本研究により肺局所における主幹部から細部にわたる気道系の形態や機能，とくに末梢気道の力学的状態を解析する技術の開発に繋がると考える．

E．結論

近年開発されたマルチスライスＸ線ＣＴ装置のボクセルデータを用いて、ヒト肺気道の３次元構造を連続的かつ自動的に解析する方法を確立した．

F．研究発表

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G．知的財産権の出願・登録状況

１．特許取得

なし。

２．実用新案登録

なし。

３．その他

なし

アレルギー性気道炎症の新たな制御機構の解明

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研究要旨

気管支喘息の本態であるアレルギー性気道炎症の病態形成には、Th2 細胞が中心的な役割を果たしている。Th2 細胞は IL-4, IL-5, IL-13 などの Th2 サイトカインを産生し、IL-4 により Th2 細胞の分化増殖と B 細胞からの IgE 産生、IL-5 により好酸球の分化、成熟および活性化、IL-13 により杯細胞分化と内皮細胞上の接着分子の発現を誘導する。そして近年、新たな Th2 サイトカインとして IL-17 ファミリーに属する IL-25 (IL-17E)が同定された。これまでの研究により IL-25 は、非 T 非 B 細胞から IL-4, IL-5, IL-13 などの Th2 サイトカインの産生を誘導し、その結果、IgE 産生や好酸球増多を誘導することが示されている。しかし、アレルギー性気道炎症における IL-25 の役割は不明であった。

本研究により我々は、IL-25 はアレルギー性気道炎症の局所で産生され、アレルギー性炎症の増強に関与していること、そして IL-25 によるアレルギー性炎症の増強には、これまで IL-25 応答には必須でないとされている CD4 陽性 T 細胞が必須であることを明らかにした。今後、IL-25 に反応する非 T 非 B 細胞の同定とともに、CD4 陽性 T 細胞が IL-25 依存的アレルギー性炎症に関与するメカニズムを解明し、アレルギー性疾患治療のターゲットとしての IL-25 の可能性を明らかにしていきたい。

A. 研究目的

気管支喘息はアレルギー性気道炎症を病態とし、その結果気道過敏性の亢進と気道閉塞が惹起される。アレルギー性気道炎症は、Th2 細胞により産生される種々のサイトカインが相加的或は相乗的に作用し惹起していることが推測されるが、その詳細は依然不明である。したがって、気管支喘息の病態解析とそれに基づく効果的かつ根本的な治療法の確立には、アレルギー性気道炎症の成立機序及びその制御機構における Th2 細胞性サイトカインの役割の解明が必須である。

近年、新たな Th2 細胞性サイトカインとして IL-17 ファミリーに属する IL-25 が単離された。IL-25 は、未だ同定されていない非 T 非 B 細胞に作用し、IL-4, IL-5, IL-13 など Th2 サイトカインの産生を誘導する非常にユニークなサイトカインであり、他の IL-17 ファミリーサイトカインが proinflammatory cytokine として機能するのとは対照的である。本研究では、アレルギー性気道炎症の新たな制御機構の解明を目的に、アレルギー性気道炎症における IL-25 の役割の解明を目指した。

B. 研究方法

1）マウス喘息モデルにおける IL-25 の役割の解明

a) アレルギー性気道炎症における IL-25 の発現. 卵白アルブミン(OVA)で腹腔内感作した野生型マウスに OVA を吸入投与し、気道における IL-25 mRNA の発現を RT-PCR 及び real-time PCR 法にて経時的に検討した。

b)アレルギー性気道炎症に対するIL-25中和の効果．IL-25 レセプター(IL-25R)の細胞外領域とヒト IgG1 の Fc 領域との融合蛋白(可溶化IL-25R(sIL-25R))を作製し、IL-25 反応性 BAF/3 細胞を用いて IL-25 に対する中和活性を評価した。sIL-25R をマウス喘息モデルの実験系に投与し、アレルギー性気道炎症に対する抑制効果を検討した。

２）肺特異的 IL-25 発現マウスの作製と解析

rat CC10 promoter の制御下で肺特異的に IL-25 を発現するトランスジェニクマウス (CC10 IL-25 マウス)を作製し、以下の解析を行った。

a) OVA で腹腔内感作した CC10 IL-25 マウス及び野生型マウスに OVA を吸入投与し、気道の好酸球及び CD4 陽性 T 細胞浸潤、サイトカイン産生や胚細胞分化を検討した。

b) OVA で腹腔内感作した CC10 IL-25 マウス及び野生型マウスに抗 CD4 抗体を投与し CD4 陽性 T 細胞を除去した。抗 CD4 抗体投与１日後に OVA を吸入投与し、その４８時間後に好酸球の気道への浸潤を評価した。

c) CC10 IL-25 マウスと Stat6 欠損マウスを交配し、Stat6 欠損 CC10 IL-25 マウスを作製した。OVA で感作した CC10 IL-25 マウス及び Stat6 欠損 CC10 IL-25 マウスに OVA を吸入投与し、その４８時間後に好酸球の気道への浸潤を評価した。

C. 結果

１）マウス喘息モデルにおける IL-25 の役割

a) 感作した BALB/c マウスでは抗原吸入８時間後をピークとする IL-25 mRNA の発現が認められた。b) sIL-25R の投与は、抗原吸入による好酸球と CD4 陽性 T 細胞の気道浸潤を有意に抑制した。

２）肺特異的 IL-25 発現マウスの作製と解析

a) CC10 IL-25マウスでは、肺特異的なIL-25 mRNAの発現が認められ、BALFを用いたELISA解析にて蛋白レベルでの発現も確認されたが、抗原非吸入のCC10 IL-25マウスでは、気道炎症は認められなかった。b) 感作したCC10 IL-25マウスに抗原を吸入投与すると気道への好酸球とCD4陽性T細胞の浸潤及びTh2サイトカインの産生が野生型マウスに比して強く認められた。c) CC10 IL-25マウスでは、抗原吸入による気道胚細胞分化とTARCの産生が野生型マウスに比して強く認められた。d) 抗CD4抗体を前投与しCD4陽性T細胞を除去すると、CC10 IL-25マウスにおいても抗原吸入による気道好酸球浸潤が強く抑制された。e) CC10 IL-25 Stat6欠損マウスでは、Stat6欠損マウスと同様に抗原吸入によるアレルギー性気道炎症がほとんど認められなかった。

D. 考察

本研究で我々は、a) 感作マウスに抗原を吸入投与すると吸入８時間後をピークとする IL-25 mRNA の発現が認められること, b) 内在性に産生された IL-25 を sIL-25R で中和するとアレルギー性気道炎症における好酸球と CD4 陽性 T 細胞の浸潤が抑制されること、c) IL-25 の肺特異的な発現はアレルギー性気道炎症を増強すること、d) IL-25 によるアレルギー性気道炎症の増強は CD4 陽性 T 細胞及び Stat6 依存的であることを明らかにした。すなわち、これまでの IL-25 蛋白の大量投与実験により、IL-25 によるアレルギー性炎症の誘導は T 細胞の非存在下でも起こることが示されていたが、より生理的に近い状況においては、IL-25 依存的なアレルギー性炎症の増強には CD4 陽性 T 細胞の関与が必須であることが明らかとなった。IL-25 によるアレルギー性気道炎症の増強に IL-25 の直接の標的細胞でない CD4 陽性 T 細胞が必須である理由は依然不明であるが、IL-25 により産生誘導される TARC をはじめとするケモカインの産生が CD4 陽性 T 細胞の局所浸潤を誘導し、その結果アレルギー性気道炎症が増強される可能性が示唆される。

E. 結論

本年度の研究において我々は、IL-25 は、アレルギー性気道炎症の局所で産生され、その発現は、CD4 陽性 T 細胞依存的にアレルギー性気道炎症を増強していることを明らかにした。今後、IL-25 を標的としたアレルギー疾患の治療法の開発を視野に、アレルギー性炎症における IL-25 産生細胞及び IL-25 反応性細胞の同定を目指したい。

F. 健康危険情報

なし

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H. 知的財産権の出願・登録状況

なし

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SHORT REPORT

Involvement of eicosanoids and surfactant protein D in extrinsic allergic alveolitis

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ABSTRACT: The pathophysiology of extrinsic allergic alveolitis (EAA) involves oxidative lung damage as well as interstitial and alveolar inflammation. Macrophages and mast cells are inflammatory components of EAA that produce both leukotrienes (LTs) and prostaglandin D_2 (PGD_2). In addition, PGD_2 is also produced by the free-radical-catalysed peroxidation of arachidonic acid during oxidative stress. Urinary 8-iso prostaglandin $F_2\alpha$ (8-isoPGF$_2\alpha$) and serum surfactant protein D (SP-D) are considered appropriate biomarkers of oxidative stress and interstitial lung disease activity, respectively. The present study aimed to assess the association of these biomarkers with the pathophysiology of EAA.

