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Suppressor of cytokine signalling-1 gene silencing in acute myeloid leukaemia and human haematopoietic cell lines

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Summary

The aim of this study was to investigate whether the suppressor of cytokine signalling (SOCS)-1 can act as a tumour suppressor when functioning as a negative regulator of the Janus family tyrosine kinases (JAKs), which have been reported to play important roles in leukaemogenesis. For this purpose, we carried out molecular analysis of the *SOCS-1* gene in human acute myeloid leukaemia (AML) and human haematopoietic cell lines. Sequencing alterations in the coding region were found in two of 90 primary AML samples and one of 17 cell lines. Hypermethylation of the *SOCS-1* gene was also observed in 72% of primary cases and 52% of cell lines and aberrant methylation strongly correlated with reduced expression. Transfection of SOCS-1 into Jurkat cells harbouring the mutation and methylation suppressed cell growth at a low serum concentration. These findings indicate that *SOCS-1* is frequently silenced in haematopoietic malignancies, mainly as a result of hypermethylation, and suggest that SOCS-1 may be able to function as a tumour suppressor.

Keywords: suppressor of cytokine signalling-1, acute myeloid leukaemia, Janus family tyrosine kinases, methylation, tumour suppressor.

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Cytokine signals play a pivotal role in the control of cell survival, proliferation, and differentiation. The signalling pathway formed by the Janus family tyrosine kinases (JAKs) and the signal transducer and activator of transcriptions (STATs) constitutes one of the major mechanisms by which cytokine receptors transduce intracellular signals (O'Shea *et al*, 2002). Binding of cytokines to their receptor causes receptor dimerization as well as the phosphorylation and activation of receptor-associated JAKs. Activated JAKs then induce tyrosine phosphorylation, dimerization and translocation into the nucleus of STATs to initiate transcription of cytokine-responsive genes. Several recent reports have suggested that JAKs play an important role in the process of malignant transformation in certain human and other species malignancies. In Drosophila, mutated JAK hopTum-1 causes leukaemia-like haematopoietic defects (Harrison *et al*, 1995; Luo *et al*, 1995). A recurrent translocation t (9;12) resulting in a TEL-JAK2 fusion transcript has been found in patients with lymphoid and myeloid leukaemias (Lacronique *et al*, 1997; Peeters *et al*, 1997). This fusion protein exerts a constitutive kinase activity

resulting from oligomerization mediated by the TEL pointed domain and can induce factor-independent growth of interleukin (IL)-3 dependent murine Ba/F3 cells (Lacronique *et al*, 2000) and fatal myelo- and lymphoproliferative disorders in mice (Schwaller *et al*, 1998). In another study, constitutive activation of the JAK–STAT pathway was detected in malignant cutaneous T-cell lymphomas (Zhang *et al*, 1996) and human T lymphotropic virus type 1 (HTLV-1)-transformed T cells (Migone *et al*, 1995). Moreover, the JAK-specific inhibitor AG490 was found to selectively block lymphoblastic leukaemia-cell growth both *in vitro* and *in vivo* (Meydan *et al*, 1996). These lines of evidence support the concept that JAKs act as oncogenes.

The suppressor of cytokine signalling (SOCS)-1/SSI-1/JAB protein functions as a negative regulator of cytokine signalling (Alexander, 2002; Fujimoto & Naka, 2003; Yoshimura *et al*, 2003). It is characterized by the central SH2 domain, which binds to multiple tyrosine-phosphorylated signalling proteins, and a highly conserved domain at the C-terminal, known as the SOCS box, which binds to a complex containing elongins BC and promotes the degradation of target proteins in a ubiquitin-dependent manner. SOCS-1 mRNA is induced by various cytokines, such as interleukin (IL)-6, IL-4, leukaemia inhibitory factor (LIF) and granulocyte colony-stimulating factor (G-CSF), resulting in production of the SOCS-1 protein. This protein then inhibits cytokine signalling transduction by binding phosphorylated JAKs and by promoting the degradation of JAKs. The function of SOCS-1 *in vivo* was examined by creating SOCS-1 deficient mice (Naka *et al*, 1998; Starr *et al*, 1998). In mice, this deletion causes growth retardation, fulminant hepatitis, macrophage infiltration of several organs, severe lymphopenia, and perinatal lethality within 3 weeks of birth. These phenomena were eliminated by introducing interferon (IFN)-γ deficiency (Alexander *et al*, 1999; Marine *et al*, 1999), suggesting that SOCS-1 inhibits IFN-γ signalling transduction *in vivo*. In addition, it has been reported that IL-2, IL-4 and IL-12 signalling transductions are dysregulated in SOCS-1 homo- and hetero-deficient mice (Fujimoto *et al*, 2002, 2004). These studies have suggested that SOCS-1 is one of the major physiological factors that negatively regulate a variety of cytokine signalling.

Of the eight SOCS family members, SOCS-1 is the most potent inhibitor of JAKs, which suggests that it may function as a tumour suppressor. Indeed, it has been reported that *SOCS-1* is frequently silenced in human hepatocellular carcinoma (HCC) (Nagai *et al*, 2001; Yoshikawa *et al*, 2001), multiple myeloma (Depil *et al*, 2003; Galm *et al*, 2003) and other neoplasms (Fukushima *et al*, 2003; Liu *et al*, 2003; Lin *et al*, 2004) as a result of aberrant methylation in exon 2 of the *SOCS-1* gene, which contains the entire coding region. SOCS-1 mRNA is abundantly expressed, particularly in haematopoietic organs, and SOCS-1 deficiency in mice causes not only lymphoid cell alterations but also myeloid cell abnormalities (Metcalf *et al*, 1999). Further, SOCS-1 is involved in the signal transduction of c-kit, FMS-like tyrosine kinase (FLT) 3, granulocyte colony-stimulating factor (G-CSF) and other substances known to be important for leukaemogenesis (De Sepulveda *et al*, 1999; Rottapel *et al*, 2002). These findings point to the possible implication of *SOCS-1* gene alterations in the pathogenesis of human haematopoietic malignancies. We therefore analysed the *SOCS-1* gene structure to examine gene deletion, mutation, and methylation in primary acute myeloid leukaemia (AML) patients and human haematopoietic cell lines.

Materials and methods

Clinical samples, cell lines and DNA extraction

A total of 90 patients who were diagnosed with AML at Osaka Univiersity Hospital was selected. Seventeen haematopoietic cell lines – Jurkat (T cell, RIKEN RCB0806), MOLT-4 (T cell), YT (natural killer (NK) cell), Ramos (Burkitt cell), Daudi (Burkitt cell), Raji (Burkitt cell), BL41 (Burkitt cell), Reh (pro B cell), NALM17 (pro B cell), NALM6 (pre B cell), RPMI8226 (myeloma cell), U266 (myeloma cell), THP-1 (myeloid cell), HL60 (myeloid cell), U937 (myeloid cell), K562 (chronic myeloid leukaemia, CML), and KU812 (CML) – were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), RIKEN cell bank (Tsukuba, Japan), or American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin. DNA was isolated from these cell suspensions by means of a DNeasy tissue kit (QIAGEN Inc., Valencia, CA, USA).

Antibodies, expression constructs and trasfection

The anti-JAK2 antibody (Ab) (clone C20) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), the anti-actin monoclonal antibody (mAb) (clone C4) from ICN pharmaceuticals (Aurora, OH, USA). The anti-SOCS-1 mAb 1262B was described in a previous report (Narazaki *et al*, 1998). The TEL-JAK2 expression vector was a gift from Dr A. Yoshimura (Kamizono *et al*, 2001). Wild-type human SOCS-1 and mutant SOCS-1 cDNA were amplified by polymerase chain reaction (PCR) from genomic DNA, inserted into the pApuro2 vector, and confirmed by DNA sequencing. DNA transfection into 293T cells with Lipofectamine (Invitrogen Corp., Carlsbad, CA, USA) has been described elsewhere (Watanabe *et al*, 2001). cDNAs of mouse wild-type SOCS-1 or R105Q mutant, which was introduced into the Arg-105 point mutation of SOCS-1 to yield Gln, were co-transfected with pSVIINeo as described previously (Narazaki *et al*, 1998) and selected in the presence of 500 μg/ml of G418 (Nakarai Tesque, Kyoto, Japan).

