sex on disease phenotype in familial rheumatoid arthritis. *Arthritis Rheum* 2006;**54**:3087–94.
9. **Gerli R,** Sherer Y, Vaudo G, Schillaci G, Gilburd B, Giordano A, *et al.* Early atherosclerosis in rheumatoid arthritis: effect of smoking on thickness of the carotid artery intima media. *Ann N Y Acad Sci* 2005;**1051**:281–90.
10. **Klareskog L,** Padyukov L, Alfredsson L. Smoking as a trigger for inflammatory rheumatic diseases. *Curr Opin Rheumatol* 2007;**19**:49–54.

Association of the toll-like receptor 4 gene polymorphisms with Behçet's disease

Behçet's disease (BD) is a multisystemic inflammatory disorder characterised by recurrent ocular symptoms, oral and genital ulcers, and skin lesions.[1 2] The aetiology of BD remains unclear, but likely both genetic and environmental factors play an important part in BD development.

We performed a whole-genome association analysis of BD using 23 465 microsatellite markers and ultimately found significant association for 147 markers (unpublished data). One of the 147 markers is located within 100 kb from the toll-like receptor (TLR) 4 gene on chromosome 9. Among the TLR family members, TLR4 is the receptor most exhaustively investigated and has been shown to recognise and interact with heat shock protein (HSP) and lipopolysaccharide (LPS),[3 4] which are regarded as antigens in BD.[5–9] Therefore, we hypothesised that *TLR4* polymorphisms may be associated with the risk of BD and conducted single-nucleotide polymorphisms (SNPs) analysis of *TLR4* in BD. To our knowledge,

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this study is the first attempt to analyse SNPs of the *TLR4* gene in BD.

Nine SNPs (fig 1) in *TLR4* were genotyped by TaqMan method, following the manufacturer's instructions, in 200 unrelated Japanese patients with BD and 102 unrelated healthy Japanese controls. Strong linkage disequilibrium (LD) existed across nine SNPs in *TLR4* (D'≥0.82). But, only in rs7037117 (named SNP8) located in the 3'-untranslated region, a significant difference was observed between cases and controls (p = 0.02) (table 1).

We analysed clinical features according to the polymorphism of SNP8 (table 1). After stratification for the effect of onset age, a highly significant association was observed between controls and 94 cases where onset age was ≤34 years (p = 0.002). When BD patients with complete type or incomplete type were compared with controls, there was statistically significant difference between 110 incomplete-type cases and controls for SNP8 (p = 0.003). Further, SNP8 polymorphism was associated with four major symptoms and two minor symptoms, and besides was strongly associated with BD with minor symptom(s) (p = 0.009). There was no significant difference in allele frequency of SNP8 between male and female or between HLA-B*51 carriers and non-carriers (data not shown). In other SNPs, minor allele frequencies of five SNPs (named SNP1, 2, 3, 4 and 5, respectively) were significantly increased in incomplete-type BD, BD where onset age was ≤34 years, and BD with minor symptom(s) ($p<0.05$, data not shown).

In haplotype analysis, the frequency of one haplotype, consisting of six minor allele of SNP1, 2, 3, 4, 5 and SNP8, was increased in the patients (23.8% vs 15.7%, OR = 1.67, 95% CI = 1.08 to 2.60, $\chi^2 = 5.29$, p = 0.03). However, this increase did not reach statistical significance after Bonferroni correction (pc>0.05). There was strong LD between these six SNPs (D'>0.97).

This study shows that one SNP in *TLR4* is associated with BD, and six SNPs have an effect on clinical features of BD. Our data are consistent with the interpretation that the immune response against TLR4 ligands, such as HSP and LPS, plays an important part in BD development. Therefore, it will be essential to identify the antigens associated with the *TLR4* sequence variant and subsequent signalling pathways in BD.

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Table 1 Association of minor allele of rs7037117, SNP for the TLR4 gene, with BD

	n	Minor allele frequency (%)	OR (95% CI)	p Value (χ^2)
Controls	102	15.7		
Patients with BD	200	24.0	1.70 (1.09 to 2.64)	0.02 (5.59)
Onset age				
≥35 years	97	20.1	1.35 (0.81 to 2.26)	0.29 (1.32)
≤34 years	94	28.7	2.17 (1.32 to 3.54)	0.002 (9.71)
Classification				
Complete BD	90	19.4	1.30 (0.77 to 2.20)	0.35 (0.94)
Incomplete BD	110	27.7	2.06 (1.28 to 3.33)	0.003 (8.96)
Major symptoms				
Oral ulcer	196	24.0	1.70 (1.09 to 2.64)	0.02 (5.54)
Skin lesion	174	23.6	1.66 (1.06 to 2.60)	0.03 (4.87)
Ocular lesion	179	22.9	1.60 (1.02 to 2.51)	0.049 (4.19)
Genital ulcer	122	24.2	1.71 (1.06 to 2.76)	0.03 (4.95)
Minor symptoms				
Arthritis	71	25.4	1.83 (1.07 to 3.11)	0.03 (4.95)
Epididymitis	15	30.0	2.30 (0.97 to 5.48)	0.07 (3.71)
Gastrointestinal lesion	22	29.6	2.25 (1.07 to 4.77)	0.049 (4.68)
Vascular lesion	7	21.4	1.47 (0.39 to 5.55)	0.48 (0.32)
Central nervous system lesion	18	30.6	2.37 (1.06 to 5.28)	0.06 (4.60)
BD with minor symptom(s)	96	26.6	1.94 (1.19 to 3.19)	0.009 (7.06)

Onset age of BD was ascertained in 191 cases, and the average was 34.5 years old. BD, Behçet's disease.

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REFERENCES

1. **Kaklamani VG**, Vaiopoulos G, Kaklamanis PG. Behçet's disease. *Semin Arthritis Rheum* 1998;**27**:197–217.
2. **Sakane T**, Takeno M, Suzuki N, Inaba G. Behçet's disease. *N Engl J Med* 1999;**341**:1284–91.

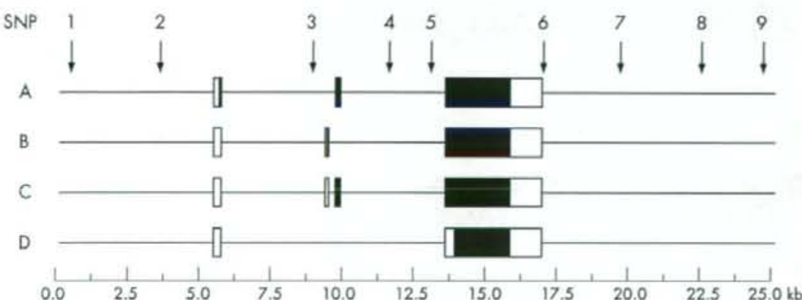

Figure 1 Toll-like receptor 4 gene structure with four transcript isoforms (A–D) and nine single nucleotide polymorphisms (SNPs) variants with minor allele frequencies >5% from the National Center for Biotechnology Information dbSNP. SNPs are indicated by the following numbers: (1) rs10759930; (2) rs1927914; (3) rs1927911; (4) rs12377632; (5) rs2149356; (6) rs11536889; (7) rs1554973; (8) rs7037117; (9) rs7045953. The black and white area in exons indicate the coding region and UTR, respectively.

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3. **Andreakos E,** Foxwell B, Feldmann M. Is targeting Toll-like receptors and their signaling pathway a useful therapeutic approach to modulating cytokine-driven inflammation? *Immunol Rev* 2004;**202**:250–65.
4. **Kang SSW,** Kauls LS, Gaspari AA. Toll-like receptors: applications to dermatologic disease. *J Am Acad Dermatol* 2006;**54**:951–83.
5. **Lehner T,** Lavery E, Smith R, van der Zee R, Mizushima Y, Shinnick T. Association between the 65-kilodalton heat shock protein, Streptococcus sanguis, and the corresponding antibodies in Behçet's syndrome. *Infect Immun* 1991;**59**:1434–41.
6. **Pervin K,** Childerstone A, Shinnick T, Mizushima Y, van der Zee R, Hasan A, *et al.* T cell epitope expression of mycobacterial and homologous human 65-kilodalton heat shock protein peptides in short term cell lines from patients with Behçet's disease. *J Immunol* 1993;**151**:2273–82.
7. **Direskeneli H,** Hasan A, Shinnick T, Mizushima R, van der Zee R, Fortune F, *et al.* Recognition of B-cell epitopes of the 65 kDa HSP in Behçet's disease. *Scand J Immunol* 1996;**43**:464–71.
8. **Kaneko S,** Suzuki N, Yamashita N, Nagafuchi H, Nakajima T, Wakisaka S, *et al.* Characterization of T cells specific for an epitope of human 60-kD heat shock protein (hsp) in patients with Behçet's disease (BD) in Japan. *Clin Exp Immunol* 1997;**108**:204–12.
9. **Mege JL,** Dilsen N, Sanguedolce V, Gul A, Bongrand P, Roux H, *et al.* Overproduction of monocyte derived tumor necrosis factor α, interleukin (IL) 6, IL-8 and increased neutrophil superoxide generation in Behçet's disease. A comparative study with familial Mediterranean fever and healthy subjects. *J Rheum* 1993;**20**:1544–9.