Two cases of acute EAA caused by the inhalation of fungi spores were reported. Eight asthmatic patients and six healthy control subjects were also enrolled in the current study.

The serum SP-D and urinary eicosanoid (LTE_4, PGD_2 metabolite (9α,11βPGF$_2$), 8-isoPGF$_2\alpha$) concentrations markedly increased during the acute exacerbation phase. These concentrations decreased following corticosteroid therapy in the EAA patients. There was a significant correlation between serum SP-D and urinary 9α,11βPGF$_2$ concentrations in the EAA patients.

In conclusion, although the present study proposes that serum surfactant protein-D and urinary eicosanoids are new biomarkers involved in the various immunological responses in extrinsic allergic alveolitis, further large-scale studies are needed to investigate the role of these compounds, not just as biomarkers, but also as biological potentiators of extrinsic allergic alveolitis.

KEYWORDS: Extrinsic allergic alveolitis, 8-iso prostaglandin $F_2\alpha$, prostaglandin D_2, surfactant protein D

Acute extrinsic allergic alveolitis (EAA) is characterised by oxidative lung damage [1]. During oxidative stress, prostaglandin D_2 (PGD_2) is nonenzymatically produced by the free-radical-catalysed peroxidation of arachidonic acid (isoprostane pathway) [2]. Briefly, isoprostanes are a unique series of PG-like compounds formed by the random oxidation of tissue phospholipids by oxygen radicals [2]. Thus, isoprostanes contain racemic mixtures of E-, D-, F-type and thromboxane-type prostane rings [3, 4]. The racemic D-ring isoprostane (12-isoPGD$_2$) subsequently undergoes rapid epimerisation to racemic PGD_2 [2].

In contrast, alveolar macrophages and mast cells produce cysteinyl-leukotrienes (CysLTs) and cyclooxygenase-dependent PGD_2 [5, 6]. Alveolar macrophages play a key role in acute EAA [7]. There is also a persistent increase in the number of alveolar mast cells in EAA patients [8]. EAA is categorised as a T-helper1-type disease and interferon (IFN)-γ plays a pivotal role in granuloma formation in EAA [7]. Interestingly, mast cells, which express the Fcγ receptor I after incubation with IFN-γ, can produce PGD_2 and CysLTs even in response to immunoglobulin (Ig)G stimulation [9].

Urinary leukotriene E4 (LTE_4) is now considered to be the most reliable analytical parameter for monitoring the endogenous synthesis of CysLTs [10, 11]. Similarly, urinary 9α,11β prostaglandin F_2 (9α,11βPGF$_2$) is a relatively stable PGD_2 metabolite and an appropriate indicator of mast cell activation [5]. Of the isoprostanes, 8-iso prostaglandin $F_2\alpha$ (8-isoPGF$_2\alpha$) is the best-characterised isomer and urinary 8-isoPGF$_2\alpha$ is considered the most accurate indicator of oxidant stress [2]. Taking this into account, it was

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Received:
September 10 2004
Accepted after revision:
August 15 2005

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

hypothesised that patients with acute EAA show increased concentrations of urinary eicosanoids (CysLTs, 9α,11βPGF$_2$ and 8-isoPGF$_2$α).

Surfactant protein D (SP-D), which is produced by alveolar type II and Clara cells [12], is an important regulatory molecule in both pulmonary surfactant homeostasis and first-line defence mechanisms against microbial or allergen challenges [13]. Krebs von den Lungen-6 (KL-6) is also produced by alveolar type II cells [14]. The measurements of serum SP-D and KL-6 concentrations contribute to the early diagnosis of interstitial lung disease (ILD) [12, 14]. EAA is an acute ILD [7]. Previous studies have demonstrated significant increases in SP-A and KL-6 concentrations in the bronchoalveolar lavage fluid (BALF) of acute EAA patients [15]. Although plasma SP-D concentration is one of the most appropriate prognostic parameters of acute respiratory distress syndrome (ARDS) [16], knowledge of the serum SP-D profile of acute EAA patients is limited [17].

The present study aims to assess the association of these biomarkers with the pathophysiology of EAA.

METHODS

Case reports

Case 1

A 61-yr-old, nonsmoking female suffered from summer-type EAA caused by *Trichosporon asahii*, the most prevalent cause of EAA in Japan [18]. Cell counts revealed that 58.9% of total BALF cells were lymphocytes and the CD4/CD8 ratio of lymphocyte surface markers was 0.6. In addition, transbronchial lung biopsy specimens exhibited lymphocytic alveolitis with granulomas. The subject was diagnosed positive for precipitin to *T. asahii* by double immunodiffusion analysis. The positive findings were confirmed during a provocation test, following which the patient was allowed to return home.

Case 2

A 48-yr-old, nonsmoking female suffered from occupational EAA caused by *Aspergillus niger*, predominantly isolated from house dust in her workplace (a linen room). In addition to being strongly positive for precipitins to both house dust and *A. niger* extract, determined by double immunodiffusion analysis, the patient was also positive for a serum-specific IgG antibody against *A. niger* (10.8 mg·dL^{-1}). High-resolution computed tomography of the patient's chest revealed supportive radiographic findings [7]. The results from both cases are shown in table 1.

Both EAA patients fulfilled the American-European Consensus Conference criteria for ARDS (table 1) [19]. Intensive corticosteroid treatment (*i.v.* administration of 1,000 mg·day^{-1} methylprednisolone for 3 days, followed by oral administration of 0.5 mg·kg^{-1} prednisone) was tapered over the 7-week period, resulting in gradual improvements of both clinical symptoms and radiographic findings.

Control subjects

Eight (six female) stable asthmatic patients (mean age (range) 58 (33–73) yrs) were enrolled as diseased control subjects. Six (three female) healthy control subjects (44 (29–58) yrs) were also enrolled for comparative analysis of urinary eicosanoid data. All the subjects were nonsmokers. Permission to conduct the study was obtained from the Ethics Committee of the National Sagamihara Hospital (Japan) and all participating subjects gave informed consent.

Measurements

Serum and spot urine samples were collected between 09:00–11:00 h. In the case of subjects with acute EAA, urine samples were collected on admission and following therapy. The samples were analysed for KL-6, SP-D and eicosanoid concentrations by methods previously described [10, 12, 17, 20]. Double immunodiffusion analysis of precipitating antibodies against 18 different fungal species was performed according to the Ouchterlony method. A specific IgG antibody against *A. niger* was performed utilising the liquid-phase immunoassay AlaSTAT microplate system (Diagnostic Products Corporation, Los Angeles, USA).

Data analysis

Serum and urinary data were expressed as mean and median, respectively. Relationships were analysed using Spearman's rank correlation test. A p-value <0.05 was regarded as statistically significant.

RESULTS

Serum KL-6 and SP-D concentrations were markedly higher in the acute EAA patients than in the eight asthmatic patients (table 1). As shown in figure 1, urinary eicosanoid concentrations in the acute EAA patients (LTE$_4$: 420 and 185 pg·mg^{-1}-creatinine; 9α,11βPGF$_2$: 658 and 382 pg·mg^{-1}-creatinine; 8-isoPGF$_2$α: 393 and 537 pg·mg^{-1}-creatinine) were markedly higher than in the healthy control subjects (LTE$_4$: 45 pg·mg^{-1}-creatinine; 9α,11βPGF$_2$: 43 pg·mg^{-1}-creatinine; 8-isoPGF$_2$α: 187 pg·mg^{-1}-creatinine). Median values

TABLE 1 Serum Krebs von den Lungen-6 (KL-6) and surfactant protein D (SP-D) concentrations on admission

Subject	Causative organism	Pa_{O_2}/Fi_{O_2} ratio	Serum	
			KL-6 U·mL^{-1}	SP-D ng·mL^{-1}
Case 1	*Tricosporon asahii*	91.4	6090	428
Case 2	*Aspergillus niger*	200	5770	396
Asthma group#			252±70	39±14

Data are presented as mean±SD. Pa_{O_2}: partial pressure of arterial oxygen; Fi_{O_2}: inspiratory oxygen fraction. #: n=8.