Analysis of direct sequencing of PCR products

The full coding sequence of the *SOCS-1* gene was amplified by nested PCR strategy. The first round of amplification was performed with primers SOCS-S4 (5′-AGC GCC CCA GCT CAC CGC TT-3′) and SOCS-AS4 (5′-GGG TAC CCA CAT GGT TCC AGG CA-3′). For re-amplification, nested primers SOCS-S3 (5′-TCA CCG CTT TGT CTC TCC CG-3′) and SOCS-AS3 (5′-AGT AAT AAC AAA ATA ACA CGG CAT CCC-3′) were used. PCR conditions consisted of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C for 35 cycles. Re-amplification was carried out under the same conditions from 1/100 μl of the first PCR mixtures as template. Reaction mixtures after re-amplification were analysed on 1% agarose gels and stained with ethidium bromide. Bands of the appropriate size were excised and purified from the gels. Direct sequencing of PCR products was carried out with cycle sequencing BigDye terminator chemistry (Applied Biosystems Japan, Tokyo, Japan) with four primers SOCS-S3, SOCS-AS3, SOCS-F (5′-CCC GGC GAC ACG CAC TTC CGC ACA-3′), and SOCS-R (5′-CGA AGC TCT CGC GGC TGC CAT CCA-3′). Mutations of the *SOCS-1* gene were confirmed with sequencing of both strands from two independent amplifications.

Bisulphite modification of genomic DNA and methylation specific PCR (MSP)

A 1 μg of genomic DNA was modified with sodium bisulphite using the CpGenome bisulphite modification kit (Intergen, Purchase, NY, USA). Modified DNA was suspended with 35 μl of 10 mmol/l Tris HCl (pH 8·5). 5 μl of suspension was subjected to MSP of CpG islands in exon 2 of the *SOCS-1* gene as described previously (Yoshikawa *et al*, 2001). *In vitro* methylated DNA (Intergen) was used as control for methylated, and DNA of normal leucocytes obtained from five healthy volunteers as that for unmethylated, DNA. PCR products were analysed on 3% NuSieve 3:1 agarose (TaKaRa, Kyoto, Japan) gels, stained with SYBR Green (TaKaRa) and visualized under ultra violet illumination.

Isolation of total RNA and reverse transcription-PCR (RT-PCR)

Total RNA was obtained with the RNeasy mini kit (QIAGEN). The RNA purity was measured spectrophotometrically at 260 nm, and 2·5 μg of the total RNA preparation was reverse transcribed with a cDNA synthesis kit (Invitrogen Corp.) using the oligo(dT) primer in a total volume of 20 μl. One microlitre of the RT reaction was then used as template for a 25 μl PCR reaction. PCR conditions for SOCS-1 cDNA were 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C for 27 cycles. The PCR primers for SOCS-1 amplification were SOCS-F and SOCS-R. The PCR conditions for β-actin cDNA were 30 s at 95°C, 30 s at 60°C, and 60 s at 72°C for 18 cycles. The PCR primers for β-actin amplification were ACTB-F (5′-GCC GAG CGG GAA ATC GTG CG-3′) and ACTB-R (5′-ACG ATG GAG GGG CCG GAC TC-3′).

5-Aza-2′-deoxycytidine assay

Cells at the density of 3–5 × 10^5/ml were treated with culture medium containing 10 μmol/l of the demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St Louis, MO, USA), with the exception of Ramos cells (5 μM 5-aza-dC). After 48 h of incubation with media changed everyday, cells were harvested for purification of RNA with or without the stimulation of 100 U/ml of IFN-γ for 2 h.

Cytotoxicity assays

Jurkat Cells (1 × 10^4) were cultured in a 96-well microtitre plate with 100 μl of culture medium. After 5 d of incubation, these cells were then assayed for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide] dye conversion with a Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan), which counts living cells by combining 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (WST-8) and 1-methoxy-phenazine methosulphate (1-methoxy-PMS). Absorbance was measured at 450 nm with an enzyme-linked immunosorbent assay immuno-reader. Triplicate samples were used for three independent experiments. Statistical analysis was performed using the unpaired *t*-test. Human wild-type (WT) and mutant SOCS-1 expression vectors were transfected into M1 cells by electroporation and three independent clones were established in a growth medium containing puromycin at 50 μg/ml. The transfectants (1 × 10^4) were then cultured in a 96-well microtitre plate with 100 μl of culture medium with or without 100 ng/ml of IL-6. After 2 d of incubation, these cells were then assayed for MTT dye conversion.

Results

Genetic alterations of the SOCS-1 gene in human haematopoietic malignancies

Genomic DNA was isolated from suspensions of a total of 90 leukaemia cell samples from patients with AML and 17 human haematopoietic cell lines. As a small deletion on chromosome 16 containing the *SOCS-1* gene had been identified in almost 40% of HCC (Nagai *et al*, 2001), we initially examined the 17 haematopoietic cell lines for gross genomic alteration of the *SOCS-1* gene. The genomic PCR methods and Southern blotting used for this purpose showed neither deletion nor rearrangement in any of the cell lines (data not shown). Our methods did not, however, enable us to rule out partial deletion, for instance of exon 1, or other complex gene rearrangements.

To determine whether the *SOCS-1* gene was mutated in haematopoietic malignancies, we performed an extensive

mutational analysis of genomic DNA by means of PCR-direct sequencing. Sequencing alterations were observed in the Jurkat cell line (Fig 1A) and in two primary samples, AML36 and AML53 (Fig 1B). In the Jurkat cell line, sequencing analysis demonstrated a 1 basepair (bp) G deletion at codon 164, resulting in a frameshift that was predicted to replace 40 amino-acid residues at the carboxyl terminus (Fig 1C, JK); in the two primary samples AML36 and AML53, PCR-direct sequencing showed a hemizygous missense mutation (C-to-T transition) at codon 198 (Fig 1B) that resulted in the substitution of Ser for Pro (P198S, Fig 1C). However, as normal tissues from the leukaemia samples and cell lines were not available for our study, we could not exclude the possibility that the missense variant P198S represented a simple polymorphism.

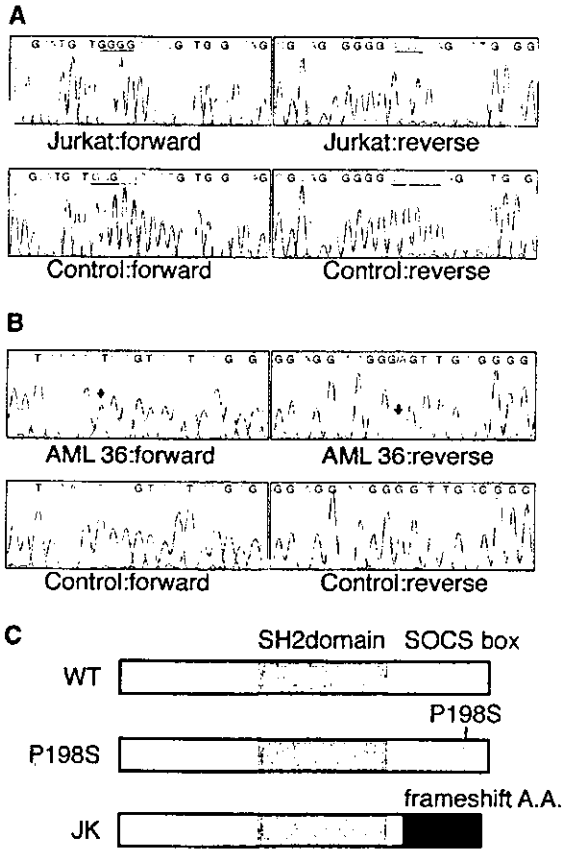

Fig 1. Sequence analysis of SOCS-1. (A and B) Upper panels show the sequences of Jurkat cells (A) or primary AML (B), and lower panels show the control sequences. Left and right panels show the forward and reverse sequences respectively. (A) Jurkat cells: homozygous 1 bp deletion (underlined) at codon 164 resulting in frame shift mutation. (B) AML36: hemizygous missense mutation (arrow) resulting in P198S at the location of the SOCS box. AML53 showed identical results. (C) Schematic representation of WT and mutant SOCS-1. P198S features a point mutation at the location of the SOCS box. Jurkat mutant (JK) has a 1 bp deletion at codon 164, resulting in absence of the SOCS box and the presence of 40 frameshift amino acids (A.A.).