Expression of high mobility group protein 1 in the sera of patients and mice with systemic lupus erythematosus

High mobility group protein 1 (HMGB1) is a non-histone nuclear protein with a dual function. Inside the cell, HMGB1 binds to DNA and modulates a variety of processes, including transcription. Outside the cell, HMGB1 can serve as an alarmin to mediate disease manifestations in animal models of sepsis and arthritis; in these models, blocking HMGB1 can attenuate disease.[1-3]

In *in vitro* experiments, HMGB1 translocation and cellular release can occur during activation as well as cell death and is present in tissue in conditions, such as rheumatoid arthritis and cutaneous lupus.[4 5] While original studies suggested that release occurs only with necrosis,[6] more recent studies have shown that HMGB1 release also occurs in late apoptosis.[7 8] As increased apoptosis and decreased clearance of apoptotic material may underlie the pathogenesis of systemic lupus erythematosus (SLE), these findings suggest that extracellular HMGB1 levels rise in this disease and promote systemic and local inflammation.

To elucidate the expression of HMGB1 in SLE, we have investigated blood levels of HMGB1, using Western blotting to analyse serum samples from a murine lupus model and patients with SLE. We obtained serum samples from MRL/MpJ-*lpr/lpr* and BALB/c mice purchased from the Jackson Laboratory (Bar Harbor, MA, USA). Human SLE serum samples were purchased from Immunovision (Springdale, AR, USA). For Western blotting, electrophoresis was performed using a 4–12% Bis-Tris sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA, USA). Protein was transferred on to PDVF membrane and blotted with a rabbit anti-HMGB1 polyclonal antiserum (gift of Dr Kevin Tracey, North-Shore Jewish Hospital, Long Island, NY, USA) followed by HRP-conjugated anti-rabbit IgG and Super Signal West Femto substrate (Pierce, Rockford, IL, USA). Images were captured with a CCD camera.

As data in fig 1 indicate, sera from patients with SLE as well as MRL/MpJ-*lpr/lpr* mice show increased levels of HMGB1 by Western blotting. To assess the extent of this increase, the density of the HMGB1 band was analysed by AlphaEasyFC version 3.1.2 and the value expressed as fold increase over control. In samples studied, for human sera, the amounts of HMGB1 increased 2.8–36-fold while, for the murine sera, the values increase 1–28-fold over controls. Furthermore, in MRL/MpJ-*lpr/lpr* mice, the HMGB1 levels rose with disease progression (data not shown). Together, these data indicate that HMGB1 release occurs with SLE and can produce increased levels in the sera similar to that occurs in sepsis and shock.[2 9] Further analysis will be need to determine the relationship to disease activity and treatment.

In the context of SLE, increased levels of other nuclear constituents in the blood (eg, DNA) have been attributed to cell death, with impaired clearance mechanisms (eg, complement deficiency) preventing normal disposal. Extracellular HMGB1 in

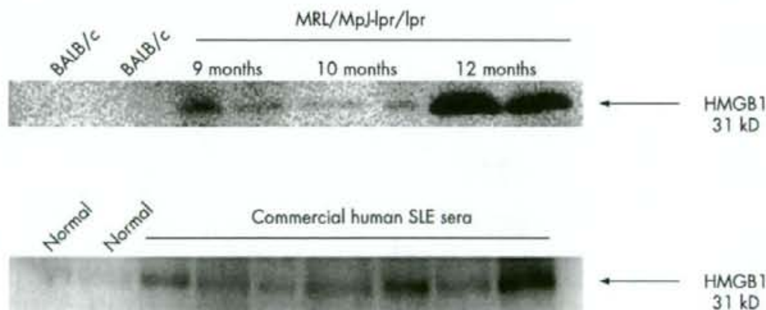

Figure 1 Detection of high mobility group protein 1 (HMGB1) in mouse and human sera. Sera from either mice (A) or human (B) were resolved on sodium dodecyl sulphate–polyacrylamide gel electrophoresis and analysed by Western blotting with an anti-HMGB1 antibody. SLE, systemic lupus erythematosus.

Research article **Open Access**

Association of reduced heme oxygenase-1 with excessive Toll-like receptor 4 expression in peripheral blood mononuclear cells in Behçet's disease

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Abstract

Introduction Toll-like receptors (TLRs) mediate signaling that triggers activation of the innate immune system, whereas heme oxygenase (HO)-1 (an inducible heme-degrading enzyme that is induced by various stresses) suppresses inflammatory responses. We investigated the interaction between TLR and HO-1 in an inflammatory disorder, namely Behçet's disease.

Methods Thirty-three patients with Behçet's disease and 30 healthy control individuals were included in the study. Expression levels of HO-1, TLR2 and TLR4 mRNA were semiquantitatively analyzed using a real-time PCR technique, and HO-1 protein level was determined by immunoblotting in peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes. In some experiments, cells were stimulated with lipopolysaccharide or heat shock protein-60; these proteins are known to be ligands for TLR2 and 4.

Results Levels of expression of HO-1 mRNA were significantly reduced in PBMCs from patients with active Behçet's disease, whereas those of TLR4, but not TLR2, were increased in PBMCs, regardless of disease activity. Moreover, HO-1 expression in PBMCs from patients with Behçet's disease was repressed in the presence of either lipopolysaccharide or heat shock protein-60.

Conclusion The results suggest that upregulated TLR4 is associated with HO-1 reduction in PBMCs from patients with Behçet's disease, leading to augmented inflammatory responses.

Introduction

Behçet's disease (BD) is an inflammatory disorder of unknown cause, characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions [1]. A close association of the human leukocyte antigen (HLA)-B51 allele with the disease suggests that genetic predisposition contributes to susceptibility to BD [2]. In addition, infections with agents such as herpes simplex virus [3,4] and *Streptococcus sanguis* [5] has been implicated in the development of BD, although no specific infectious agent has been identified as its cause [6]. Rather, several reports have suggested that ubiquitous antigens presented by micro-organisms, such as heat shock proteins (HSPs), trigger crossreactive autoimmune responses through molecular mimicry machinery, which results in BD [6].

Not just acquired but also innate immune systems are activated in BD, because hyperfunction of neutrophils is a hallmark of the disease [7]. However, the immunopathological mechanisms remain uncertain. Toll-like receptors (TLRs), which are expressed on phagocytes and other cells, recognize

BD = Behçet's disease; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HLA = human leukocyte antigen; HO = heme oxygenase; HSP = heat shock protein; LPS = lipopolysaccharide; PBMC = peripheral blood mononuclear cell; PCR = polymerase chain reaction; PMN = polymorphonuclear leukocyte; RA = rheumatoid arthritis; TLR = Toll-like receptor; TNF = tumor necrosis factor.

'pathogen-associated molecular patterns' in microbes and mediate inflammatory signal transduction [8,9]. TLR2 and TLR4 recognize lipoproteins and lipopolysaccharide (LPS), respectively. Furthermore, both receptors also bind to the endogenous 60 kDa HSP (HSP60), leading to cell activation [10,11]. It is becoming clear that TLRs are involved in systemic autoimmune disorders, because it was recently demonstrated TLR2 and TLR4 are involved in rheumatoid arthritis (RA) [12-14] and TLR9 in systemic lupus erythematosus [15,16]. These findings have led to the hypothesis that microbial antigens not only trigger autoimmune responses through specific T-cell receptors but they also activate the innate immune system through the TLRs, leading to the inflammation that is characteristic of BD [17].