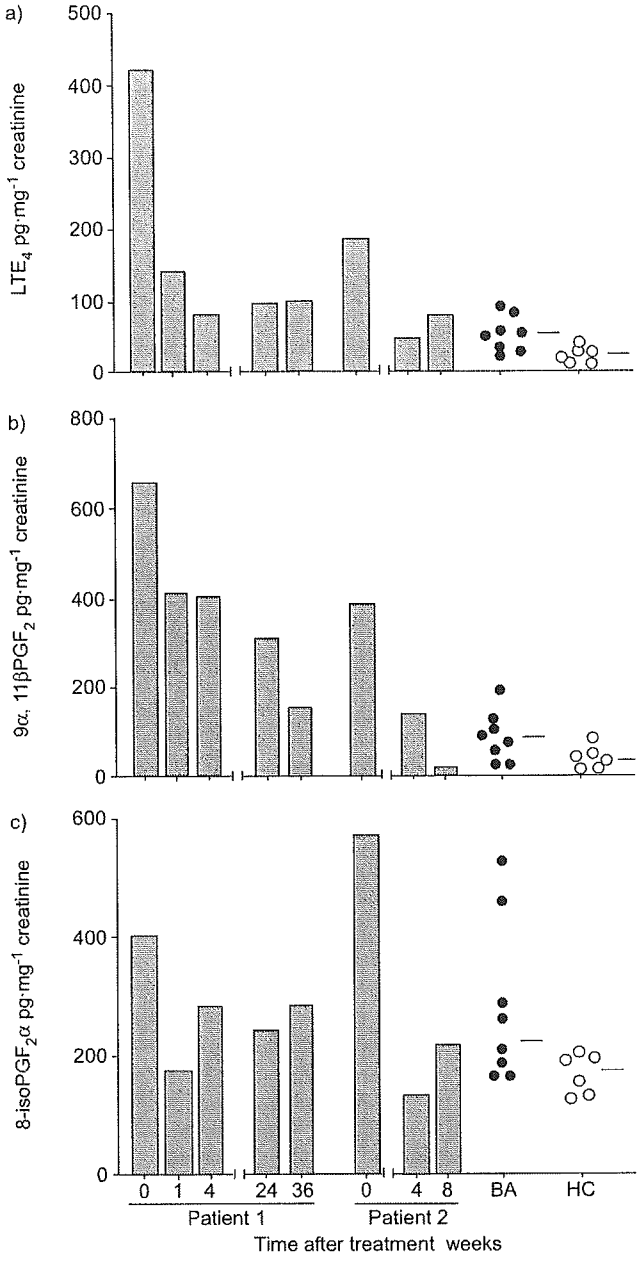

FIGURE 1. a) Urinary leukotriene E_4 (LTE_4), b) 9α,11β prostaglandin F_2 (9α,11βPGF$_2$), and c) 8-iso prostaglandin F_2α (8-isoPGF$_2$α) concentrations in the extrinsic allergic alveolitis patients. BA: asthmatic patients; HC: healthy control subjects. Horizontal bars indicate median values.

of urinary LTE_4, 9α,11βPGF$_2$ and 8-isoPGF$_2$ concentrations in the asthmatic patients were 55, 79 and 235 pg·mg^{-1}-creatinine, respectively. The serum SP-D, KL-6 and urinary eicosanoid concentrations decreased following corticosteroid therapy in the EAA patients (figs 1 and 2). There was a significant correlation between serum SP-D and urinary 9α,11βPGF$_2$ concentrations in the EAA patients ($p<0.05$, $rs=1$; fig. 2).

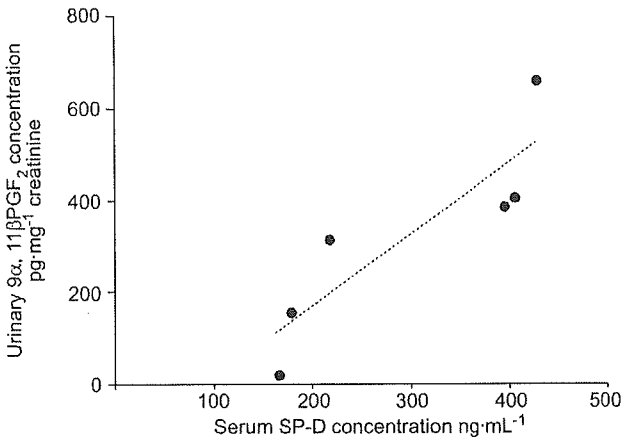

FIGURE 2. A significant correlation between serum surfactant protein D (SP-D) and urinary 9α,11β prostaglandin F_2 (9α,11βPGF$_2$) concentration was found in the extrinsic allergic alveolitis patients. $rs=1$; $p<0.05$.

DISCUSSION

The present authors demonstrated, for the first time, that acute EAA is characterised by eicosanoid overproduction. Although increased urinary LTE_4 concentration has been reported in patients with ARDS [21], to the current authors' knowledge this is the first report demonstrating a PGD_2 overproduction in patients with ARDS or even acute EAA. The CysLT and PGD_2 overproduction may well be associated with the increased cyclooxygenase activity of both alveolar macrophages [6] and mast cells [5]. However, the higher urinary 9α,11βPGF$_2$ concentrations in EAA patients are mainly considered to be a reflection of mast cell activation [5, 22]. Interestingly, mast cells activated by aggregated IgG, following IFN-γ-induced up-regulation of Fcγ receptor I, can produce both PGD_2 and CysLTs [9], which is consistent with the pathophysiology of EAA.

8-isoPGF$_2$α is an accurate biomarker of oxidative stress *in vivo* [2]. The present study demonstrated that urinary 8-isoPGF$_2$α concentration is increased in acute EAA patients, suggesting a central role for oxidant stress in the pathogenesis of acute EAA. The isoprostane pathway also contributes to the PGD_2 overproduction in acute EAA [2], although the current approach was unable to ascertain its relative contribution to PGD_2 production. It has recently been discovered that isoprostanes containing D- or E-type prostane rings are excreted into the urine as conjugates with *N*-acetyl cysteine sulfoxide, suggesting that these metabolites may be used as biomarkers to estimate whole-body production of D- or E-type isoprostanes [23]. Future experiments using this methodology will hopefully provide even more answers.

Increased 8-isoPGF$_2$α concentrations have been reported in the breath condensate of patients with ARDS [24], ILD [25] and asthma [26]. Recently, WOOD *et al.* [27] demonstrated that despite high variability, sputum 8-isoPGF$_2$α concentrations were significantly increased in patients with severe persistent asthma. The present study also demonstrated similar findings in that the two asthmatic patients with extremely high urinary 8-isoPGF$_2$α concentrations (458 and 525 pg·mg^{-1}-creatinine,

respectively) were characterised by severe persistent asthma and hypereosinophilia (11.2 and 17.2%, respectively).

These eicosanoids are also known to possess various other biological activities, such as being potent constrictors of pulmonary vascular smooth muscle and causing plasma exudation [4–6]. Although the full extent of the biological activity of the eicosanoids in acute EAA remains to be determined, the various components of the eicosanoid metabolic pathways may become therapeutic targets in acute EAA.

Consistent with a previous case report by TANAKA *et al.* [17], serum SP-D concentrations in the acute EAA patients were markedly increased. In contrast, serum SP-D concentrations in the asthmatic patients were low, which is in accordance with data by KOOPMANS *et al.* [28] Interestingly, the serum SP-D concentrations subsequently decreased and showed a significant correlation with urinary $9\alpha,11\beta PGF_2$ concentrations in the EAA patients. Serum SP-D is a biomarker of ILD activity [12] and SP-D plays a protective role in pulmonary inflammation [13]. PGD_2 and its metabolite, 15-d-PGJ_2, have the potential to serve as downregulators of lung injury induced by bleomycin [29]. Taken together, these findings suggest that both SP-D and PGD_2 appear to be important regulatory factors in the pathophysiology of EAA.

In conclusion, the present study proposes that serum surfactant protein D and urinary eicosanoids are new biomarkers involved in various immunological responses in extrinsic allergic alveolitis. Further large-scale studies are needed to investigate the role of these compounds, not just as biomarkers, but also as potentiators of extrinsic allergic alveolitis.