Jurkat mutant SOCS-1 exhibits instability and impaired inhibition of cytokine signalling

As we could not conclude whether these variations represented somatic mutations or polymorphisms, and as the frequency of P198S in leukaemia samples (2/90) and DNA from normal individuals (0/15) was not high enough to perform statistical analysis, the significance of the coding alterations remained elusive. The identified variations were, however, closely related to the SOCS box, which is the structural motif thought to be principally involved in promoting the degradation of oncogenic tyrosine kinase TEL-JAK2 and stabilization of the SOCS-1 protein (Kile *et al*, 2002). To clarify the pathogenetic status of these mutations, WT, P198S variant, and Jurkat mutant (JK) expression vectors were constructed (Fig 1C). First, to examine the function of the promotion of TEL-JAK2 degradation, serial dilutions of WT and mutant SOCS-1 cDNA were transiently transfected into 293T cells with the TEL-JAK2 expression vector. As shown in Fig 2A, both WT and P198S, but not JK, reduced the expression level of TEL-JAK2 in the 293T cells in a dose-dependent manner. We also investigated the stability of the mutant SOCS-1 in 293T cells as described previously (Hanada *et al*, 2001). In the case of WT (Fig 2B, top panel), the protein level of SOCS-1 remained stable and showed no reduction during a 5-h incubation with cycloheximide (CHX). However, only a 1-h incubation with CHX reduced the protein level of JK mutant SOCS-1, which lacked the SOCS box because of a frameshift mutation at codon 164 (Fig 2B, third panel). P198S, the missense variation in the SOCS box, showed slight instability in comparison with WT (Fig 2B, second panel). In addition to the co-expression system, we also used stable SOCS-1 expressing transfectant to determine the effect of mutant SOCS-1 on protein stability and the cytokine-mediated signalling pathway. To this purpose, WT and mutant SOCS-1 expressing vectors were transfected into mouse M1 cells and three independent expressing clones were established. In these stable transfectants, JK mutant SOCS-1 protein showed a weaker expression compared to that of WT and P198S SOCS-1 (Fig 2C, top panel). As shown in Fig 2D, the stimulation of IL-6 for 2 d induced the cell death of M1/mock cells and the viable cell number of these cells was 3 ± 5% in comparison with that of unstimulated cells. However, IL-6-induced cell death was not observed in both M1/WT cells (105 ± 18%) and M1/P198S cells (85 ± 13%). On the other hand, the viable cell number of M1/JK cells showed a significant decrease (27 ± 17%) when stimulated with IL-6, possibly due to the partial inhibition of IL-6 signalling by JK mutant SOCS-1 in these cells (Fig 2D). These results were consistent with those of previous studies using the SOCS box-deleted mutant (Narazaki *et al*, 1998) (Hanada *et al*, 2001) and indicated that the mutation observed in Jurkat cells was likely to be a pathogenic alteration.

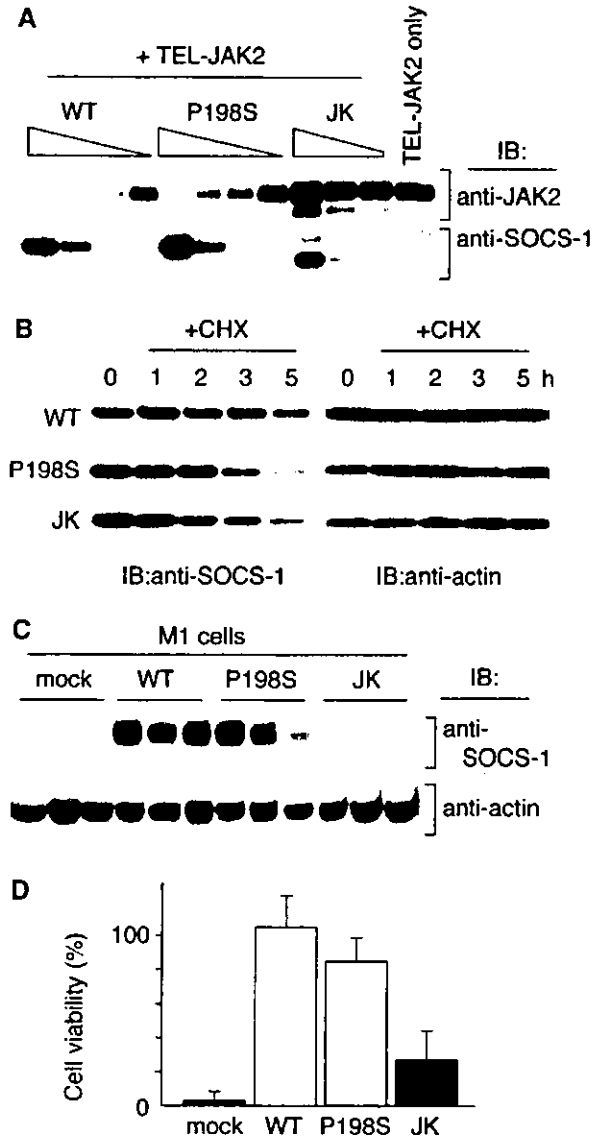

Fig 2. Fuctional analysis of mutant SOCS-1. (A) Serial dilutions of WT and mutant SOCS-1 vectors (lanes 1, 5, and 9 were 5 μg; lanes 2, 6, and 10 were 1 μg; lanes 3, 7 and 11 were 0·1 μg; lanes 4, and 8 were 0·01 μg) were transfected into 293T cells with 1 μg of plasmid carrying TEL-JAK2 as indicated at the top. After a 48-h incubation, whole cell lysates were harvested, divided into three samples, and subjected to immunoblotting (IB) to visualize expression levels of TEL-JAK2 by using the anti-JAK2 Ab (top panel) and of SOCS-1 by using the anti-SOCS-1 mAb, which recognizes the N-terminal portion (lower panel). (B) WT and mutant SOCS-1 vectors were transfected into 293T cells. Transfected cells were divided into five samples and incubated with 100 μg/ml cyclocheximde (CHX) for the indicated periods. Whole cell lysates were blotted with the anti-SOCS-1 mAb to analysis the stability of SOCS-1 (top panel). An equal amount of cell lysate was confirmed by immunoblotting with anti-actin mAb (lower panel). (C) SOCS-1 expression in stable transfectants of M1 cells was visualized by immunoblotting using the anti-SOCS-1 Ab (top panel). (D) Effects of WT and mutant SOCS-1 on IL-6 signalling in M1 cells. Each stable transfectant was cultured with or without 100 ng/ml of IL-6 as described in Materials and methods. Mock cells were transfected with the pApuro2 vector only and used as control cells. Bars indicates mean + SD of cell viability from three IL-6 stimulated transfectants relative to unstimulated cells ($n = 3$, mean + SD).

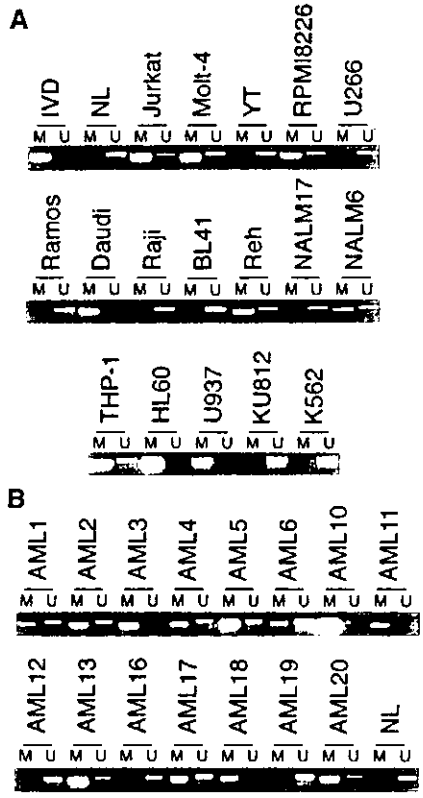

Fig 3. Methylation status of the *SOCS-1* gene. MSP results for CpG islands in exon 2 are shown in cell lines (A) and primary AML DNA (B). *In vitro* methylated DNA (IVD) and DNA from normal leucocytes (NL) were used as control for MSP. M and U indicate methylated and unmethylated DNA respectively.