Few studies have been conducted to investigate the role played by the regulatory systems in inflammatory diseases of humans, including BD. We are interested in heme oxygenase (HO)-1, because accumulating evidence suggests that HO-1 protects the host in a variety of pathologic conditions [18,19]. Our laboratory has demonstrated the beneficial role of HO-1 in inflammatory lung disease [20] and lupus nephritis [21]. On the other hand, a deficiency in HO-1 expression is associated with severe chronic inflammation, as demonstrated by studies conducted in HO-1 knockout mice [22] and observations in a patient with HO-1 deficiency [23]. These findings are consistent with the notion that HO-1 plays a physiologic role in protecting against inflammation. Furthermore, our recent studies [24-26] have demonstrated substantial pathologic roles of HO-1 in rheumatic diseases. Abundant expression of HO-1 was identified in synovial tissues of patients with RA, in the absence of elevated serum HO-1 levels [24,25]. Further analysis using RA synovial cell lines suggests that HO-1 plays a regulatory role in RA inflammation [25]. Our recent study [26] showed that tumor necrosis factor (TNF) suppresses HO-1 expression in human monocytes, leading to augmentation of inflammatory responses, and that clinical efficacy of anti-TNF therapy is associated with restoration of HO-1 expression in circulating monocytes from patients with RA [26]. In another study [20], HO-1 gene therapy successfully ameliorated lung injury induced by LPS, which stimulates the innate immune system through TLR4. It is thus of interest to study the relationship between TLRs, as activating factors, and HO-1, as a regulatory factor of inflammatory responses in inflammatory disorders.

In the present study, mRNA expression levels of HO-1, TLR2, and TLR4 in circulating leukocytes from BD patients were determined. The data suggest that activation signals through essentially over-expressed TLR4 cause reduction in HO-1 expression in peripheral blood mononuclear cells (PBMC), resulting in an augmentation of inflammatory responses in BD.

Materials and methods

Patients and healthy donors

Thirty-three patients with BD, who met the International Study Group criteria for diagnosis of BD [27], were enrolled in the study. Their mean age was 47.7 ± 15.0 years, and 13 were male and 20 were female.

All of the patients were under the care of the Yokohama City University Hospital. As previously described [28], 13 patients with one or more lesions (including genital ulcers, uveitis, erythema nodosum, arthritis, gastrointestinal lesions, central nervous system lesions, and/or C-reactive protein >10 mg/l) were regarded to have active disease during the study.

The patients had been treated with a combination of the following agents: colchicines (17 patients), corticosteroids (13 patients), nonsteroidal anti-inflammatory drugs (14 patients), sulfasalazine (two patients), and cytotoxic drugs such as methotrexate (one patient), cyclosporine (four patients), tacrolimus (one patient) and cyclophosphamide (one patient). Thirty healthy age- and sex-matched individuals were also included as a control group. HLA-B type was determined by SRL Inc. (Tokyo, Japan) using lymphocyte cytotoxicity assay or a PCR reverse sequence specific oligonucleotides method. All experiments were conducted after written informed consent has been obtained, which was approved by the local institutional review board.

Reagents

Reagents were obtained from the following manufactures: recombinant human TNF-α (R&D; Minneapolis, MN, USA), polymyxin B and LPS *Escherichia coli* O111: B4 (Calbiochem; La Jolla, CA, USA), low endotoxin recombinant human HSP60 (Stressgen; Victoria, Canada), and $IgG_1\kappa$ (Serotech; Oxford, UK). Infliximab was kindly provided by Tanabe Seiyaku (Osaka, Japan).

Cell preparation and culture

PBMCs and polymorphonuclear leukocytes (PMNs) were isolated by centrifugation over two Ficoll-Hypaques gradients of specific gravities 1.077 (ICN; Aurora, OH, USA) and 1.119 (Nacalai; Kyoto, Japan). Purity of the separated neutrophils, which were determined by flow cytomeric scattergram, was typically above 97% [7]. Monocytes were negatively selected by magnetic cell sorting (Miltenyi Biotec; Gladbach, Germany) using a monocyte isolation kit (Miltenyi Biotec). More than 95% of the obtained monocytes expressed CD14, based on flowcytomeric analysis [26].

The cells were incubated in hepes modified RPMI1640 (Sigma-Aldrich; Saint Louis, MO, USA) containing 10% fetal calf serum (Equitech-bio; Kerrville, TX, USA), 2 mmol/l L-glutamine (Sigma-Aldrich), 100 U/ml penicillin plus 100 μg/ml streptomycin (Sigma-Aldrich) in a 5% carbon dioxide in an air incubator at 37°C. To determine HO-1 expression at mRNA

and protein levels, cells were cultured in the presence or absence of LPS (10 ng/ml) or HSP60 (3 μg/ml) for 6 to 24 hours.

Transfection

Purified monocytes (1×10^6) were transfected with 3 μg of human HO-1 expression vector or control vector by using Nucleofector (Amaxa Biosystems; Gaithersburg, MD, USA) and human monocyte Nucleofector kit (Amaxa Biosystems) [25,26]. Twenty four hours later, the cells were used for further experiments.

Reverse transcription PCR and Real-time PCR

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) [21,24-26]. One microgram of total RNA served as a template for single-stranded cDNA synthesis in a reaction using oligo (dT) primers and SuperScript II (Invitrogen). For the reverse transcription PCR, 1 μl cDNA was incubated with 9.375 μl de-ionized distilled water, 2 μl dNTP, 2.5 μl 10 × PCR buffer, 0.125 μl Taq polymerase (Takara, Ohtsu, Japan), and primer pairs for target genes. The primers used in the study are summarized in Table 1.

Cycling conditions included 35 cycles of amplification for 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, and a final extension phase consisting of one cycle of 10 minutes at 72°C. The primers and probes for human HO-1, TLR2, TLR4, CD14, TNF-α, MD-2 (Myeloid differentiation factor-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used in the real-time PCR were purchased from PE Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed using a BD Qtaq DNA polymerase (BD Bioscience), and the data were analyzed by the ABI prism 7700 sequence detection system (PE Applied Biosystems, Franklin Lakes, NJ, USA). Briefly, 1/50 of cDNA derived from 1 μg total RNA, 200 nmol/l probe, and 800 nmol/l primers were incubated in 25 μl at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The analysis system determined the number of cycles at which the amplified DNA in the sample exceeded the threshold during the PCR. Gene expression levels of the individual samples were calculated on standard curves of each cDNA generated by serial dilutions of the PCR amplified products. The data on HO-1, TLR2, TLR4, and TNF-α were standardized to the expression of GAPDH in the same samples, using multiplex PCR technique. Expression level of HO-1 mRNA in a sample is indicated as arbitrary units.

Immunoblot analysis

The expression of HO-1 protein was determined by immunoblotting as described previously [25]. Briefly, cells were treated with lysis buffer (137 mmol/l NaCl, 20 mmol/l Tris-HCl, 50 mmol/l NaF, 1 mmol/l EDTA, and Triton-X), supplemented with a protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes on ice, and the supernatants were recovered by centrifugation at 15,000 rpm for 30 minutes. For TLR2 and TLR4 immunoblotting, after addition of lysis buffer, cells were homogenized for 15 minutes by ultrasonifier (Branson Japan, Kanagawa,

Table 1

Primers used in the study

Primer	Sense/antisense	Sequence
HO-1	Sense	CAGGCAGAGAATGCTGAG
	Antisense	GCTTCACATAGCGCTGCA
TLR2	Sense	TGACTGCTCGGAGTTCTCCC
	Antisense	GTCAGCACCAGAGCCTGGAG
TLR4	Sense	GCGGCTCGAGGAAGAGAAGA
	Antisense	AGGCTCTGATATGCCCCATC
GAPDH	Sense	ACAGTCAGCCGCATC
	Antisense	AGGTGCGGCTCCCTA
TNF-α	Sense	ATGAGCACTGAAAGCATGATC
	Antisense	GGCGATGCGGCTGATGGT
CD14	Sense	CGGCCGAAGAGTTCACAAGT
	Antisense	AGTGCAGTCCTGTGGCTTC
MD-2	Sense	TAAATCTTTTCTGCTTACTGA
	Antisense	TACTCAATTTATTCTAATTTGAAT

HO, heme oxygenase; MD, Myeloid differentiation factor-2 ; TLR, Toll-like receptor; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Japan). The samples were resolved electrophoretically on a 4% to 20% gradient of polyacrylamide gel (Daiichi Kagaku, Tokyo, Japan) and transferred onto a polyvinyldene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk/Tris-buffered saline overnight at 4°C, the membrane was incubated with optimally diluted anti-HO-1 monoclonal antibody (Stressgen), anti-TLR2 and anti-TLR4 (Imgenex, San Diego, CA, USA) monoclonal antibody, or anti-actin goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature or overnight at 4°C, and subsequently for 45 minutes with horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Life Sciences, Piscataway, NJ, USA) or rabbit anti-goat IgG horseradish peroxidase conjugate (Zymed, South San Francisco, CA, USA). The signals were developed by using the enhanced chemiluminescence detection system (Amersham Life Sciences). The amount of blotted protein was measured densitometrically by using Scion image analysis and image processing software (NIH Image Engineering, Bethesda, MD, USA).