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Hum Genet (2005) 117: 16–26
DOI 10.1007/s00439-005-1285-0

ORIGINAL INVESTIGATION

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Functional promoter polymorphism in the *TBX21* gene associated with aspirin-induced asthma

Received: 25 November 2004 / Accepted: 25 January 2005 / Published online: 2 April 2005

Abstract Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. T-bet, a Th1-specific transcription factor of T-box family, has been found to control interferon-γ (IFN-γ) expression in T cells. Mice lacking the T-bet gene (*tbx21*) demonstrate multiple physiological and inflammatory features reminiscent of human asthma. In order to examine whether polymorphisms in the candidate gene, *TBX21*, located on chromosome 17q21.32, are related to the risk of human asthma phenotypes, we have searched for genetic variations in the human *TBX21* gene and identified 24 single nucleotide polymorphisms (SNPs), including five novel SNPs, by direct sequencing in Japanese subjects. Among asthma phenotypes, a promoter −1993T → C SNP, which is in linkage disequilibrium with a synonymous coding 390A → G SNP in exon 1, is significantly associated with a risk of aspirin-induced asthma (AIA; $P=0.004$, $P_c=0.016$). This association has also been confirmed in additional independent samples of asthma with nasal polyposis ($P=0.008$), regardless of aspirin hypersensitivity. Furthermore, our data indicate that the −1993T → C substitution increases the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position, resulting in increased transcriptional activity of the *TBX21* gene. Thus, in addition to the antigen-driven excess Th2 response, increased T-bet (and subsequent IFN-γ)

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production in human airways of individuals with the −1993T → C polymorphism could contribute to the development of certain asthma-related phenotypes, such as AIA.

Introduction

Asthma is defined as a chronic inflammatory lung disease that is characterized by airway hyperreactivity, eosinophil inflammation, and mucus hypersecretion resulting in intermittent airway obstruction (Busse and Lemanske 2001). A considerable increase has been noted in the incidence of allergic diseases including asthma in industrialized societies over the past three decades (Bach 2002; Woolcock and Peat 1997). The etiology of asthma is complex and multifactorial; development of the disease is controlled by both host genetic factors and a variety of environmental exposures. Although environmental influences, particularly a decrease in infections because of improved hygiene, might have increased allergic diseases, at least a dozen polymorphic genes have been calculated to regulate asthma, by controlling the inflammatory response, immunoglobulin E (IgE), cytokine, and chemokine production, and airway remodeling (Cookson 1999; Fahy et al. 2000; Umetsu et al. 2002).

Asthma is thought to arise from an imbalance in T helper type 1 (Th1)-Th2 immune regulation, resulting in the driving of the development of Th2-biased immune responses and the overproduction of cytokines such as interleukin 4 (IL-4), IL-5, IL-9, and IL-13, which mediate allergic inflammation (Renauld 2001; Umetsu et al. 2002). In contrast, Th1-type cytokine interferon-γ (IFN-γ) is essential for macrophage activation in cellular defense mechanisms, and IFN-γ-producing Th1 cells have been suggested to protect against allergic responses by dampening the activity of Th2 effector cells (Renauld 2001). However, the evidence from other in vivo studies of asthma conflicts with this hypothesis, suggesting a contribution of IFN-γ to asthmatic airway inflammation (Busse and Lemanske 2001; Salvi et al. 2001).

T-bet is a member of the T-box family of transcription factors that has been found to be expressed in IFN-γ-producing Th1, but not in Th2, cells. T-bet is a transcriptional regulator essential for the lineage commitment of Th1 cells by directly activating Th1-associated genetic programs and repressing Th2 cytokine production (Szabo et al. 2000). Recently, evidence has shown decreased numbers of $CD4^+$ T cells expressing T-bet in the airways of patients with allergic asthma, relative to control subjects (Finotto et al. 2002). Furthermore, deletion of the T-bet gene, *tbx21*, in mice results in airway eosinophilia, Th2 cytokine production, airway hyperresponsiveness (AHR), and changes of airway remodeling without allergen sensitization and challenge. Thus, T-bet-deficient mice demonstrate multiple physiological and inflammatory features reminiscent of human asthma (Finotto et al. 2002).

The human T-bet gene (*TBX21*) is located on chromosome 17q21.32, a region near to that linked with asthma in a genome screen for asthma and skin tests (Dizier et al. 2000; Zhang et al. 1999). Moreover, the region on mouse chromosome 11 that is syntenic to human chromosome 17q12-q22 has been linked to AHR (Zhang et al. 1999). So far, to our knowledge, there have been no reports showing disease-related polymorphism(s) in the *TBX21* gene. Based on these observations, we propose that genetic polymorphism contributes to susceptibility to human asthma and/or related phenotypes. To test this hypothesis, we have searched for polymorphisms in *TBX21* and then conducted a genetic association study in the Japanese population. Finally, we have investigated the functional consequences of disease-related polymorphisms.

Materials and methods

Subjects

We recruited 361 patients with childhood asthma (mean age 9.7 years, range 4–15 years, mean total serum IgE level, 1021 U/ml; 92% of whom were atopic), 313 adult patients with atopic asthma (mean age 49 years, range 20–81 years; mean total IgE, 775.7 U/ml), and 88 adult patients with non-atopic asthma (mean age 59 years, range 42–75 years; mean total IgE, 174.8 U/ml) from the Osaka Prefectural Habikino Hospital and the Miyatake Asthma Clinic. Patients with aspirin-induced asthma (AIA; mean age 53 years, range 24–73 years; 54% of whom were atopic; ≥50% had nasal polyposis) were recruited from the National Sagamihara Hospital. All patients with asthma were diagnosed according to the criteria of the National Institutes of Health, with minor modifications (National Heart, Lung, and Blood Institute, National Institutes of Health, 1997, http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm). The diagnosis of atopic asthma was based on the positive immunoassay test to common allergens (at least one of the following: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Aspergillus fumigatus*) or a higher total serum IgE of ≥400 kU/l, as used in our previous study (Mao et al. 1996). The criteria for a diagnosis of non-atopic asthma was a total serum IgE of < 400 kU/l and the absence of allergen-specific IgE (≤ 0.35 kU/l). All patients with AIA were documented to have histories of asthmatic attacks, such as severe bronchoconstriction and nasal symptoms, following the ingestion of more than two different kinds of non-steroidal anti-inflammatory drugs (NSAIDs) or to have had a positive reaction to aspirin systemic challenge. Of 72 AIA patients (58%), 42 were diagnosed on the basis of the aspirin challenge test, as previously described (Kawagishi et al. 2002; Mita et al. 2001). We also

independently recruited 42 asthmatic patients with nasal polyposis (AS/NP; mean age 54 years, range 23–75 years; 73% of whom were atopic). The NP was diagnosed on the basis of history, including nasal symptoms, clinical examination, nasal endoscopy, and sinus computerized tomography scanning. All adult asthmatics, except AIA patients, had no past history of aspirin hypersensitivity. Controls were 640 randomly selected healthy individuals with an age range 18–83 years from the same geographic areas who had neither respiratory symptoms nor history of asthma-related diseases and aspirin hypersensitivity. All subjects in this study were ethnically Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama.

Screening for polymorphisms and genotyping

To identify single-nucleotide polymorphisms (SNPs) in the human *TBX21* gene, we sequenced all six exons, including a minimum of 200 bases of flanking intronic sequence, 2.2 kb of the 5′ flanking region, and 2.5 kb continuous to the 3′ flanking region of the sixth exon from 24 asthmatic subjects (12 unrelated children and 12 adults). Eighteen primer sets were designed on the basis of the *TBX21* genomic sequence from the GenBank database (accession number AC003665; Table 1). For each polymerase chain reaction (PCR), 5 ng genomic DNA was amplified in a total reaction volume of 10 μl containing 12.5 pmol each primer, 3.9 mM $MgCl_2$, 1.25 mM each dNTP, 0.5 U *Taq* polymerase. Cycling conditions were an initial 95°C for 2 min, followed by 37 cycles at 94°C for 30 s, 58°C or 60°C for 30 s, and 72°C for 3 min, with a final extension of 7 min at 72°C. Each fragment amplified by PCR was sequenced by using the BigDye Terminator (Applied Biosystems, Foster City, Calif., USA) on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). The sequences were analyzed, and polymorphisms were identified by using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, Mich., USA). Four selected SNPs, viz., −1993T → C, 99C → G, 1298T → C, and 7725T → C, were genotyped by three methods: PCR-RFLP (PCR-restriction fragment lenght polymorphism; for −1993T → C and 1298T → C), Invader assay (for 99C → G), and direct sequencing (for 7725T → C). For PCR-RFLP analysis, we used mismatched primers for the −1993T → C SNP (5′-GGTCTTACTGAAAGCTCTCA-3′ and 5′- TCTCCTCCCCAACACCTTACGC-3′) and for the 1298T → C SNP (5′-GGCTAGTGCAGTAAAGCTTG-3′ and 5′-GGTTTTCACTGGACCAGCCGC-3′) where the changed nucleotides are underlined. The amplified products were digested with *Hha*I (−1993T → C) or *BstU*I (1298T → C) restriction enzymes (New England Biolabs, Bevery, Mass., USA) according to the manufacturer's instructions and were separated by electrophoresis on 4% agarose gels. Based on information available from the public JSNP database (http://snp.ims.u-tokyo.ac.jp), we generated the 99C → G SNP (IMS-JST000934) genotypes by using the Invader assay as previously described (Ohnishi et al. 2001). For the 7725T → C SNP, we performed direct sequencing with primers 5′-TTATCCAGGGTCATAGGGTAG-3′ and 5′-CCTCAGCCTTTAGAGAAGTTG-3′.