The SOCS-1 gene is frequently methylated in human haematopoietic malignancies

As mentioned earlier, *SOCS-1* is not a common target for gene deletion and mutation in human haematopoietic malignancies. In the light of reports that *SOCS-1* is silenced in HCC (Nagai *et al*, 2001; Yoshikawa *et al*, 2001), multiple myeloma (Depil *et al*, 2003; Galm *et al*, 2003) and other organ neoplasms (Fukushima *et al*, 2003; Liu *et al*, 2003; Lin *et al*, 2004) by hypermethylation, we next examined the methylation status of the *SOCS-1* gene in AML and human haematopoietic cell lines. MSP, a method first reported by Herman *et al* (1996), was used to demonstrate hypermethylation of *p16*, *VHL*, *E-cadherin*, and other genes. With this method, sodium bisulphite treatment and MSP were performed on the DNA from 17 cell lines and 88 primary samples. Aberrant methylation of the *SOCS-1* gene was observed in nine of 17 (52%) cell lines (Fig 3A) and 64 of 88 (72%) primary cases (Fig 3B, only representative data shown). There was no methylation in the DNA of normal leucocytes (NL) from five healthy volunteers. RT-PCR analysis was used to examine mRNA levels in 12 of the cell lines in order to determine alterations in SOCS-1 gene expression caused by aberrant hypermethylation. As shown

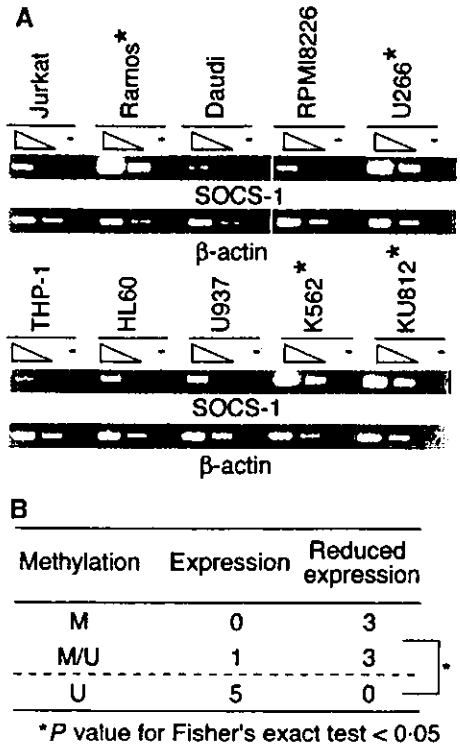

Fig 4. Expression analysis of SOCS-1 mRNA (A). RT-PCR was carried out as described in Materials and methods (lanes 1, 4, 7, 10 and 13). All RT-PCR products were separated on 2% agarose gels and identified by staining with ethidium bromide. To perform semi-quantitative analysis of RT-PCR, a fivefold dilution of the RT reaction (lanes 2, 5, 8, 11 and 14) was used as templates for PCR. To exclude the amplification from contaminated genomic DNA, an RT negative reaction (lanes 3, 6, 9, 12 and 15) was also used. Asterisks indicate the cell lines that showed the unmethylated *SOCS-1* gene. (B) Correlation between methylation and reduced expression.

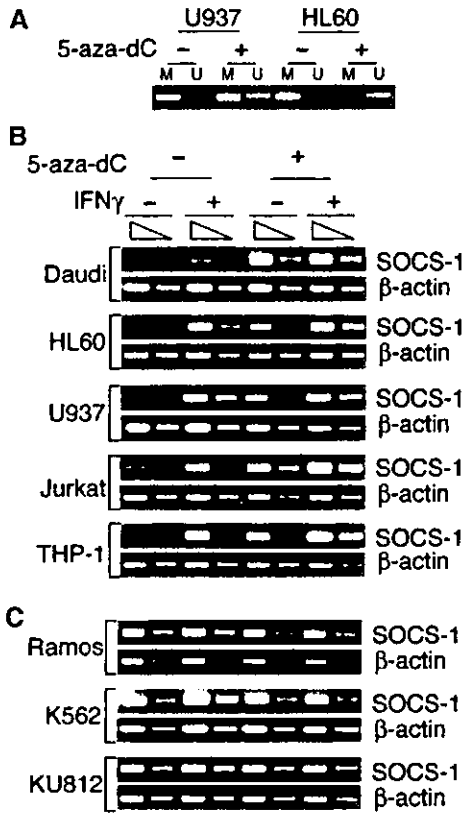

Fig 5. Effects of 5-aza-dC on methylation status of the *SOCS-1* gene (A) and expression of SOCS-1 mRNA in SOCS-1 expressing (B) or reduced expression cells (C). Indicated cell lines were treated with 10 μmol/l of 5-aza-dC for 48 h, except for Ramos cells (5 μmol/l), and with 100 U/ml of IFN-γ for 2 h in the indicated combinations. DNA and RNA were isolated and subjected to MSP (A) and semi-quantitative RT-PCR of *SOCS-1* mRNA as described in Materials and methods (B and C, lanes 1, 3, 5 and 7). A fivefold dilution of the RT reaction was also used as templates for PCR (B and C, lanes 2, 4, 6 and 8).

in Fig 4A, unmethylated cell lines showed a greater increase in expression than methylated cell lines; indeed, a strong correlation between methylation and reduced expression was observed (Fig 4B; $P < 0{\cdot}05$). In addition, cells displaying either SOCS-1 expression or reduced expression were treated with the demethylating agent, 5-aza-dC. Since SOCS-1 is not constitutively expressed in normal cells, but is rapidly induced by various stimuli such as cytokines, we also examined SOCS-1 expression following stimulation with IFN-γ, which is known to be the most potent inducer. As shown in Fig 5A, treatment with 5-aza-dC demethylated the CpG islands in the *SOCS-1* gene. In many methylated cells, SOCS-1 was induced by IFN-γ (Fig 5B). However, either with or without IFN-γ stimulation, the expression level of SOCS-1 was enhanced by treatment with 5-aza-dC (Fig 5B). On the other hand, SOCS-1 was constitutively expressed in unmethylated cells and its expression level remained almost unchanged following IFN-γ stimulation or 5-aza-dC treatment (Fig 5C). Taken together, these findings suggest the possibility that *SOCS-1* is frequently silenced by hypermethylation.

Ectopical expression of SOCS-1 in Jurkat cell line suppresses cell growth at a low serum concentration

To clarify the tumour-suppressor activity of SOCS-1, vectors of wild-type SOCS-1 were transfected into Jurkat cells harbouring the mutation and methylation of the *SOCS-1* gene to create stable transfectants. For a negative control, its SH2 domain mutant (R105Q) was also transfected because R105Q completely lacked the biological effect of SOCS-1 (Narazaki *et al*, 1998; Nicholson *et al*, 1999). Expression of SOCS-1 was confirmed by Western blot analysis (Fig 6A). We examined cell-growth activity at low (Fig 6B) and normal (Fig 6D) FCS concentrations. Compared with mock and R105Q-expressing cells, the WT SOCS-1-expressing cells exhibited a lower viable cell count after 4 and 5 d incubation in 0·75% FCS, although the two transfectants showed the same growth rate in culture medium containing 10% FCS. This result was confirmed with an MTT assay (Fig 6C). One of the major targets of SOCS-1 in Jurkat cells seems to be JAKs, which are essential for many types of cytokine and growth-factor signalling. To establish

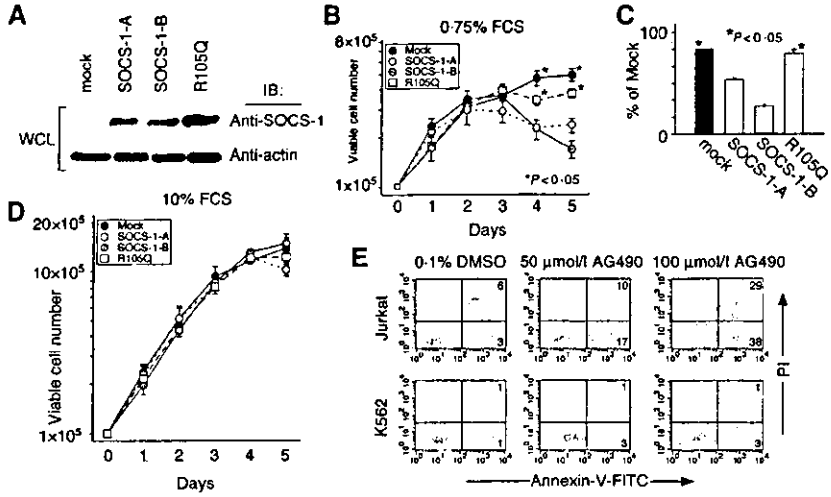

Fig 6. Ectopical expression of SOCS-1 in a Jurkat cell line suppressed the cell growth at a low serum concentration. (A) SOCS-1 expression in independent established clones. (B) The indicated stable transfectants (1×10^5 cells) were plated in 24-well plates and grown in 0·75% FCS. Absolute numbers of viable cells were determined by trypan blue exclusion assay on days 1–5. The results are shown as mean ± SE from three independent experiments. Statistical analysis was performed with the unpaired *t*-test. (C) MTT assay analysis was carried out after a 5-d incubation in 0·75% FCS culture medium. Bars indicate the relative absorbance in 450 nm of SOCS-1 transfectants with the value for mock cells set at 100 ($n = 3$, mean + SE). (D) SOCS-1 transfectants grown in 10% FCS. (E) Analysis of AG490-induced apoptosis. Jurkat and K562 cells were cultured with 50 or 100 μmol/l of AG490 or 0·1% dimethylsulphoxide in 1% FCS growth medium. After a 24-h incubation, cells were stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) and then analysed by flow cytometry.

whether this is true, Jurkat and K562 cells were treated with the JAK specific inhibitor AG490 at a low serum concentration and subjected to flow cytometry to determine the percentage of apoptotic cells. As shown in Fig 6E, AG490 induced apoptosis in Jurkat cells, but not in K562 cells, which expressesSOCS-1. These results indicate that SOCS-1 has a tumour-suppressor effect and that JAKs seems to show one of the major targets of SOCS-1 on Jurkat cells.