Statistical analysis

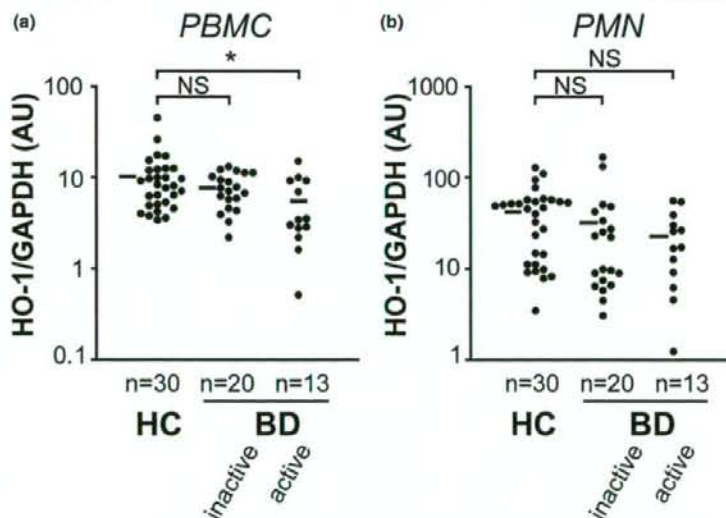
Mann-Whitney U-test, Kruskal-Wallis test with post-hoc Scheffe's test, paired *t*-test, and regression analysis were used to test for differences. *P* values less than 0.05 were considered statistically significant.

Results

Reduced HO-1 mRNA expression in PBMCs from patients with active BD

HO-1 mRNA expression level was determined in circulating leukocytes from BD patients by using a real-time PCR technique (Figure 1). A good correlation between HO-1 mRNA and protein levels has been demonstrated [26]. Consistent with previous findings [24], we found no significant difference in HO-1 mRNA expression in PBMCs between BD patients (including both patients with active and those with inactive disease) and healthy control individuals (data not shown). A more detailed analysis based on disease activity, however, revealed that PBMCs from patients with active BD, but not those with inactive disease, expressed significantly lower HO-1 mRNA levels than did PBMCs from healthy control individuals (Figure 1a). Because HO-1 is preferentially expressed by monocytes among PBMCs, amounts of HO-1 mRNA may depend on the proportion of monocytes detected [26]. CD14 mRNA levels determined by real-time PCR were comparable between BD and healthy control individuals, indicating that there was no difference between groups in the proportion of monocytes among circulating leukocytes (data not shown). Moreover, no significant difference was found in absolute counts of monocytes between patients with active BD and those with inactive disease (active 497.9 ± 218.8/μl versus inactive 462.7 ± 182.4/μl; $P = 0.77$, by Mann-Whitney U-test), indicating that HO-1 expression was reduced in individual cells from patients with active disease. As shown in Figure 1b, HO-1 mRNA lev-

Figure 1



HO-1 mRNA expression in PBMCs and PMNs from patients with BD. **(a)** Peripheral blood mononuclear cell (PBMC) heme oxygenase (HO)-1 mRNA expression in healthy controls (HC), and patients with active and inactive Behçet's disease (BD) were determined semiquantitatively by real-time PCR. Horizontal bars represent mean values of HO-1 mRNA. **(b)** Polymorphonuclear leukocyte (PMN) HO-1 mRNA expression levels of HC, and patients with active and inactive BD. **P* < 0.05, as determined using the Kruskal-Wallis test with post-hoc Scheffe's test. AU, arbitrary unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

els in PMNs were not significantly different between BD patients and control individuals (Figure 1b).

No particular clinical manifestations, including ocular lesions (Table 2) and treatments (data not shown), were associated with the reduction in HO-1 mRNA expression in PBMCs. There were no differences in mRNA expression levels of HO-1 and TLRs between HLA-B51-positive and -negative patients (Table 2). Levels of HO-1 mRNA expression were not altered by treatment with colchicine or prednisolone in the patients (data not shown).

Increased TLR4, but not TLR2, expression by PBMCs from BD patients

Because HSP60 has been implicated in the pathogenesis of BD [17], levels of mRNA for TLR2 and TLR4 (both of which recognize HSP60 as a ligand) were examined in PBMCs and PMNs from patients with BD (Figure 2). In preliminary experiments, the relationship between levels of TLR mRNA and protein in circulating leukocytes was examined. Briefly, after fractionating PBMCs into CD14-positive cells and CD14-depleted cells by means of magnetic cell sorting, mRNA and protein levels of TLRs were compared by using real-time PCR and immunoblotting techniques, respectively. TLR4 was preferentially expressed on CD14-positive cells, but not CD14-depleted cells, at both mRNA and protein levels (Additional file 1). Moreover, TLR4 and TLR mRNA levels correlated well with protein levels.

No significant differences were found in levels of TLR2 mRNA expression in PBMCs between patients with active BD, patients with inactive BD, and healthy control individuals (Figure 2a). On the other hand, TLR4 mRNA expression levels were elevated in PBMCs from patients, irrespective of disease activity (Figure 2b) and HLA-B51 phenotype (data not shown). However, no significant differences in levels of mRNA expression for CD14 and MD-2, which are critically involved in LPS-mediated signal transduction of TLR, were found between patients and control individuals (data not shown). There was no abnormality in mRNA expression of TLR2 and TLR4 in PMNs (Figure 2c,d). The results indicate that TLR4 mRNA expression is constitutively increased in PBMCs from BD patients.

Inverse correlation between HO-1 and TLR4 mRNA in PBMCs from BD patients

TLR4 signaling triggers activation of the innate immune system, whereas HO-1 plays a regulatory role in inflammatory response. Analysis of the relationship between the two molecules showed that TLR4 mRNA was inversely correlated with HO-1 mRNA in PBMCs from BD patients (Additional file 2; $P < 0.05$, $r = -0.42$, by regression analysis). Because LPS (a TLR4 ligand) has been shown to suppress interleukin-10-dependent HO-1 expression in human PBMCs [29], it is plausible that excessively expressed TLR4 contributes to defective HO-1 expression in PBMCs from BD patients. As expected, the immunoblotting study revealed that stimulation with LPS reduced HO-1 expression in PBMCs from patients with BD, irrespective of the presence or absence of interleukin-10 (Figure 3a). The suppressive effect on HO-1 expression was completely abrogated by a LPS neutrizer, namely polymyxin B (Figure 3a). Similarly, real-time PCR analysis revealed a reduction in HO-1 mRNA levels in LPS-stimulated PBMCs (Figure 4a) when TNF mRNA expression was elevated (Figure 4b). The magnitude of LPS-induced HO-1 suppression (calculated as the gap in HO-1 mRNA between PBMCs subjected to 6 hours of LPS treatment and untreated PBMCs [ΔHO-1]) was significantly correlated with TLR4 mRNA expression levels in untreated PBMCs (Figure 4c).

In our previous study [26] we demonstrated that TNF enhances HO-1 mRNA degradation, resulting in a reduction in HO-1 expression in human monocytes. Because TLR4 signaling leads to synthesis of TNF, which may be involved in the reduction in HO-1 expression in PBMCs from patients with BD. However, levels of TNF mRNA did not correlate with those of HO-1 in PBMCs from patients with BD (data not shown). Moreover, although the preliminary experiments confirmed that 10 ng/ml LPS efficiently stimulated PBMCs to produce substantial amounts of TNF protein, anti-TNF-α antibody infliximab did not eliminate the suppressive effect of LPS on HO-1 expression in monocytes *in vitro* (Additional file 3). The findings indicated that the effect is not solely dependent on TNF (Figure 3d).