Luciferase assay

We generated luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, by cloning three concatenated copies of a 20-bp fragment of the *TBX21* gene into pGL3-Basic vector (Promega, Madison, Wis., USA) in the *Nhe*I site. The 20-bp primer sets carrying −1993T or −1993C alleles were 5′- CTAGCGGAGAAATGGTGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCACCCATTCCA AGATC-5′ (reverse) or 5′- CTAGC

Table 1 Primer sequences used in screening for SNPs of the human *TBX21* gene

F1	5′-TTTCCAGTAATAGCCGCTCCT-3′	R1	5′-CACAGCCTAGACACTGGTTC-3′
F2	5′-TTGCATAGTTACCATCCACCG-3′	R2	5′-GACCTTGGGATCCTTCACTAC-3′
F3	5′-AAGACTCCATTTGATCTTCAAC-3′	R3	5′-TTCACTCCACAAGGTGTCATG-3′
F4	5′-GTCAGGCTGGGACAGAAATG-3′	R4	5′-TGAGTTGGCTGCATCTTGTAG-3′
F5	5′-CTGGCTGCTGCTGATGCAG-3′	R5	5′-TGTCACTAGAGTCGCAGCGC-3′
F6	5′-AGTACTCGCCAAGAGCGTAG-3′	R6	5′-AAAAACAGACGAGACGTTCTTG-3′
F7	5′-TCGCGCTCAACAACCACCTG-3′	R7	5′-CTCAAAGTAAGACCGGAAAGG-3′
F8	5′-GGCTAGTGCAGTAAAGCTTG-3′	R8	5′-GACCAGAAGCTTGGGCTGTG-3′
F9	5′-CTCTGTTGTGTGGTCAGGAG −3′	R9	5′-TGAGAAGGTATGGAGGTAACC-3′
F10	5′-TTGAAGGAGGCAGTGGCTC-3′	R10	5′-AACACAGCTACCCAAAGTTATC-3′
F11	5′-TTATCCAGGGTCATAGGGTAG-3′	R11	5′-CCTCAGCCTTTAGAGAAGTTG-3′
F12	5′-TAACTTCCTCTACTTTTCCTGG-3′	R12	5′-AAACATCCTGTAGTGGCTGG-3′
F13	5′-TGCCTGGGCACTGTTGCAG-3′	R13	5′-GAAAAACGAACCTTCCTTCCTG-3′
F14	5′-CAACAATGTGACCCAGGTAG-3′	R14	5′-CAAGCTTTCCAACTCCAGTG-3′
F15	5′-GCCCTGTTTGTGCTGATACC-3′	R15	5′-CACAAGCAGAACCAGTCACC-3′
F16	5′-TGGGTTCAACTCAGCTTTGGT −3′	R16	5′-CTTTCATCATGTCATCTGCTC-3′
F17	5′-GCGAAGGAGACTCTAAGAGG-3′	R17	5′-TCTTGCTTCTTGAGATGTGGG-3′
F18	5′-CACGTATGTTATAACCATCAGC-3′	R18	5′-AGAGATAAAGGTGGAGGGCTG-3′

GGAGAAATGGCGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCGCCCATTCCA AGATC-5′ (reverse), respectively, where the added nucleotides for the *Nhe*I site are underlined. HEK293 or HeLa cells were cultured in growth medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Subconfluent cells cultured in 12-well plates were transiently co-transfected with 0.5 μg pGL3-Basic vector DNA or each reporter construct (pGL3/−1993T or pGL3/−1993C) and 10 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, by using 1.5 μl FuGENE six transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we then lysed the cells and measured firefly and *Renilla* luciferase activities in a luminometer by using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility shift assay

Nuclear extracts were prepared from HEK293 and HeLa cells as described previously (Dignam et al. 1983). Double-stranded oligonucleotides −1993T and −1993C were obtained by annealing three concatenated copies of 5′-GAAATGGTGGGTAAG-3′ and 5′-GAAATGGC-GGGTAAG-3′ with their respective complementary oligonucleotides. Electrophoretic mobility shift assay (EMSA) analysis was performed by using DIG gel shift kit (Roche). We prepared digoxigenin (DIG)-labeled double-stranded oligonucleotides corresponding to the sequence at position −2000 to −1986 of the *TBX21* promoter containing the −1993 polymorphism. For each binding reaction, we incubated DIG-labeled probes with nuclear extract (2–5 μg) in 1× binding buffer (20 mM HEPES, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitole, 30 mM KCl), 1 μg poly (dI-dC), and 0.1 μg poly L-lysine for 30 min on ice. For competition studies, we incubated unlabeled double-stranded oligonucleotide (100-fold molar excess) during preincubation. Reaction products were separated on 6% non-denaturing polyacrylamide gels in 0.3× TBE buffer (1× TBE buffer = 0.09 M TRIS-borate, 0.002 M EDTA, pH 8.3) and visualized by chemiluminescent detection. We scanned results into an LAS-3000 CCD camera system (Fuji Photo Film, Tokyo, Japan) and quantified each band intensity by using image analysis software Image Gauge Version 2.0 (Fuji Photo Film).

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium by using a χ^2 goodness of fit test at each locus. We then compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom or Fisher exact test and calculated odds ratios (ORs) with 95% confident intervals (95% CI). For multiple comparisons, *P*-values were corrected by the Bonferroni method. The linkage disequilibrium (LD) statistic *D′* was calculated by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described elsewhere (Nakajima et al. 2002). Comparisons in reporter assays and EMSA experiments were performed with the Student's *t* test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Screening for common polymorphisms in TBX21

Direct DNA sequencing of the indicated regions in 12 asthmatic and 12 healthy subjects (total 24 subjects) identified 24 biallelic SNPs in *TBX21*: three in the 5′ flanking region, three in the coding region (one non-synonymous and two synonymous), three in the 3′ untranslated region, and 15 in the intron (Table 2, Fig. 1). Five of these 24 SNPs (532G → C, 729G → T, 2839G → A, 9408C → A, and 10143C → A) are novel, and another 14 have been reported recently in Korean (Chung et al. 2003) and Finnish (Ylikoski et al. 2004) populations. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG), and the positions for other SNPs are relative to the ATG on genome contig AC003665. A graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene is given in Fig. 1. Since most of the SNPs were of relatively low frequency and in view of their location and LD with other sites, further genotyping and association studies in our asthma population focused on four SNPs: −1993T → C, 99C → G, 1298T → C, and 7725T → C. The distributions of all four SNPs were in Hardy–Weinberg equilibrium in the control group ($P > 0.05$). We calculated both *D′* and r^2 as statistical values for LD pair-wise between each SNP (Fig. 2). One of the three promoter SNPs (−1993T → C) and one synonymous coding SNP (390A → G, G130G) in exon one, were shown to be in strong LD.

TBX21 genotyping and association studies in asthma and related phenotypes

Initially, the association study was carried out on four clinical groups: child patients with asthma ($n = 361$), adult patients with atopic asthma ($n = 313$), adult patients with non-atopic asthma ($n = 88$), and adult patients with AIA ($n = 72$). Adult asthmatics, except AIA patients, had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Allele frequencies of each selected SNP were compared between the patients and the normal controls by using a χ^2 test with 1 d.f. (Table 3). After correction for the number of SNPs investigated (Bonferroni correction), we found a significant association between the promoter SNP at −1993 and AIA in our Japanese cohort ($P = 0.004$;

Table 2 Locations and allele frequencies of *TBX21* SNPs in Japanese (*UTR* untranslated region)

Number	SNP	Location	Amino acid substitution	Minor allele frequency (%)	Primers
1	−1993T → C[a]	Promoter	–	8.3	F3R3
2	−1514T → C	Promoter	–	7.1	F4R4
3	− 1499G → A	Promoter	–	2.4	F4R4
4	99C → G[a]	Exon 1	H33Q	6.3	F6R6
5	390A → G	Exon 1	GI30G	8.3	F6R6
6	532G → C	Intron 1	–	2.2	F7R7
7	729G → T	Intron 1	–	4.2	F7R7
8	1298T → C[a]	Intron 1	–	16.7	F8R8
9	1662C → A	Intron 1	–	2.1	F8R8
10	2608G → A	Intron 1	–	2.1	F9R9
11	2839G → A	Intron 1	–	4.2	F9R9
12	7725T → C[a]	Intron 1	–	18.8	F11R11
13	8381A → T	Intron 1	–	16.7	F12R12
14	8762G → C	Intron 1	–	4.2	F12R12
15	9408C → A	Intron 2	–	2.3	F13R13
16	9882C → T	Intron 3	–	4.2	F14R14
17	9898C → T	Intron 3	–	4.2	F14R14
18	10143C → A	Intron 3	–	2.1	F14R14
19	10150T → C	Intron 3	–	4.2	F14R14
20	11268C → T	Intron 5	–	4.2	F16R16
21	11755G → A	Exon 6	P485P	2.1	F16R16
22	12077T → C	3′UTR	–	4.2	F17R17
23	12211G → A	3′UTR	–	2.1	F17R17
24	12403A → C	3′UTR	–	4.2	F17R17