Discussion

In the present study, we have identified *SOCS-1* as a target gene for mutation and methylation in human haematopoietic malignancies. The coding region of the *SOCS-1* gene for mutations was firstly investigated and two types of gene alteration were observed. P198S, that is a missense variation, was identified in secondary leukaemias from two patients preceded by Shwachman syndrome (AML36) and myelodysplastic syndrome (MDS, AML53). Shwachman syndrome is an autosomal-recessive disorder characterized by exocrine pancreatic insufficiency, neutropenia and other organ dysfunction, whose disease-associated mutations have been recently identified in an uncharacterized gene (*SBDS*) (Boocock *et al*, 2003). These syndromes often show neutropenia, are treated with G-CSF therapy, and accompanied with an increased risk for developing AML. Since somatic G-CSF receptor mutations in cases of severe congenital neutropenia (Dong *et al*, 1994) and abnormalities of the molecules involved in G-CSF signal transduction are thought to be involved in leukaemogenesis, coding alterations of P198S may be related to the development of leukaemia in Shwachman syndrome and MDS. However, as we could not conclude whether this variation is a somatic mutation or polymorphism and our results suggest that P198S seems to function normally in 293T and M1 cells, the significance of P198S misssense variation remains elusive. In addition to the P198S variation, SOCS-1 mutation was identified in the Jurkat cells. This mutation resulted in loss of the SOCS box, which is homologous to the region of the VHL protein (Kile *et al*, 2002). This protein binds to the elongin BC complex and promotes the ubiquitination and degradation of hypoxia-inducible transcription factors 1α and 2α via the SOCS box. Germline mutations within the *VHL* tumour suppressor gene cause von Hippel–Lindau (VHL) disease, which is characterized by a dominantly inherited multisystemic family cancer syndrome. Inherited mutations in the SOCS box of VHL protein are frequently observed in VHL disease (Kishida *et al*, 1995). In previous studies using co-expression systems, deletion of the SOCS box from SOCS-1 had little impact on the inhibition of cytokine signal transduction compared to mutant SOCS-1 in the central SH2 domain or kinase inhibitory region, which show a complete loss of their biological effect (Narazaki *et al*, 1998; Nicholson *et al*, 1999). The results of our experiments with the JK mutant of SOCS-1 and mutational analysis of VHL gene suggest, however, that ubiquitination mediated by the SOCS box may be significant for the process of malignancy.

There is mounting evidence that tumour suppressor genes can be inactivated by cancer-specific methylation of their 5′ end region (promoter, untranslated region or exon 1), and that this modification of tumour suppressor genes may be more

common than amino acid sequence alterations (Baylin *et al*, 2001). Our results presented here also suggest that SOCS-1 may be silenced by tumour-specific methylation. However, two questions need to be dealt with. First, the primer location of MSP existed in the exon 2 of the *SOCS-1* gene, but not in the 5′ end region. The *SOCS-1* gene contains two exons (Saito *et al*, 2000). Exon 1 has a short sequence while exon 2 contains the entire coding region, which shows an unexpected high GC rich sequence (about 70%). This implies that exon 2 of the *SOCS-1* gene is not detached from the CpG island in the 5′ end region. Although it remains to be determined whether critical methylation sites exist in the 5′ end region, hypermethylation of exon 2 could cause changes in chromatin conformation and lead to gene inactivation. It should also be noted that IFN-γ could induce expression of SOCS-1 mRNA in some methylated cell lines in spite of their methylation. It is possible that this induction is related to the location of gene methylation because, if methylation is specific to exon 2, but not the promoter region, STAT1 and IRF-1, the transcription factors activated by IFN-γ, can interact with their response elements in the promoter region (Saito *et al*, 2000). This interaction could then overcome methylation-mediated silencing, as was previously reported in the case of a progesterone receptor gene that was induced by an oestrogen receptor without the need for demethylation of the progesterone receptor gene CpG island (Ferguson *et al*, 1998). These observations are not consistent with those typical of methylation-mediated gene silencing. Nevertheless, it is clear that SOCS-1 gene methylation is tumour-specific and correlates with reduced expression.

It has been reported that SOCS-1 blocks many signal transductions, such as those of IL-6, G-CSF, and granulocyte-macrophage colony-stimulating factor, so that the tumour-suppressor activity of SOCS-1 may be mediated by alterations of these signal transductions (Alexander, 2002; Fujimoto & Naka, 2003; Yoshimura *et al*, 2003). In a recent report, Galm *et al* (2003) reported that SOCS-1 was frequently silenced in multiple myeloma and aberrant SOCS-1 methylation was found in IL-6 dependent myeloma cell lines. Although this report indicates that IL-6 is a candidate for signal transduction that SOCS-1 negatively regulates in the malignant cells, IL-6 signal transductions were inhibited by SOCS-1 only in re-constituted systems in which it was forcibly-expressed. It is true that SOCS-1 was identified by functional cloning as a negative regulator of IL-6 (Starr *et al*, 1997), but no abnormalities of IL-6 signal transduction have been found in SOCS-1 deficient mice (Alexander *et al*, 1999). Moreover, a conditional knockout strategy has demonstrated that SOCS-3, but not SOCS-1, negatively regulates IL-6 signalling *in vivo* (Croker *et al*, 2003; Lang *et al*, 2003; Yasukawa *et al*, 2003). We should thus be careful to determine which molecules that are located upstream activate JAKs.

In the present study, we identified *SOCS-1* as a target gene for mutation and methylation in human haematopoietic malignancies. The detection of SOCS-1 methylation in 72% of primary leukaemias suggests that the inactivation of the *SOCS-1* gene is a frequent event in the process of leukaemogenesis. Methylation in exon 2 of the *SOCS-1* gene was associated with reduction of SOCS-1 gene expression in haematopoietic cell lines. Moreover, transfection of SOCS-1 suppressed the growth of the Jurkat cell line at a low serum concentration. These results suggest that *SOCS-1* silencing may confer a growth advantage on some haematopoietic malignancies, and hence that *SOCS-1* may be a tumour-suppressor gene. Recently, Chen *et al* (2003) reported that the incidence of methylation in SOCS-1 gene was significantly higher in AML with t(15;17) than that in AML with t(8;21), although the reason for this was not clearly examined. To establish the function of SOCS-1 as a tumour suppressor and clarify the contribution of SOCS-1 methylation to the development of malignant diseases, we are now investigating the role of SOCS-1 in DNA damage response with the aid of SOCS-1 knockout mice.

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PEDIATRICS

Methylenetetrahydrofolate Reductase Polymorphism in Patients with Bronchial Asthma

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ABSTRACT

Objective: **Bronchial asthma is a chronic inflammatory condition of the respiratory tract. The C677T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is reported to confer susceptibility to cardiovascular diseases and inflammatory conditions. We hypothesized that TT genotype of MTHFR may influence the development of bronchial asthma and thus examined the C677T polymorphism in our Japanese asthmatic patients.**

Design: **Clinical Investigation.**

Methods: **Genotypes for MTHFR were determined in 461 asthmatic patients (male/female ratio: 248/213) by the polymerase chain reaction and restriction fragment length polymorphism method and the results were compared with those obtained from 1430 healthy subjects (male/female ratio: 939/491).**

Results: **For the male population, the frequency of the TT genotype in asthmatic patients was significantly higher than in healthy subjects (16.9% vs. 11.0%, odds ratio = 1.65, 95% confidence interval: 1.12-2.44, P = 0.011). For the female population, the frequency of the TT genotype in atopic asthmatic patients was insignificantly higher than in non-atopic asthmatic patients (17.3% vs. 11.8%).**

Conclusion: **Our findings suggest that the TT genotype of MTHFR is a probable genetic risk factor for the development of bronchial asthma in Japanese males.**

KEY WORDS

bronchial asthma, homocysteine, methylenetetrahydrofolate reductase, oxidative stress, polymorphism

INTRODUCTION

Bronchial asthma is a condition characterized by episodic reversible airway obstruction, airway hyperresponsiveness and allergic inflammation of the airway. Multiple inflammatory cells, cytokines and other mediators participate in the pathogenesis of bronchial asthma[1]. Several lines of evidence suggest that oxidative stress contributes to airway inflammation and epithelial damage and to alterations in the immune system, and that individuals with lowered antioxidant capacity are at increased risk for developing asthma[2,3]. Therefore, genetic, environmental and dietary factors that diminish antioxidant defenses could potentially increase the vulnerability to oxidative stress and thus the risk for developing asthma. Accordingly, study of

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genetic polymorphism related to oxidative stress may be of help to our understanding of the complex pathophysiology of asthma, and could lead to the development of new and effective management protocols for this common disease.