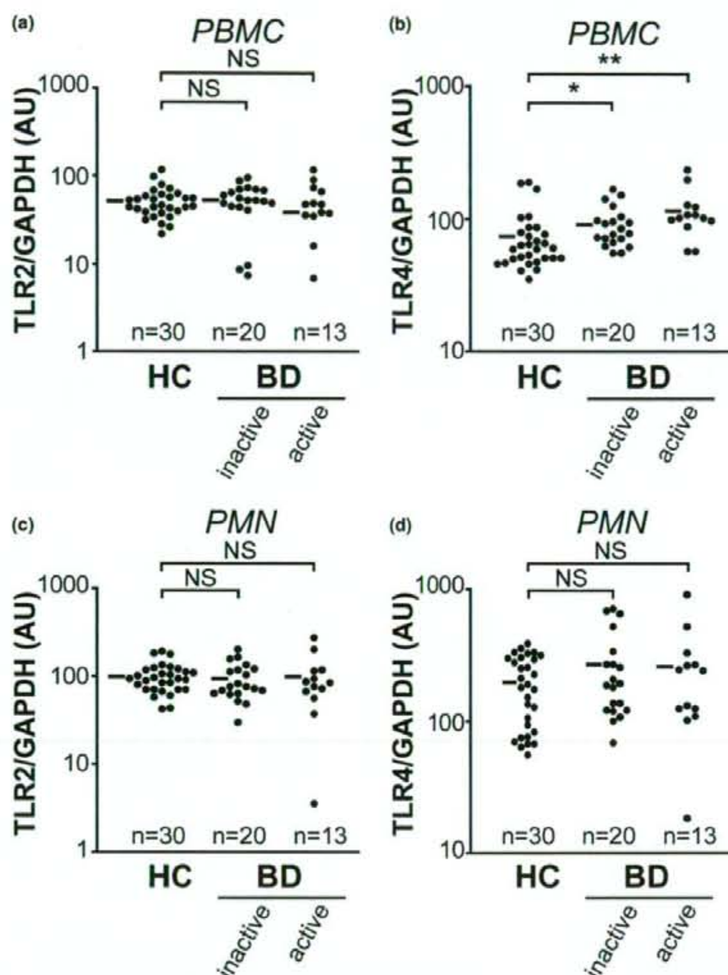
Table 2

HO-1 mRNA expression in patients with BD

	PBMCs/PMNs	Ocular involvement		HLA-B51	
		- ($n = 17$)	+ ($n = 16$)	- ($n = 10$)	+ ($n = 14$)
HO-1 (AU)	PBMCs	7.7 ± 3.1	5.7 ± 4.1	8.1 ± 3.9	5.6 ± 3.7
	PMNs	32.9 ± 46.1	31.6 ± 34.1	31.6 ± 38.0	19.9 ± 18.0

Values are expressed as mean ± standard deviation. AU, arbitrary unit; BD, Behçet's disease; HLA, human leukocyte antigen; HO, heme oxygenase; PBMC, peripheral blood mononuclear cells; PMN, peripheral blood multinuclear cells.

Figure 2



TLR2 and TLR4 mRNA expression in PBMCs and PMNs from patients with BD. Expression levels in peripheral blood mononuclear cells (PBMCs) of **(a)** Toll-like receptor (TLR)2 and **(b)** TLR4 mRNA in healthy controls (HC), and patients with active and inactive Behçet's disease (BD) were determined semiquantitatively by real-time PCR. Horizontal bars represent mean values of HO-1 mRNA. Expression levels in polymorphonuclear leukocytes (PMNs) of **(c)** TLR2 and **(d)** TLR4 in HC, and patients with active and inactive BD. $^{*}P < 0.05$, $^{**}P < 0.01$, as determined using the Kruskal-Wallis test with post-hoc Scheffe's test. AU, arbitrary unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

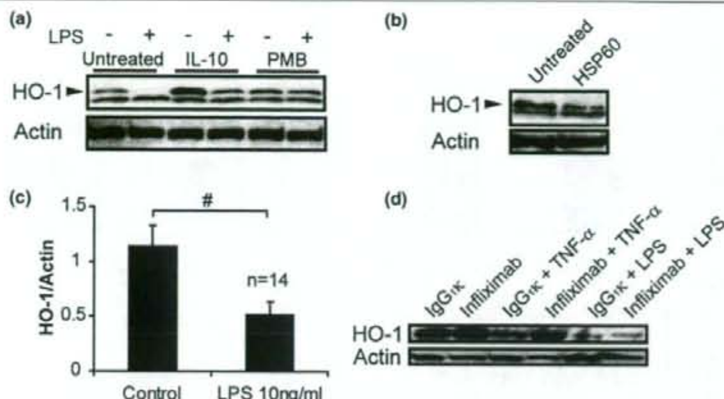
No effect of forced HO-1 expression on TLR2 and TLR4 mRNA in human PBMCs

Because our previous study demonstrated bidirectory interactions between HO-1 and TNF [26], we also examined effects of HO-1 upregulation on TLR levels in monocytes. Overexpression of HO-1 protein was confirmed by immunoblotting analysis 24 hours after transfection with pHO-1 (human HO-1 expression vector) into monocytes. Under these conditions, no differences were found in expression levels of TLR2 and TLR4 between HO-1 cDNA transfected monocytes and controls (Figure 5). Taken together, our findings implicate the involvement of excessive TLR4 expression in low levels of HO-1 mRNA expression in PBMCs from patients with BD.

Discussion

In the present study we found endogenous HO-1 expression to be decreased in PBMCs from patients with active BD. Dysregulation of HO-1 expression is associated with some rheumatic diseases. Our previous studies [24,25] have demonstrated elevated serum HO-1 levels in patients with

Figure 3



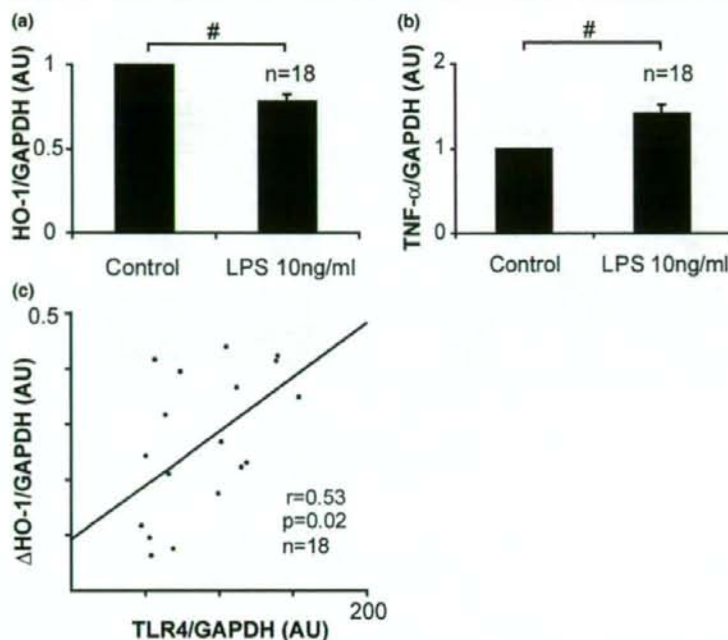
Effects of HSP60 and LPS on HO-1 protein expression in PBMCs from patients with BD. **(a)** Effect of lipopolysaccharide (LPS) stimulation on heme oxygenase (HO)-1 and actin protein expression in peripheral blood mononuclear cells (PBMCs) from patients with Behçet's disease (BD). PBMCs from a BD patient were stimulated with LPS in the presence or absence of 10 ng/ml interleukin (IL)-10 and 100 μg/ml polymyxin B (PMB). Representative immunoblotting data for HO-1 protein in the cells are shown. The arrowhead indicates 32 kDa molecular weight HO-1 specific band. **(b)** Effect of heat shock protein (HSP)60 (3 μg/ml) stimulation on endogenous HO-1 protein expression in PBMCs from patients with BD. The arrowhead indicates 32 kDa HO-1 specific band. A representative of three independent experiments is shown. **(c)** Mean and standard error of the mean (SEM) values of HO-1 and actin protein expression in PBMCs stimulated by LPS (1 ng/ml) for 24 hours in patients with BD ($n = 14$). $^{\#}P < 0.001$, as determined using paired *t*-test. **(d)** Effect of infliximab (10 μg/ml) or $IgG_1\kappa$ (10 μg/ml) on HO-1 expression in LPS (10 ng/ml) or tumor necrosis factor (TNF; 1 ng/ml) treated PBMCs.

adult onset Still's disease and hemophagocytic syndrome, and aberrant expression of HO-1 in synoviocytes from patients with RA. However, reduced HO-1 levels in leukocytes have not been demonstrated in other rheumatic diseases. Evidence suggests that increased expression of HO-1 can benefit the host in a variety of pathologic conditions, including inflammatory changes, whereas a deficiency in HO-1 expression is associated with vigorous inflammation, as demonstrated by studies of HO-1 knockout mice and observed in a patient with HO-1 deficiency [22,23]. In RA, HO-1-expressing cells were located in the lining and sublining layers, but not in the cartilage-pannus junction, where bone and cartilage are actively destroyed [25,30,31]. Furthermore, our previous report [26] demonstrated that selective knockdown of HO-1 expression by using specific small interfering RNA resulted in upregulation of synthesis of proinflammatory cytokines, including interleukin-6, interleukin-8 and TNF, which have been shown to be elevated in sera from BD patients [6]. This suggests that leukocyte function is regulated by HO-1 expressed in the cells [26]. Thus, defective expression of HO-1 may be involved in the inflammation characteristic of BD, especially in patients with active disease.