[a]These SNPs were genotyped in a larger population

corrected P, $P_c = 0.016$). There was an increased risk for AIA associated with a C allele (OR = 1.93; 95% CI 1.22–3.06). Association analysis also demonstrated that a significant difference in allele frequency of −1993 SNP between AIA and other adult asthmatics who had no past history of aspirin hypersensitivity ($P = 0.001$). No other statistically significant association between disease status and genotype or any specific allele was detected from any of the other three case-control disease-association comparisons. We further analyzed the genotype and allele frequencies of the SNP at position 390, which was found to be in LD with SNP at −1993, in the AIA and control groups and then calculated the LD coefficient D' and r^2 between the −1993T → C and 390A → G SNPs ($D' = 0.92$; $r^2 = 0.85$). ORs of developing AIA at −1993T → C and 390A → G were 2.15 (95% CI 1.26–3.64) and 2.19 (95% CI 1.27–3.77), respectively, when the TC and TT (AG and GG) genotypes were compared with the wild-type TT (AA) genotype (Table 4). To determine whether these two SNPs in *TBX21* could also be associated with another AIA-related phenotype, we analyzed −1993T → C and 390A → G SNPs in 42 samples from independent adult AS/NP who either had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Interestingly, comparison of the genotype and allele frequencies also revealed significant differences between the AS/NP group and the normal control group ($P = 0.008$ and 0.012, respectively). Furthermore, in the AIA case group, the C-allele frequency in AIA patients with NP tended to be much higher than that in AIA patients without NP (data not shown). Thus, although the sample size was small, we confirmed the *TBX21* SNP effect by using independent samples of AS/NP, regardless of aspirin hypersensitivity. We further analyzed two-loci haplotype distributions constituting the −1993T → C and 390A → G SNPs in the control, AIA, and AS/NP samples. Haplotype −1993T-390A was the

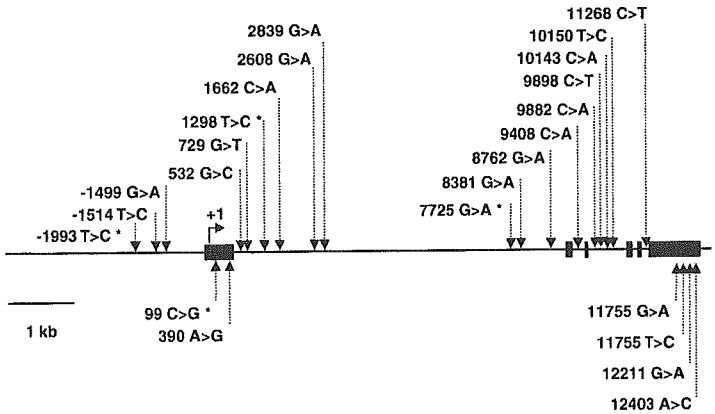

Fig. 1 Graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene (*black boxes* five coding exons, *asterisks* SNPs genotyped in a larger population). Positions for SNPs are relative to the translation start site (+1)

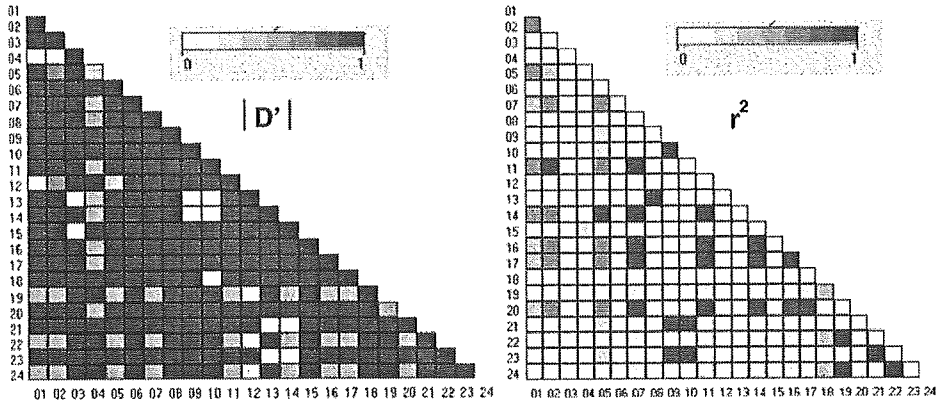

Fig. 2 Pair-wise linkage disequilibrium (*LD*) was measured by $|D'|$ and r^2 among the all of the SNPs identified in 24 sequenced samples. The blocks are *shaded* corresponding to the values obtained from the LD analysis program, SNP Alyze

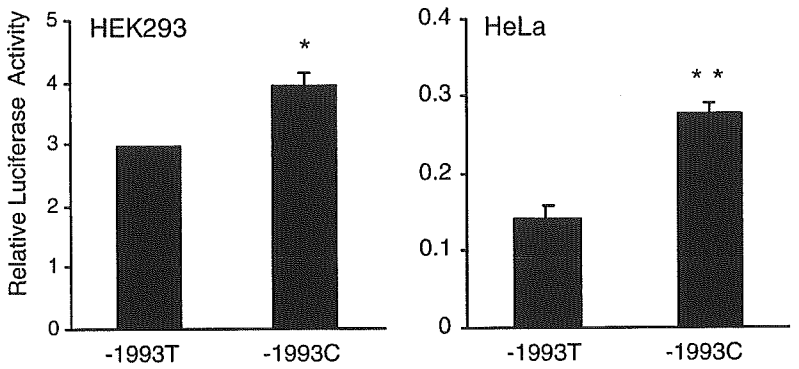

Fig. 3 Effect of the −1993T → C SNP on the transcription activity of the human *TBX21* promoter. HEK293 cells or HeLa cells were transiently cotransfected with pGL/−1993T or pGL/−1993C and pRL-TK vector. The relative luciferase activity of the *TBX21* reporter constructs is represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for three independent experiments. $*P < 0.001$; $**P < 0.005$, as determined by the Student's *t* test

most common, followed by −1993C-390G and −1993C-390A. The two major haplotypes −1993T-390A and −1993C-390G were named haplotypes 1 and 2, respectively. The overall distribution of two loci haplotypes was not different between cases and controls (3 d.f.; $P = 0.10$ for AIA, and $P = 0.09$ for AS/NP), although the frequencies of two major haplotypes, haplotypes 1 and 2, were significantly different in a χ^2 test (1 d.f.; $P = 0.014$ for AIA, and $P = 0.016$ for AS/NP) (Table 4).

Transcriptional effect of TBX21 −1993T → C polymorphism

In functional assays, since the 390A → G polymorphism is a synonymous substitution, which is less likely to be directly associated with disease in general, we focused on the promoter SNP at position −1993, viz., −1993T → C. To understand the role of the T/C polymorphism at −1993 in the transcriptional regulation of the human *TBX21* gene, we performed transient expression of the −1993T and −1993C luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, in HEK293 and HeLa cells. Luciferase activity in cell extracts was analyzed after 24 h of transfection and was standardized against the internal control (*Renilla* activity). The results of this experiment showed that the −1993C construct had significantly higher luciferase reporter activity compared with the wild-type −1993T construct (33%–98% increase; $P < 0.005$). These results suggest that the −1993C allele may be associated with the increased transcriptional activity of the *TBX21* gene in human lungs.