There is general interest in hyperhomocysteinemia as a risk factor for vasculopathy, which is thought to exert its effects through oxidative damage[4,5]. Recently, a common C to T mutation at nucleotide position 677 (C677T) has been identified in the gene coding for 5,10-methylenetetrahydrofolate reductase (MTHFR), which is involved in the remethylation of homocysteine to methionine[6,7]. The C677T mutation causes a valine-for-alanine substitution, which decreases MTHFR activity and tends to be associated with elevated blood homocysteine levels. Other studies suggest that the TT genotype of MTHFR is significantly associated with coronary artery disease and ischemic stroke[8,9]. It has also been reported that the TT genotype may represent a genetic risk factor for the development and aggravation of inflammatory conditions such as inflammatory bowel disease, Kawasaki disease and chronic glomerulonephritis[10-13].

Based on the above considerations, we hypothesized that the TT genotype of MTHFR may influence the development of bronchial asthma and thus examined the C677T polymorphism in Japanese asthmatic patients. This is the first report to demonstrate the association between MTHFR C677T mutation and development of bronchial asthma.

METHODS

Subjects

We studied 461 Japanese patients with bronchial asthma who had been seen at the outpatient clinics of Fukui Medical University Hospital (Fukui), Tenri Hospital (Nara), Osaka University Hospital (Osaka), Hokkaido University Hospital (Hokkaido), Gifu University Hospital (Gifu) and their affiliated hospitals. They included 248 males and 213 females and their age ranged from 1 to 85 years (mean 33 years; median 28 years). The diagnosis of asthma was based on the criteria of the National Asthma Education and Prevention Program, Expert Panel Report II[14]. Atopy was defined by the presence of high levels of specific serum IgE ($\geq$ 0.35 KU_A/l) to at least one aeroallergen (such as house dust mite) using the CAP radioallergosorbent test fluoro enzyme immunoassay system (Pharmacia Upjohn Diagnostics, Tokyo, Japan)[15]. As age-matched healthy controls, 1430 subjects (939 males and 491 females) were derived from studies conducted by our group (n = 339)[11] (unpublished results) and other groups (n = 1091)[8,16,17]. Informed consent was obtained from our subjects (461 patients and 339 healthy controls) and/or their parents before blood samples were collected. This study protocol was approved by the ethics committees of all the hospitals that participated in the present study.

Genetic analysis

Genomic DNA was extracted from peripheral-blood leukocytes. Identification of the C677T mutation in the MTHFR gene was performed by polymerase chain reaction (PCR) using primers described by our group[11]: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'(Toagosei, Tsukuba, Japan). PCR thermal cycling conditions were a 10-min denaturation period at 94℃ and 43 cycles of the following: 94℃ for 1 min, 55℃ for 30 s and 72℃ for 1 min. This was followed by a 10-min extension at 72℃ (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan). The amplified products were digested with *Hinf*I (TaKaRa Biomedicals, Ohtsu, Japan) at 37℃ for 4 hours. The *Hinf*I-treated PCR fragments were electrophoresed in 5% polyacrylamide gel and visualized with ethidium bromide. The C to T mutation creates a *Hinf*I recognition site that leads to digestion of the 198-bp PCR product into 175- and 23-bp fragments. Heterozygote subjects show three fragments (198 bp, 175 bp and 23 bp) and a homozygous C to T mutation results in the production of two fragments of 175 bp and 23 bp.

Although we did not examine blood homocysteine levels in our subjects, data from another group on healthy Japanese individuals showed that the mean homocysteine level was significantly higher in subjects with the TT genotype (15.7 μM) than in those carrying the CC (11.6 μM) or CT (11.8 μM) genotype[18].

Statistical analysis

The analysis of genotype involved comparing the number of subjects carrying the TT genotype with the number of subjects carrying the other genotypes. Statistical analysis was also performed on the numbers of C677 alleles and T677 alleles. The differences were examined by the chi-squared test or Fisher's exact test where appropriate. Statistical significance was inferred when the P value was < 0.05.

RESULTS

The genotype frequencies of MTHFR in healthy subjects and asthmatic patients are listed in Table 1. The frequencies of the C677T polymorphism in healthy subjects were CC: CT: TT = 579 (40.5%): 681 (47.6%): 170 (11.9%). The frequency of the TT genotype was higher in both male and female asthmatic patients (16.9% vs. 11.0%, 16.0% vs. 13.6%, respectively) and the difference was significant for the male population (odds ratio = 1.65, 95% confidence interval: 1.12-2.44, P = 0.011). The allele frequency of the T mutation was significantly higher in male asthmatics than in male healthy controls (41.3% vs. 34.3%; odds ratio = 1.35, 95% confidence interval: 1.10-1.65, P = 0.004).

According to the criteria described in the METHODS section, 378 patients (82%) were classified as "atopic" asthmatics and 83 (18%) were "non-atopic" asthmatics. The distribution of the MTHFR C677T polymorphism was examined according to atopy (Table 2). For the female population, the frequencies of the TT genotype and the T677 alleles were higher in atopic asthmatics than those in non-atopic asthmatics (17.3% vs. 11.8%, 40.7% vs. 34.3%, respectively), but the differences were statistically insignificant.

Table 1. Genotype distribution of the MTHFR gene in healthy subjects and asthmatic patients

	MTHFR		
	CC	CT	TT
Healthy subjects			
Male (n = 939)	398 (42.4%)	438 (46.6%)	103 (11.0%)
Female (n = 491)	181 (36.9%)	243 (49.5%)	67 (13.6%)
Asthmatic patients			
Male (n = 248)	85 (34.3%)	121 (48.8%)	42 (16.9%)*
Female (n = 213)	80 (37.6%)	99 (46.5%)	34 (16.0%)

MTHFR, 5,10-methylenetetrahydrofolate reductase. Numbers in parentheses represent percentage of individuals.
*P = 0.011 vs. corresponding healthy subjects.

Table 2. Genotype distribution of the MTHFR gene in patients with atopic and non-atopic asthma

	MTHFR		
	CC	CT	TT
Atopic asthmatic patients			
Male (n = 216)	72 (33.3%)	107 (49.5%)	37 (17.1%)
Female (n = 162)	58 (35.8%)	76 (46.9%)	28 (17.3%)
Non-atopic asthmatic patients			
Male (n = 32)	13 (40.6%)	14 (43.8%)	5 (15.6%)
Female (n = 51)	22 (43.1%)	23 (45.1%)	6 (11.8%)

MTHFR, 5,10-methylenetetrahydrofolate reductase. Numbers in parentheses represent percentage of individuals.

DISCUSSION

Advances in asthma management are likely to depend on a better understanding of how genetic and environmental factors influence susceptibility to, and outcome in, this disease. The implication of the MTHFR C677T polymorphism in the pathogenesis of bronchial asthma is entirely novel. The MTHFR gene is located on chromosome 1 at 1p36.3. The complementary DNA sequence is 2.2 kilobases long and consists of 11 exons[6,7]. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine. In recent years, accumulating evidence suggests that mild to moderate hyperhomocysteinemia and its genetic promoter, MTHFR C677T mutation, may be associated with the development and progression of cardiovascular and inflammatory diseases[6-13].

Reactive oxygen species (ROS) are implicated in the cellular toxicity of hyperhomocysteinemia[4,5]. ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), are generated during oxidation of the sulfhydryl group of homocysteine. Excessive O_2^- inactivates nitric oxide (NO) with the formation of the adduct peroxynitrite ($ONOO^-$). These oxidants are thought to account for the homocysteine-induced cytotoxicity. Furthermore, homocysteine both inhibits glutathione peroxidase activity *in vitro* and leads to a marked reduction in mRNA levels for the intracellular isoform; the inhibition of glutathione peroxidase is unique to homocysteine compared with other biologic thiols. Homocysteine significantly decreases the intracellular thiol redox state, as measured by the ratio [glutathione]/[glutathione disulfide], through these oxidative mechanisms.