Although a pathogenic role of anti-HSP60 specific autoimmune responses has been suggested in BD, abnormal activation of the innate immune system has also been identified in the disease [1,6]. Furthermore, involvement of TLRs has been shown in other systemic autoimmune diseases [16]. In the present study, expression levels of TLR2 and TLR4 were examined because both TLRs recognize HSP60 as ligands [10,11]. Actually, HSP60 was reported to be expressed in PBMCs, and in intestinal and mucocutaneous lesions from BD patients [32,33]. Our findings demonstrated that levels of TLR4 mRNA, but not of TLR2 mRNA, are constitutively increased in PBMCs from patients with BD, regardless of disease activity. The data suggest possible involvement of TLR4 in BD, although TLR4 has been also implicated in other rheumatic diseases [13,34]. Abnormal expression of TLR4 can predispose to defective HO-1 expression in BD PBMCs, because TLR4 may be a putative HO-1 repressor in hepatic ischemia/reperfusion injury mouse model [35]. Indeed, HO-1 expression was suppressed in PBMCs stimulated with LPS [29]. Moreover, elevated soluble CD14 in plasma of BD patients may further facilitate LPS binding to TLR4 [36]. Interestingly, LPS-induced lung injury in a mouse model was rescued by administration of an HO-1 adenovirus vector [20]; this suggests that HO-1 supplementation may have utility as a strategy for countering TLR4-related inflammation. Such a strategy may also be applicable to BD.

TNF plays a critical role in the development of BD [1,37,38]. Several studies, including ours, have demonstrated that TNF is excessively produced in patients with active BD [28,38]. Indeed, anti-TNF therapy is effective in the disease, especially for management of ocular lesions [39]. In our previous study [26] we showed that TNF suppresses HO-1 expression levels in human peripheral monocytes, thereby accelerating inflammatory responses; this suggests that excessive TNF levels contribute to defective HO-1 expression. However, no association was found between HO-1 and TNF mRNA levels in cir-

Figure 4



Effect of LPS on HO-1 mRNA expression in PBMCs from BD patients. Expression of **(a)** heme oxygenase (HO)-1 and **(b)** tumor necrosis factor (TNF) mRNA in peripheral blood mononuclear cells (PBMCs) from patients with Behçet's disease (BD; $n = 18$). Values presented are mean and standard error of the mean (SEM) change, regarding 1 to be the value of untreated cells. #$P < 0.001$, as determined using paired *t*-test. **(c)** Relationship of endogenous Toll-like receptor (TLR)4 mRNA with gap in HO-1 mRNA between PBMCs subjected to 6 hours of treatment with lipopolysaccharide (LPS) and untreated PBMCs (ΔHO-1). $P = 0.02$, $r = 0.53$, as determined by regression analysis. AU, arbitrary unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

culating PBMCs from patients with BD. In addition, the suppressive effect of LPS on HO-1 was not abrogated by anti-TNF antibody, at least *in vitro*, although significant synthesis of TNF in response to LPS was confirmed in the experiments (Additional file 3). These data, rather, suggest that the effect of LPS is mainly mediated by a pathway distinct from TNF. However, TNF may also contribute to defective HO-1 expression *in vivo*, because other types of cells also produce TNF in BD. Taken together, our findings suggest that highly expressed TLR4 might contribute to reduced HO-1 expression, leading to an activation of the innate immune system in BD, although other factors including TNF may be involved in the defective HO-1. Because TLRs other than TLR4 are also likely to be involved in the pathogenesis BD [17], further investigation of molecular mechanisms, including interactions between TLRs and HO-1, are required, especially those that distinguish BD from other inflammatory diseases.

Conclusion

Based on the data presented, we hypothesize that HSP60 stimulates not only antigen-specific autoimmune responses but also the innate immune system through constitutively overexpressed TLR4, which mediates HO-1 reduction in PBMCs, leading to inflammation in BD. Restoration of HO-1 expression might be a promising therapeutic strategy in the disease. Alternatively, specific intervention in TLR4-mediated signals that lead to HO-1 reduction may also be of benefit in BD.

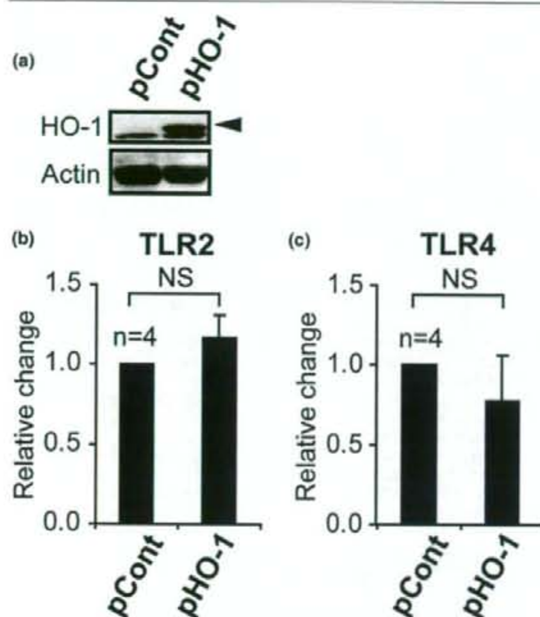
Competing interests

The authors have received no financial support or other benefits from commercial sources for the work reported here, and the authors have no other financial interests that could create a potential conflict of interest or the appearance of a conflict of interest with regard to the present study.

Authors' contributions

YI designed and organized the study. YK, MT, RW, SM, and MK conducted the laboratory work. YK, MT, RW, SM, MK, AI, HI, SO, AU, NM, and YI were involved in the analysis and interpretation of data. YK, MT, and YI were involved in writing the report. All authors read and approved the final manuscript. The authors thank Mr Tom Kiper for his review.

Figure 5



Effect of forced HO-1 expression on Toll-like receptor (TLR)2 and TLR4 mRNA expression in peripheral monocytes. **(a)** Immunoblotting analysis of heme oxygenase (HO)-1 and actin in pHO-1 (human HO-1 expression vector) or pCont (control vector) transfected monocytes. The arrow represents HO-1 protein. **(b)** Real-time PCR analysis of TLR2 and TLR4 mRNA expression in pHO-1 transfected peripheral blood mononuclear cells (PBMCs). NS, not significant.

Additional files

The following Additional files are available online:

Additional file 1

The Protein and mRNA TLR2, TLR4 and HO-1 expression levels in PBMCs. (A) TLR2, TLR4, HO-1, and actin protein expression in PBMCs and CD14+/- cells from a healthy control individual (HC). (b) TLR2, TLR4, CD14, and β-actin mRNA expression levels in CD14+/- cells from HCs. (C) Correlation between densitometrically analyzed HO-1 protein levels and semiquantatively evaluated HO-1 mRNA expression by real-time PCR in PBMCs and CD14+/- cells from a HC.
See http://www.biomedcentral.com/content/supplementary/ar2367-S1.TIFF

Additional file 2

The correlation between HO-1 and TLR4 mRNA levels in PBMCs from patients with BD.
See http://www.biomedcentral.com/content/supplementary/ar2367-S3.TIFF

Additional file 3

The effect of LPS, PMB, and infliximab on TNF-α production by PBMCs. TNF-α levels in supernatants of PBMC-cultured media recovered after 24 hours of stimulation with LPS, with or without PMB and/or infliximab, as determined by ELISA.
See http://www.biomedcentral.com/content/supplementary/ar2367-S2.TIFF