EMSA analysis

To examine whether −1993T → C affected interaction of a nuclear factor(s) with the *TBX21* sequence around −1993, we then performed EMSAs. We prepared 2000/−1986 double-stranded oligonucleotide probes containing either the T or the C allele at −1993 bp. HEK293 nuclear extract contained nuclear proteins binding specifically to this region of the *TBX21* promoter, resulting in the formation of one major and one minor complex. Competition with 100-fold to 200-fold excess of unlabeled −1993T of −1993C probes resulted in complete inhibition of complex formation. The single band corresponding to the −1993C allele was significantly more intense than that corresponding to the −1993T allele (21% increase; $P = 0.02$ by Student's *t* test), suggesting the two different alleles had different affinities for a

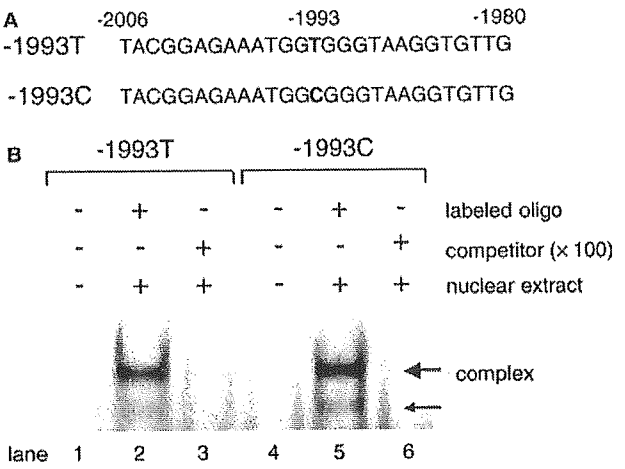

Fig. 4 EMSAs with nuclear extracts prepared from HEK293 cells. Extracts were incubated with DIG-labeled 27-bp double-stranded oligonucleotides corresponding to the −1993T or −1993C alleles of *TBX21*. Competition studies were performed by preincubating with a 100-fold excess of the unlabeled −1993T or −1993C double-stranded competitor oligonucleotides. **a** Oligonucleotide sequences containing T or C at −1993 bp (*bold*) and that were used as a probe or a competitor are shown. **b** Unknown nuclear protein of HEK293 nuclear extracts formed a much stronger complex with the −1993C oligonucleotide compared with the −1993T oligonucleotide (compare *lane 2* vs. *lane 5*; $P = 0.02$ by Student's t test). Binding complex was specifically competed by excess of unlabeled −1993T or C oligonucleotide (*lanes 3, 6*). Band intensity was quantified by using the LAS-3000 camera system and image analysis software Multi Gauge Version 2.0 (Fuji Photo Film). A representative result of three independent experiments is shown

particular nuclear factor. The same trend was also observed in HeLa cells (data not shown). Computer analysis of sequences covering −1993 bp, by using NSITE, available at http://www.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter, indicated that the −1993T → C SNP is situated on a putative binding site for the E2F-1 transcription factor. To identify whether these putative consensus sites were involved in the transcriptional regulation of the *TBX21* gene, we performed a gel shift assay in the presence of specific anti-E2F-1 antibody (C-20; Santa Cruz Biotechnology, Calif., USA). However, preincubation with anti-E2F-1 antibody did not result in a supershift of the DNA-protein complexes (data not shown), suggesting that this protein (family) was not present in the complex binding to this region under these conditions. Together, these data indicate that the −1993T → C SNP in the human *TBX21* gene increases the affinity of an unknown nuclear protein to the binding site around −1993, leading to increased transcriptional activity and a higher expression of the T-bet protein.

Discussion

In the adaptive immune system, $CD4^+$ Th cells differentiate into at least two classes of effector cells, Th1 and Th2, in response to different pathogen-derived antigens. Th1 cells mediate cellular immunity and provide protection against intracellular pathogens and viruses, whereas Th2 cells produce IL-4, IL-5, and IL-13 and eradicate helminthes and other extracellular parasites (Mosmann and Coffman 1989). T-bet, a T box expressed in T cells, has recently been described as a master transcriptional regulator specific to IFN-γ-expressing lineages and is sufficient to induce IFN-γ and IL-12 receptor β2 expression, even under Th2-polarizing conditions (Afkarian et al. 2002; Szabo et al. 2000). Recent experiments have found that, without any allergic sensitization or challenge, the bronchi in mice lacking the T-bet gene, *tbx21*, are infiltrated with eosinophils and lymphocytes and exhibit signs of the airway remodeling and AHR to methacholine that are typical of allergic asthma (Finotto et al. 2002).

In order to examine whether polymorphisms in the candidate gene *TBX21* are related to the risk of human asthma phenotypes, we have characterized sites of

Table 3 Allele frequencies of *TBX21* SNPs in Japanese patients from different asthma groups and controls. Values are the number (%) of successfully genotyped chromosomes

Allele	Healthy controls ($n=640$)	Child patients with asthma ($n=361$)	P^a	Adult patients with					
				Atopic asthma ($n=313$)	P^a	Non-atopic asthma ($n=88$)	P^a	AIA ($n=72$)	P^a
−1993T → C									
T	1149 (89.8)	624 (89.1)		565 (90.3)		161 (93.6)		118 (81.9)	
C	131 (10.2)	76 (10.9)	0.67	61 (9.7)	0.74	11 (6.4)	0.11	26 (18.1)	0.004[b]
99C → G									
C	1127 (88.5)	617 (88.6)		509 (85.1)		143 (88.3)		116 (85.3)	
G	147 (11.5)	79 (11.4)	0.9	89 (14.9)	0.04	19 (11.7)	0.94	20. (14.7)	0.28
1298T → C									
T	1073 (83.8)	603 (83.5)		541 (87.0)		149 (84.7)		125 (86.8)	
C	207 (16.2)	119 (16.5)	0.86	81 (13.0)	0.07	27 (15.3)	0.78	19 (13.2)	0.35
7725G → A									
G	1062 (83.4)	577 (79.9)		502 (82.6)		139 (81.8)		122 (84.7)	
A	212 (16.6)	145 (20.1)	0.05	106 (17.4)	0.67	31 (18.2)	0.6	22 (15.3)	0.68

[a] P-value for the comparison with controls
[b] P-value statistically significant after Bonferroni correction (corrected $P=0.016$)

Table 4 Genotype, allele, and haplotype frequencies in Japanese AIA, AS/NP cases, and controls for the *TBX21* SNPs at—1993 and 390

Locus	Haplotype number		Controls ($n=640$)	AIA ($n=72$)	Uncorrected P	Odds ratio (95% CI)	AS/NP ($n=42$)	Uncorrected P	Odds ratio (95%)
−1993T → C		Genotype TT	519 (81.1)	48 (66.7)		1.0	27 (64.3)		1.0
		Genotype TC+CC	121 (18.9)	24 (33.3)	0.004	2.15 (1.26–3.64)	15 (35.7)	0.008	2.38(1.23–4.62)
		Allele T	1149 (89.8)	118 (81.9)		1.0	68 (81.0)		1.0
		Allele C	131 (10.2)	26 (18.1)	0.004	1.93 (1.22–3.06)	16 (19.0)	0.012	2.06(1.16–3.66)
390A → G		Genotpye AA	533 (83.3)	50 (69.4)		1.0	29 (69.0)		1.0
		Genotype AG+GG	107 (16.7)	22 (30.6)	0.004	2.19 (1.27–3.77)	13 (31.0)	0.019	2.23(1.12–4.44)
		Allele A	1165 (91.0)	120 (83.3)		1.0	70 (83.3)		1.0
		Allele G	116 (9.0)	24 (16.7)	0.004	2.01 (1.25–3.24)	14 (16.7)	0.021	2.01 (1.10–3.68)
[−1993]-[390]	1	T-A	1148 (89.7)	120 (83.3)			68 (80.9)		
	2	C-G	114 (8.9)	22 (15.3)	0.014[a]		14 (16.7)	0.016[a]	
	3	C-A	17 (1.3)	2 (1.4)			2 (2.4)		
	4	T-G	1 (0.1)	0 (0.0)	0.10[b]		0 (0.0)	0.09[b]	

[a] *P*-value for the comparison of the frequencies of haplotype 1 and 2
[b] *P*-value for the overall distribution of two loci haplotypes

genetic variation in selected genomic regions of *TBX21*. Among 24 SNPs identified (five are novel), four polymorphic sites were selected for further analysis. All SNPs fulfilled Hardy–Weinberg expectations in both asthmatic and non-asthmatic subjects, and our study showed a significant association between AIA and a SNP in the regulatory region −1993T → C of the human *TBX21* gene ($P_c=0.016$); this was found to be in strong LD with a synonymous coding SNP, 390A → G, located in exon 1 ($D'=0.92$; $r^2=0.85$). Consistent with recent data (Chung et al. 2003; Ylikoski et al. 2004), these four *TBX21* SNPs lack association with any other asthma phenotype in Japanese subjects.