The reported frequency of homozygotes for the mutation, having the TT genotype, varies in different populations from one geographic area to another (ranging from 5 to 20%)[6,7]. Our results showed that among Japanese healthy subjects the frequency of the TT genotype was 11.9% (170/1430), which was comparable to that in Caucasians.

The present study is the first to demonstrate the association between the TT genotype and development of bronchial asthma in male subjects. The mechanism(s) of the predisposition of the TT genotype to asthma remains speculative. The contribution of oxidative stress induced by this genotype is one possibility. Oxidative stress has been implicated in the initiation and worsening of asthma[2,3]. Increased release of ROS has been reported in exhaled condensates[19] and from circulating neutrophils and monocytes, bronchoalveolar lavage cells of patients with asthma[20]. ROS can cause cellular damage by oxidizing membrane lipids, proteins and nucleic acids. Furthermore, oxidative stress can activate the transcription factor, nuclear factor kappa-B (NF-κB), and previous studies reported high expression of this factor in airway cells of patients with asthma[21,22]. Increased NF-κB expression and DNA binding may underlie the increased expression of several inflammatory proteins in the asthmatic airway, including tumor necrosis factor-α, interleukin (IL)-1β; RANTES, eotaxin, macrophage chemotactic protein-1, macrophage inflammatory protein-1α; granulocyte-macrophage colony stimulating factor; inducible NO synthase, inducible cyclooxygenase; and intercellular adhesion molecule-1, vascular cell adhesion molecule-1[21-25]. It seems reasonable to consider that these changes create feed-forward amplifying loops that could form the basis for the development and progression of the chronic inflammatory process in asthma and that the MTHFR TT genotype enhances these pathological events especially in male subjects.

It is of note that more males than females develop asthma during childhood, probably because of narrower air-

ways, increased airway tone and possibly higher IgE in boys[14]. For the male asthmatic patients, the frequency of the TT genotype in children was insignificantly lower than in adults (patients aged < 16 years: 15.3% [20/131] vs. patients aged ≥ 16 years: 18.8% [22/117]). In addition, the frequency of the TT genotype in atopic asthmatics was almost comparable to that in non-atopic asthmatics (17.1% vs. 15.6%) (Table 2).

It needs to be taken into account that some proportion of the healthy controls reported by other groups (n = 1091)[8,16,17] may have allergic diatheses including bronchial asthma. The prevalence of asthma in Japanese children and adults is reported to be 3%[26]. If these individuals of the above healthy controls (3% of the total of 1091 subjects) were assumed to have the same distribution of the C677T polymorphism as the patient group, the frequency of the TT genotype would remain higher in both male and female asthmatics than that of the corresponding "non-asthmatic" healthy controls (16.9% vs. 10.8%, 16.0% vs. 13.6%, respectively). Under this condition, the difference would remain significant for the male population (odds ratio = 1.68, 95% confidence interval: 1.14-2.47, P = 0.007).

Atopy is a significant predisposing factor for the development of asthma[14]. Certain genetic and environmental factors drive the development of a Th2 lymphocyte-predominant immune response, which is associated with atopy and IgE-mediated inflammation. Th2 lymphocytes generate cytokines, including IL-4, IL-5 and IL-13, which play a primary role in B cell switching to IgE synthesis. In contrast, Th1 lymphocytes, which play a primary role in interferon-γ production, inhibit B-cell IgE synthesis. Th1- and Th2-type cytokines reciprocally regulate the Th1/Th2 immune responses. Recent *in vitro* studies have suggested that oxidative environments may act differentially on activated human Th cells by inhibiting Th1 cytokine production but promoting the expression of Th2 cytokines; thiol antioxidants such as N-acetyl-L-cysteine and glutathione decrease IL-4 production in human T cells and IgE production by B cells[27,28]. Moreover, glutathione depletion in antigen presenting cells inhibits Th1 cytokines and/or favors Th2 responses[29]. In studies using human B cell lines, Yanagihara *et al.*[30] showed that N-acetyl-L-cysteine regulates the IgE isotype switching by inhibiting the activation of NF-κB. It was expected that altered redox status induced by the TT genotype may drive the development of the Th2-predominant immune responses to environmental stimuli, which is associated with atopy and allergic inflammation in the airway. For the female population, the frequency of the TT genotype in atopic asthmatics was higher than in non-atopic asthmatics, although the difference was statistically insignificant. Since the number of non-atopic asthmatics was rather small, further studies using larger population samples may be required to confirm this contention.

In conclusion, our results suggest that the MTHFR TT genotype is a probable genetic risk factor for the development of bronchial asthma, possibly through the oxidative mechanism, in male Japanese subjects. Although further investigations are required to clarify the molecular mechanisms governing whether a Th1 or Th2-immune response predominates in the hyperhomocysteinemic state, the findings presented here should provide new insights into the pathophysiology of bronchial asthma. The homocysteine-lowering effects of folate and vitamins B_6 and B_{12} have been anticipated for individuals carrying the TT genotype[7]. The other relevant subject of exploration may be the investigation of the possible favorable effects of these vitamins on the development and worsening of asthma.

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Review

The Role of Interleukin-4 and Interleukin-13 in the Non-Immunologic Aspects of Asthma Pathogenesis[1)]

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Bronchial asthma is a complex disease characterized by airway inflammation involving a Th2-cytokine, interleukin (IL)-13. A substantial body of evidence has accumulated pointing to the pivotal role of IL-13 in the pathogenesis of bronchial asthma. The evidence is categorized as (i) analyses of mouse models, (ii) expression of these cytokines in the bronchial lesions, and (iii) genetic association of the signaling molecules of these cytokines. In addition, the molecular mechanism of the signal transduction of IL-13 has also been well characterized. We have applied microarray analyses to human bronchial epithelial cultures to search for genes regulated by IL-13 and have identified a subset of disease-relevant genes by comparison with cDNA libraries derived from normal and asthmatic bronchial biopsies. Expression of squamous cell carcinoma antigen-1 (SCCA1) and SCCA2, the cysteine and serine protease inhibitors, respectively, was the highest in the bronchial epithelial cells stimulated by IL-4 and IL-13 and was augmented in the asthmatic cDNA library. Furthermore, serum levels of SCCA were also elevated in asthmatic patients. Taken together, it was supposed that SCCA may play some role in the pathogenesis of bronchial asthma, and measuring its serum level may be relevant for diagnosing or monitoring the status of bronchial asthma. Clin Chem Lab Med 2003; 41(7):860–864

Key words: Bronchial asthma; Allergy; Interleukin-13; Receptor; Squamous cell carcinoma antigen.

Abbreviations: AHR, airway hyperresponsiveness; BECs, bronchial epithelial cells; Ig, immunoglobulin; IL, interleukin; IL-4Rα; the IL-4 receptor α chain; IL-13Rα1, the IL-13 receptor α1 chain; IL-13Rα2, the IL-13 receptor α2 chain; serpin, serine proteinase inhibitor; SCCA, squamous cell carcinoma antigen; SNP, single nuclear polymorphism.

Introduction

The incidence of allergic diseases has dramatically increased in recent decades, especially in urban and industrialized areas. It has been reported that, at present, up to half of the population in Japan suffers from bronchial asthma, atopic dermatitis, or allergic rhinitis (1). The medical cost for treating such patients is huge and on the increase. Thus, it is important socially as well as medically to establish more useful strategies to overcome allergic disorders.

Bronchial asthma is a complex disorder involving a combination of genetic and environmental factors (2). Together, these result in infiltration of Th-2 lymphocytes, mast cells, and eosinophils into asthmatic airways with downstream mediator release and disordered airway function. Cytokines derived from the Th-2 lymphocytes are considered to orchestrate the asthmatic phenotype, irrespective of atopy (2).

Although among Th-2 cytokines, paradigmatic ones, interleukin (IL)-4 and IL-5, had been thought to be the primary regulators of bronchial asthma, it has been recently suggested that IL-13 is a central mediator of bronchial asthma. In this Review, we summarize the pathophysiological roles of IL-13 in bronchial asthma and the trials to clarify the molecular mechanism for IL-13 to induce bronchial asthma.

1. Significance of IL-13 in the Pathogenesis of Bronchial Asthma

Key roles for IL-13 in the pathogenesis of bronchial asthma are supported mainly based on analyses of mouse models; however, some evidence to prove that IL-13 is also involved in the pathogenesis of human asthma patients has been demonstrated.