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References

1. Sakane T, Takeno M, Suzuki N, Inaba G: **Behcet's disease.** *N Engl J Med* 1999, **341:**1284-1291.
2. Ohno S, Ohguchi M, Hirose S, Matsuda H, Wakisaka A, Aizawa M: **Close association of HLA-Bw51 with Behcet's disease.** *Arch Ophthalmol* 1982, **100:**1455-1458.
3. Sezer FN: **The isolation of a virus as the cause of Behcet's diseases.** *Am J Ophthalmol* 1953, **36:**301-315.
4. Lee S, Bang D, Cho YH, Lee ES, Sohn S: **Polymerase chain reaction reveals herpes simplex virus DNA in saliva of patients with Behcet's disease.** *Arch Dermatol Res* 1996, **288:**179-183.
5. Anonymous: **Skin hypersensitivity to streptococcal antigens and the induction of systemic symptoms by the antigens in Behcet's disease: a multicenter study. The Behcet's Disease Research Committee of Japan.** *J Rheumatol* 1989, **16:**506-511.
6. Direskeneli H: **Behcet's disease: infectious aetiology, new autoantigens, and HLA-B51.** *Ann Rheum Dis* 2001, **60:**996-1002.
7. Takeno M, Kariyone A, Yamashita N, Takiguchi M, Mizushima Y, Kaneoka H, Sakane T: **Excessive function of peripheral blood neutrophils from patients with Behcet's disease and from HLA-B51 transgenic mice.** *Arthritis Rheum* 1995, **38:**426-433.
8. Akira S, Takeda K: **Toll-like receptor signalling.** *Nat Rev Immunol* 2004, **4:**499-511.
9. Kawai T, Akira S: **Toll-like receptor downstream signaling.** *Arthritis Res Ther* 2005, **7:**12-19.
10. Ohashi K, Burkart V, Flohe S, Kolb H: **Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex.** *J Immunol* 2000, **164:**558-561.
11. Vabulas RM, Ahmad-Nejad P, da Costa C, Miethke T, Kirschning CJ, Hacker H, Wagner H: **Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells.** *J Biol Chem* 2001, **276:**31332-31339.
12. Seibl R, Birchler T, Loeliger S, Hossle JP, Gay RE, Saurenmann T, Michel BA, Seger RA, Gay S, Lauener RP: **Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium.** *Am J Pathol* 2003, **162:**1221-1227.
13. Frasnelli M, So A: **Toll-like receptor 2 and toll-like receptor 4 expression on CD64+ monocytes in rheumatoid arthritis: com-**

ment on the article by Iwahashi et al. *Arthritis Rheum* 2005, **52**:2227-2228.

14. Iwahashi M, Yamamura M, Aita T, Okamoto A, Ueno A, Ogawa N, Akashi S, Miyake K, Godowski PJ, Makino H: **Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis.** *Arthritis Rheum* 2004, **50**:1457-1467.
15. Papadimitraki ED, Choulaki C, Koutala E, Bertsias G, Tsatsanis C, Gergianaki I, Raptopoulou A, Kritikos HD, Mamalaki C, Sidiropoulos P, Boumpas DT: **Expansion of toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process.** *Arthritis Rheum* 2006, **54**:3601-3611.
16. Marshak-Rothstein A: **Toll-like receptors in systemic autoimmune disease.** *Nat Rev Immunol* 2006, **6**:823-835.
17. Direskeneli H, Saruhan-Direskeneli G: **The role of heat shock proteins in Behcet's disease.** *Clin Exp Rheumatol* 2003, **21**:S44-S48.
18. Otterbein LE, Soares MP, Yamashita K, Bach FH: **Heme oxygenase-1: unleashing the protective properties of heme.** *Trends Immunol* 2003, **24**:449-455.
19. Morse D, Choi AM: **Heme oxygenase-1: the 'emerging molecule' has arrived.** *Am J Respir Cell Mol Biol* 2002, **27**:8-16.
20. Inoue S, Suzuki M, Nagashima Y, Suzuki S, Hashiba T, Tsuburai T, Ikehara K, Matsuse T, Ishigatsubo Y: **Transfer of heme oxygenase 1 cDNA by a replication-deficient adenovirus enhances interleukin 10 production from alveolar macrophages that attenuates lipopolysaccharide-induced acute lung injury in mice.** *Hum Gene Ther* 2001, **12**:967-979.
21. Takeda Y, Takeno M, Iwasaki M, Kobayashi H, Kirino Y, Ueda A, Nagahama K, Aoki I, Ishigatsubo Y: **Chemical induction of HO-1 suppresses lupus nephritis by reducing local iNOS expression and synthesis of anti-dsDNA antibody.** *Clin Exp Immunol* 2004, **138**:237-244.
22. Poss KD, Tonegawa S: **Reduced stress defense in heme oxygenase 1-deficient cells.** *Proc Natl Acad Sci USA* 1997, **94**:10925-10930.
23. Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S: **Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency.** *J Clin Invest* 1999, **103**:129-135.
24. Kirino Y, Takeno M, Iwasaki M, Ueda A, Ohno S, Shirai A, Kanamori H, Tanaka K, Ishigatsubo Y: **Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia.** *Arthritis Res Ther* 2005, **7**:R616-R624.
25. Kobayashi H, Takeno M, Saito T, Takeda Y, Kirino Y, Noyori K, Hayashi T, Ueda A, Ishigatsubo Y: **Regulatory role of heme oxygenase 1 in inflammation of rheumatoid arthritis.** *Arthritis Rheum* 2006, **54**:1132-1142.
26. Kirino Y, Takeno M, Murakami S, Kobayashi M, Kobayashi H, Miura K, Ideguchi H, Ohno S, Ueda A, Ishigatsubo Y: **Tumor necrosis factor alpha acceleration of inflammatory responses by down-regulating heme oxygenase 1 in human peripheral monocytes.** *Arthritis Rheum* 2007, **56**:464-475.
27. Anonymous: **Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease.** *Lancet* 1990, **335**:1078-1080.
28. Misumi M, Hagiwara E, Takeno M, Takeda Y, Inoue Y, Tsuji T, Ueda A, Nakamura S, Ohno S, Ishigatsubo Y: **Cytokine production profile in patients with Behcet's disease treated with infliximab.** *Cytokine* 2003, **24**:210-218.
29. Ricchetti GA, Williams LM, Foxwell BM: **Heme oxygenase 1 expression induced by IL-10 requires STAT-3 and phosphoinositol-3 kinase and is inhibited by lipopolysaccharide.** *J Leukoc Biol* 2004, **76**:719-726.
30. Zwerina J, Tzima S, Hayer S, Redlich K, Hoffmann O, Hanslik-Schnabel B, Smolen JS, Kollias G, Schett G: **Heme oxygenase 1 (HO-1) regulates osteoclastogenesis and bone resorption.** *FASEB J* 2005, **19**:2011-2013.
31. Chu CQ, Field M, Feldmann M, Maini RN: **Localization of tumor necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis.** *Arthritis Rheum* 1991, **34**:1125-1132.
32. Ergun T, Ince U, Eksioglu-Demiralp E, Direskeneli H, Gurbuz O, Gurses L, Aker F, Akoglu T: **HSP 60 expression in mucocutaneous lesions of Behcet's disease.** *J Am Acad Dermatol* 2001, **45**:904-909.
33. Imamura Y, Kurokawa MS, Yoshikawa H, Nara K, Takada E, Masuda C, Tsukikawa S, Ozaki S, Matsuda T, Suzuki N: **Involvement of Th1 cells and heat shock protein 60 in the pathogenesis of intestinal Behcet's disease.** *Clin Exp Immunol* 2005, **139**:371-378.
34. Raffeiner B, Dejaco C, Duftner C, Kullich W, Goldberger C, Vega SC, Keller M, Grubeck-Loebenstein B, Schirmer M: **Between adaptive and innate immunity: TLR4-mediated perforin production by CD28null T-helper cells in ankylosing spondylitis.** *Arthritis Res Ther* 2005, **7**:R1412-1420.
35. Shen XD, Ke B, Zhai Y, Gao F, Busuttil RW, Cheng G, Kupiec-Weglinski JW: **Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury.** *Am J Transplant* 2005, **5**:1793-1800.
36. Sahin S, Lawrence R, Direskeneli H, Hamuryudan V, Yazici H, Akoglu T: **Monocyte activity in Behcet's disease.** *Br J Rheumatol* 1996, **35**:424-429.
37. Sayinalp N, Ozcebe OI, Ozdemir O, Haznedaroglu IC, Dundar S, Kirazli S: **Cytokines in Behcet's disease.** *J Rheumatol* 1996, **23**:321-322.
38. Mege JL, Dilsen N, Sanguedolce V, Gul A, Bongrand P, Roux H, Ocal L, Inanc M, Capo C: **Overproduction of monocyte derived tumor necrosis factor alpha, interleukin (IL) 6, IL-8 and increased neutrophil superoxide generation in Behcet's disease. A comparative study with familial Mediterranean fever and healthy subjects.** *J Rheumatol* 1993, **20**:1544-1549.
39. Ohno S, Nakamura S, Hori S, Shimakawa M, Kawashima H, Mochizuki M, Sugita S, Ueno S, Yoshizaki K, Inaba G: **Efficacy, safety, and pharmacokinetics of multiple administration of infliximab in Behcet's disease with refractory uveoretinitis.** *J Rheumatol* 2004, **31**:1362-1368.

A Mouse Model of Allogeneic Corneal Endothelial Cell Transplantation

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Purpose: Corneal endothelial cell (CEC) transplantation should become clinically applicable in the near future. However, the immunologic changes after allo-CEC transplantation are poorly understood at present. We tried to establish a mouse model of allogeneic CEC transplantation for immunologic studies.