The percent of the −1993C or 390G allele was much higher in AIA patients than normal controls. In an attempt to extend and support these findings, we further genotyped the −1993T → C and 390A → G SNPs in independent adult AS/NP patients and also found a significant association between these SNPs and AS/NP for the allele and genotype frequencies ($P=0.008$). Furthermore, our data indicated that the single base substitution corresponding to the −1993 *TBX21* polymorphic site produced differences in the transcriptional activity of the *TBX21* gene. Unexpectedly, the *TBX21*/−1993C reporter construct was transcriptionally more active than the wild-type −1993T construct in HEK293 and HeLa cells. In addition, EMSA analysis demonstrated that the −1993T → C substitution increased the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position.

AIA refers to the development of bronchoconstriction following the ingestion of aspirin and other NSAIDs. This clinically distinct syndrome is characterized by aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis, commonly called the "aspirin triad". AIA affects 5%–20% (about 10%) of adult asthmatics with a higher prevalence in women and is infrequently found in asthmatic children (Babu and Salvi 2000; Szczeklik and Stevenson 1999). Chronic persistent inflammation is the hallmark of patients with AIA. Recently, the importance of arachidonic acid metabolites in the pathogenesis of AIA has become apparent. The cyclo-oxygenase (COX) theory is widely accepted: AIA attacks are triggered by the specific inhibition of COX in the respiratory tract, which is followed by a reduction of prostaglandin E2 (a brake on leukotriene synthesis) and an overproduction of cysteinyl leukotrienes. Thus, cysteinyl leukotrienes have been recognized as the key mediators of AIA, but the precise molecular mechanism involved in AIA remains unclear.

Surprisingly, our results have shown a significant increase in the −1993C allele, the putative higher expression of T-bet, among patients with AIA or AS/NP, compared with controls in our Japanese cohort. An inappropriate or excess Th2-biased immune response to environmental antigens has generally been considered to play a crucial role in the development of asthma. Whereas Th2 cells promote asthmatic inflammation, Th1 cells, which secret IFN-γ, have been proposed to protect against asthma by dampening the Th2 response. However, the evidence from many studies of asthma in human and animal models conflicts with this interpretation (Busse and Lemanske 2001; Salvi et al. 2001). For example, IFN-γ production is elevated in the serum of patients with asthma (Corrigan and Kay 1990), in supernatants of bronchoalveolar lavage (BAL) cells (Cembrzynska-Nowak et al. 1993), in T cells themselves in BAL (Krug et al. 1996), and in whole blood culture (Magnan et al. 2000). By using an adaptive transfer system in mice, previous reports have shown that antigen-specific Th1 cells cause considerable airway inflammation instead of attenuating Th2-mediated lung disease (Hansen et al. 1999; Li et al. 1998; Randolph et al. 1999). IFN-γ has been demonstrated to activate eosinophils in vitro, not only with an increased expression of Fcγ receptors, CD69, HLA-DR, and intercellular adhesion

molecule-1, but also with increased viability (Busse and Lemanske 2001; Krug et al. 1996). Furthermore, therapy with IL-12, a Th1-inducing cytokine, fails to reduce AHR or the late asthmatic reaction (Bryan et al. 2000). These and other recent data (Ford et al. 2001; Sugimoto et al. 2004) suggest that IFN-γ contributes to the augmentation of allergic lung inflammation partly through the activation of eosinophils, highlighting the importance of both Th1 and Th2 cytokines in the development of asthma. Thus, the classification of allergic inflammation in asthma as a Th2-mediated disease is too simplistic (Busse and Lemanske 2001), and, as pointed out by recent work (Sugimoto et al. 2004), we propose that asthma may be classified roughly into at least two subgroups, Th2-type asthma and Th1/Th2 mixed-type asthma, including AIA.

Previous reports have suggested that Th1 cells can actually cooperate with Th2 cells in vivo and enhance Th2, eosinophil, and neutrophil recruitment by increasing the expression of TNF-α, chemokines, and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) in the lungs (Randolph et al. 1999; Takaoka et al. 2001). Actually, aspirin sensitivity and AS/NP often coexist with severe asthma, and the airways of AIA patients with NP show signs of persistent inflammation with marked eosinophilia and enhanced VCAM-1 expression (Hamilos et al. 1996). The continuous airway inflammation in AIA could result from a non-IgE-mediated reaction to specific endogenous or exogenous antigens such as a virus (Szczeklik 1988; Szczeklik and Stevenson 1999). A latent or chronic viral infection has been shown to alter the expression of many cellular genes, including several constituents of the arachidonic acid pathway (Zhu et al. 1998), and virally infected cells are more prone to drug and drug-metabolite-related toxicity (Levy 1997; Nakagawa et al. 2001). Moreover, an antiviral drug, acyclovir, is reported to inhibit analgesic-induced bronchoconstriction and decrease the urinary levels of leukotrieneE4 in patients with AIA (Yoshida et al. 1998). Based upon these observations of AIA, virus-specific Th1 cells responding to a respiratory tract infection could alter the local lung environment sufficiently to increase Th2 and eosinophil recruitment, leading to strong Th2 responses to inhaled antigens induced by IL-4, 5, 13, and other mediators. We postulate that the −1993T → C SNP in the *TBX21* promoter causes a functional difference in T-bet expression, resulting in increased T-bet production and (viral-induced) an excessive Th1 inflammatory reaction in the lungs.

Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. Although we find no associations of *TBX21* SNPs with other asthma groups except for AIA, our data also indicate that the presence of the −1993C allele increases the risk of AS/NP, regardless of aspirin sensitivity. Thus, in asthma phenotypes, the *TBX21* SNPs are probably not strictly associated with aspirin sensitivity itself. NP is a chronic inflammatory disease of the paranasal sinus mucosa, leading to the protrusion of edematous polyps into the nasal cavities (Mygind 1990). NP is commonly found in association with non-atopic asthma and aspirin sensitivity, and this association of NP with asthma might reflect the shared pathophysiology of these disorders of the upper and lower airways, respectively. Furthermore, previous studies have shown that NP-infiltrating T cells expressed a mixed Th1/Th2 pattern of cytokines (Hamilos et al. 1995; Sanchez-Segura et al. 1998). Together, our present data suggested that, in a variety of asthma-related conditions, the amplification of either side of the Th1/Th2 pathway, or both, could be adverse to the host. Churg-Strauss syndrome (CSS), also known as allergic granulomatosis and angiitis, is another asthma-related disorder characterized by systemic small vessel vasculitis. Indeed, analysis of the cytokine profile of T cell lines from patients with CSS has shown both type-1 cytokine and type-2 cytokine responses (Kiene et al. 2001). Of note, clinical signs of autoimmunity such as vasculitis have been observed in some patients with AIA (Szczeklik et al. 1995, 1997).

The human *TBX21* gene is located on chromosome 17q21.32, which has previously been linked with asthma and skin tests (Dizier et al. 2000). Moreover, the region on mouse chromosome 11, a region that has been linked to AHR, is syntenically homologous to human chromosome 17q12-q22 (Zhang et al. 1999). *TBX21* is likely to be a novel candidate gene in this region, in addition to other candidate genes such as eotaxin (*CCL11*). However, our data cannot exclude the possibility that −1993T → C is in LD with another polymorphism in *TBX21* or a neighboring gene. Further studies in larger or other populations will be required to confirm the effect of the *TBX21* polymorphism. To date, several candidate genes of the enzymes in the arachidonic pathway, such as *LTC4S* and *ALOX5*, have been proposed to increase the susceptibility to AIA (Choi et al. 2004; Kawagishi et al. 2002; Sanak et al. 1997); indeed many other genes, in addition to *TBX21*, probably contribute to the pathogenesis of AIA. Genetic epidemiology on larger numbers of AIA patients is an important future requirement in order to clarify the importance of our findings.

In conclusion, we have identified 24 SNPs (five novel) in the *TBX21* gene, and our studies demonstrate that the −1993T → C SNP in the *TBX21* promoter is likely to be associated with an increased risk for AIA in Japanese. This is the first report demonstrating a relationship between the *TBX21* SNPs and clinical features of human asthma. Furthermore, we have shown that the −1993T → C polymorphism affects the transcriptional activity of the gene and may contribute to an increase in T-bet expression. In certain asthma subgroups, such as AIA and AS/NP, this promoter SNP may cause inappropriate Th1 responses in the airway, leading to severe airway inflammation, in combination with antigen-specific Th2 responses. Our present data shed light on an important area of further study regarding the precise phenotype classification of asthma by using genotypes and also focus on the Th1 response in the pathogenesis of AIA.

Acknowledgements This work was supported by grants-in-aid from the Ministry of Health, Labor, and Welfare, the Japan Science and Technology Corporation, and the Japanese Millennium project. We thank all participants in the study. We also thank Hiroshi Sekiguchi and Miki Kokubo for technical assistance and Chinatsu Fukushima for providing data on the patients.

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