Methods: Benzalkonium chloride was injected into the anterior chamber of the eyes of recipient BALB/c mice to create bullous keratopathy. Full-thickness corneal transplantation was performed by using 4 types of corneas: BALB/c corneas (isograft group), BALB/c corneas denuded of CEC (no endothelium group), C3H/He mouse corneas (allograft group), and corneas reconstituted by seeding immortalized C3H/He mouse CECs onto BALB/c corneas denuded of endothelium (CEC allograft group). Eyes were observed with an operating microscope for 4 weeks after transplantation and were subjected to histologic examination and fluorescein microscopy.

Results: All corneal grafts were transparent in the isograft group (n = 12), whereas none of the grafts were clear by 4 weeks after transplantation in the no endothelium group (n = 13). Corneal grafts were transparent at 4 weeks in 75% of the CEC allograft group (n = 12). The histologic rejection rate was 0% in the CEC allograft group, which was significantly lower than in the allograft group (67%; n = 18; $P < 0.01$).

Conclusions: We established a mouse allo-CEC transplantation model by using cultured cells. This model should be useful for studying the immunologic processes after CEC transplantation.

Key Words: corneal transplantation, corneal endothelial cell transplantation, cultured corneal endothelial cells, immunological rejection

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Penetrating keratoplasty (PK) is a standard surgical intervention for many corneal disorders. The leading cause of corneal diseases that require PK is endothelial dysfunction, including bullous keratopathy and graft failure, which only needs to be treated by replacement of the corneal endothelium.[1,2] Because stromal incisions have to be made in the corneal surface and corneal sutures have to be placed for PK, many complications occur after surgery, including irregular astigmatism and/or suture-induced problems.[3]

Deep lamellar endothelial keratoplasty was originally reported by Ko et al,[4] who performed it in animals, and was first performed in humans by Melles et al.[5] It is a selective endothelial replacement procedure that avoids incisions of the corneal surface or sutures and dramatically reduces postoperative refractive errors.[1,5–8] Descemet stripping and automated endothelial keratoplasty is a method of transplanting a corneal lenticle, which consists of the endothelium, the Descemet membrane, and part of the posterior stroma.[9] With these techniques, however, the final corrected visual acuity is still not satisfactory, presumably because of irregularity at the interface between host and donor corneal stroma. Moreover, all of these corneal transplantation techniques require fresh human corneas, but there is a severe worldwide shortage of donors.

To solve the problems of donor cornea shortage and postoperative refractive errors, surgical procedures that replace the endothelium by using cultured corneal endothelial cells (CECs) have been introduced as cell- and sheet-transplantation techniques in animal models. Iron-endocytosing CECs[10,11] or CEC precursors,[12,13] as well as CECs on a thin collagen sheet[14] or a bioengineered human corneal endothelial cell sheet fabricated from a thermoresponsive support,[15] have been used for injection into the anterior chamber or for sheet transplantation, respectively. These cell or sheet transplantation techniques using cultured CECs without corneal trephination can provide a solution to both the donor shortage and the problem of postoperative refractive errors. Therefore, allotransplantation with cultured CECs is likely to come into clinical use in the near future. However, animal models for analysis of the allogeneic immune reaction after CEC transplantation have not yet been reported, and the immunologic response to allo-CEC transplantation is still unknown.

In this study, we created a mouse bullous keratopathy model, because CEC transplantation is used for corneas affected by bullous keratopathy. We established a cultured allo-CEC transplantation model in mice with bullous keratopathy

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to allow examination of the immunologic changes that occurred after CEC transplantation.

MATERIALS AND METHODS

Animals

Inbred strains of BALB/c (H-2^d) and C3H/HeN (H-2^k) male mice weighing 25–30 g (8–12 weeks old) were purchased from Clea Japan Co. (Tokyo, Japan). Both major and minor histocompatibility antigens differ among the BALB/c and C3H strains. BALB/c mice were used as recipients for transplantation, whereas C3H/He and BALB/c mice were both used as donors. Each mouse was anesthetized by intramuscular injection of a mixture of 4.0 mg of ketamine and 1.0 mg of xylazine before the surgical procedures. Animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research (Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research).

Culture and Fluorescence Labeling of Mouse CECs

An immortalized C3H CEC cell line infected with SV40 virus[16–18] was kindly provided by Prof. Niederkorn from the University of Texas Southwestern Medical School. Culture was done in Eagle minimum essential medium (EMEM; Sigma, St. Louis, MO) with 10% fetal bovine serum. The medium was changed every 3 days, and cultures were passaged every 2 weeks after treatment with 0.025% trypsin (Sigma). Cultured mouse CECs were labeled with fluorescent cell linker (PKH26-GL; Zynaxis, Malvern, PA) according to the manufacturer's instructions. In brief, mouse CECs (from 5×10^6 to 10×10^6 cells) were placed into serum-free medium, spun, and resuspended in the diluent provided with the dye. A 4×10^{-6} dilution of the dye was added to the cells, and incubation was performed at room temperature for 10 minutes. The reaction was stopped by addition of serum at a final concentration of 10%. The cells were washed 3 times in serum-containing medium, and fluorescence was observed under a fluorescent microscope (models BH2-RFLT3 and BX50; Olympus, Tokyo, Japan) with excitation at 420 nm and emission at 480 nm. The Zynaxis dye used for staining is unable to transfer fluorescence to other cells, so we can conclude that all fluorescent cells represent original donor cells repopulating the Descemet membrane.

Reconstitution of Denuded Corneas With Fluorescein-labeled Mouse CECs

Corneas of BALB/c mice were removed with a 2.0-mm trephine, and CECs were gently scraped off with a sterile cotton swab. The corneas were rinsed three times with sterile saline and immersed for 5 minutes in saline containing 50 mg/mL gentamicin (Sigma). Subsequently, the denuded posterior surface of each cornea was coated with 0.01% human plasma fibronectin (Invitrogen) to promote firm adherence of CECs. After labeling, the CECs were washed twice with phosphate-buffered saline (PBS) and resuspended in 100 μL of low-glucose EMEM with 6% dextran (Sigma). Next, the C3H CEC cell line (immortalized by infection with SV40) stained with a fluorescent dye (1.0×10^6 cells in 100 μL of culture medium) was applied to the posterior surface of each cornea, after which the corneas were placed in the wells of 24-well plates and centrifuged at 1000 rpm (176g) for 10 minutes to promote the attachment of mouse CECs to the Descemet membrane. The corneas were maintained in the culture medium for a further 2 days.

Surgery

All procedures were performed under an operating microscope. An endothelial injury model was created as described elsewhere for rabbits, with some modifications.[19,20] In brief, benzalkonium chloride of 0.05% was injected into the anterior chamber with a 30-gauge needle to remove CECs and to suppress their regeneration (Terumo, Tokyo, Japan). After 10 seconds, the anterior chamber was rinsed with saline for 20 seconds. Full-thickness corneal grafts were excised from normal donor eyes by using a 2.0-mm trephine (Inami, Tokyo, Japan), and 1.5-mm-diameter grafts were transplanted into the recipient corneas with 8 or 10 interrupted 11-0 nylon sutures (Mani, Tochigi, Japan). The sutures were removed at 9 days after surgery.

Corneal Transplantation Groups

Four experimental groups were created by transplanting different donor corneas into recipient BALB/c mice. (1) Full-thickness corneal grafts from BALB/c mice were transplanted (isograft group). (2) Mouse corneas denuded of endothelium, but not treated with fibronectin, were preserved in 100 μL of low-glucose EMEM with 6% dextran for 2 days by the same method as that for reconstituted corneas (no endothelium group). (3) Full-thickness corneal grafts from C3H mice were transplanted (allograft group). (4) BALB/c-corneas denuded of endothelium were reconstituted with immortalized C3H-CECs and were transplanted (CEC allograft group). After transplantation, the corneal grafts were evaluated for stromal opacification and edema.

Observations and Exclusion Criteria

Mice were followed for 4 weeks, and their grafts were observed by slit-lamp biomicroscopy once a week. Animals with complications such as corneal wound rupture and those that died under anesthesia were excluded from this study. A total of 64 mice underwent surgery, and 9 mice were excluded because of corneal wound rupture ($n = 4$) or death caused by anesthesia ($n = 5$). Eight corneas per group were enucleated for histologic examination.

Definition of Endothelial Graft Failure and Immunologic Graft Failure Caused by Rejection

We tried to distinguish endothelial graft failure without an immunologic reaction from immunologic graft failure caused by allo-rejection. Grafts that developed edema without opacity caused by cellular infiltration were defined as showing endothelial failure. Graft edema associated with moderate opacity (pupil margins and iris vessels were still visible) caused by cellular infiltration was defined as immunologic rejection. Slit-lamp biomicroscopy was used to differentiate

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