1.1 Analyses of mouse models

Analyses of mice null for components of the IL-13 signal transduction pathways, including IL-13, the IL-4 receptor α chain (IL-4Rα), and STAT6, have revealed that IL-13, as well as IL-4, plays a pivotal role in induction of airway hyperresponsiveness (AHR), a characteristic feature of asthma (3–5). Furthermore, epithelial overexpression of an IL-13 transgene or administration of IL-13 in mice have shown that IL-13 induces an asthma-like phenotype independent of lymphocytes (6–8). It is of importance to note that these analyses indicated that IL-13 acts directly on non-immune cells in bronchial tissue, evoking the phenotypic changes. Actually,

[1)] Based on a presentation at the 18th International Congress of Clinical Chemistry and Laboratory Medicine, Kyoto, Japan, 20–25 October 2002.

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it has been very recently reported that reconstitution of STAT6 only in bronchial epithelial cells into STAT6-disrupted mice restores IL-13-induced AHR and mucous production, but not inflammation or fibrosis (9), showing the importance of IL-13's direct action on bronchial epithelial cells (BECs).

1.2 Expression of IL-13 in the bronchial lesions

Expression of IL-13 is higher at the baseline and greatly up-regulated by allergen challenge in bronchial tissues or bronchoalveolar lavage fluids derived from asthma patients (10, 11). Thus, expression of IL-13 is predominant, compared to IL-4. It is well-known that IL-4 and IL-13 have common activities (12); however, these results suggest that the biological activities of IL-4 and IL-13 are skewed toward immune cells and peripheral tissues, respectively, *in vivo*.

1.3 Genetic association of the IL-13 signaling molecules

Genetic predisposition to allergic diseases is thought to be polygenic and the genetic factors are thought to exist among polymorphisms (13). Extensive efforts have been made to identify such factors by genome-wide search and candidate-gene study (14). Based on candidate-gene study, it has been reported that several polymorphisms of genes encoding IL-13 signaling molecules such as *IL13, IL4RA, IL13RA1, STAT6,* and *BCL6* are genetically associated with asthma or atopy (15). Among the single nuclear polymorphisms (SNPs) that are demonstrated to be genetically associated with bronchial asthma or atopy, we have analyzed the functional properties of variants of *IL4RA* (Ile50Val) and *IL13* (Gln110Arg). The variant of IL-4Rα, whose amino acid at 50 is isoleucine, up-regulates receptor responses to IL-4, leading to increased STAT6 activation and immunoglobulin E (IgE) synthesis, compared to the valine type (16, 17). It remains undetermined whether this SNP affects the IL-13 signal. The variant of IL-13 whose amino acid at 110 is glutamine has less affinity with the IL-13 receptor α2 chain (IL-13Rα2), a decoy receptor, and an enhanced stability, compared to the arginine type, causing up-regulation of the IL-13 concentration *in vivo* (Figure 1) (18, 19).

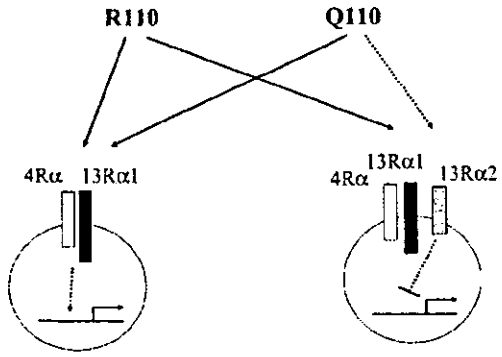

Figure 1 Association between the SNP on the *IL13* gene (Arg110Gln, R110Q) and the IL-13 receptors. Q110 and R110 have the same affinity with the heterodimer composed of IL-13Rα1/IL-4Rα, whereas Q110 has lower affinity with IL-13Rα2 than R110, transducing stronger IL-13 signal.

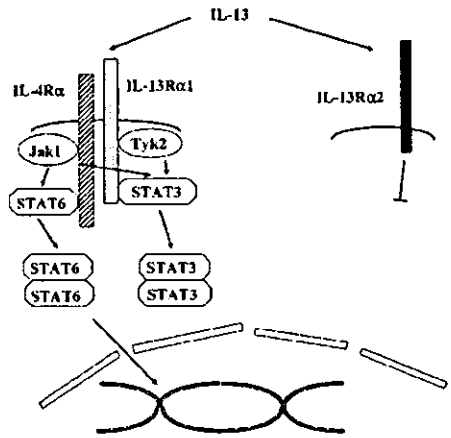

Figure 2 The receptor structure and signal transduction mechanism of IL-13. IL-13 binds to either IL-13R, composed of IL-13Rα1 and IL-4Rα, or IL-13Rα2. Engagement of IL-13R causes signal transduction, mainly through the JAK-STAT pathway.

2. Structure of the IL-13 Receptor and Signal Transduction Mechanism of IL-13

IL-13 exerts its biological activities by binding to its receptor on the cell surface as well as other cytokines (Figure 2). IL-13R is composed of two components, IL-4Rα and the IL-13R α1 chain (IL-13Rα1) (20–23). IL-4 can also bind to this receptor, denoted as type II IL-4R (12, 24). There exists another IL-13-binding unit, IL-13Rα2 (25, 26). As the cytoplasmic domain of IL-13Rα2 is short, IL-13Rα2 is thought not to transduce the IL-13 signal but to block it, acting as a decoy receptor. Actually, we have recently observed that IL-13 completely diminished STAT6 phosphorylation in the IL-13Rα2-expressing BEAS-2B cells, a human bronchial epithelial cell line, whereas IL-4 did not influence it, supporting this idea (Yasunaga S, *et al.*, unpublished data).

Upon engagement of the receptor by IL-13, its signal is transduced intracellularly mainly *via* the JAK-STAT and the phosphatidylinositol-3 kinase/insulin receptor substrate-1/2 pathways (12). The JAK-STAT pathway activated by IL-13 corresponds to activation of JAK1 and TYK2 followed by activation of STAT6, a critical transcription factor for IL-13 to exert its biological activities, and STAT3 (23, 27); however, thus far, the biological role of STAT3 in the IL-4 and IL-13 signals remains to be clarified.

3. Tissue Distribution of the Receptors of IL-13

In hematopoietic and immune cells, B cells and monocytes are the main target cells for IL-13 (28). Although IL-13Rα1 is not expressed on the cell surface at the rest-

ing stage of B cells, activation by anti-IgM antibodies and anti-CD40 antibodies enhances expression of IL-13Rα1, which enabled the cells to respond to IL-13, causing class switching to IgE and CD23 expression (23, 29). Thus, the IL-13 signal is regulated by expression of IL-13Rα1 in B cells. In contrast, we revealed that IL-13Rα1 is widely expressed in non-hematopoietic cells, including human BECs (18, 30). It has been reported that fibroblasts and smooth muscle cells, in addition to BECs, express the functional IL-13R, so that IL-13 induces production of eotaxin, C3, and β1-integrin in these cells (31–35).

Tissue distribution and expression regulation of IL-13Rα2 had remained obscure. It has been confirmed that immune cells do not express IL-13Rα2 (29). We have recently observed that either IL-4 or IL-13 induced expression of IL-13Rα2, but not of IL-13Rα1 or IL-4Rα in BECs (Yasunaga S *et al.*, unpublished data). Induction of IL-13Rα2 by IL-4 or IL-13 was STAT6-dependent, although it required *de novo* protein synthesis. Furthermore, IL-13Rα2 was expressed in lung tissues of ovalbumin-induced asthma model mice, in which both IL-4 and IL-13 were highly expressed. These results suggested that IL-13Rα2 is induced by its ligand, down-regulating the IL-13 signal in BECs, which functions as a negative-feedback system for the cytokine signal (Figure 3).

4. Identification of IL-13-Inducible Genes in Human BECs

To study potential downstream consequences of IL-13 on human BECs, we first employed microarrays (HuGeneFL Array, Affymetrix, Santa Clara, CA, USA) to identify genes induced by IL-13 in human BECs (36). Human BECs derived from three different donors were exposed to IL-13 for 24 h and subjected to microarray analysis. We limited target identification to those genes whose expression was up-regulated > 2-fold by IL-13. Of the 5600 annotated genes present on the arrays, IL-13 affected 47, 63, and 49 genes. Expression of 12 genes was up-regulated also by IL-4 (Table 1). Among these genes, two members of the serine proteinase inhibitor (serpin) family of protease inhibitors, squamous cell carcinoma antigen-1 (SCCA1) (SERPINB3) and SCCA2 (SERPINB4), showed the highest increase (~20-fold). No other investigated cytokine, including IL-5, IL-9, tumor necrosis factor-α (TNF-α), and IL-1β, induced expression of these two genes.

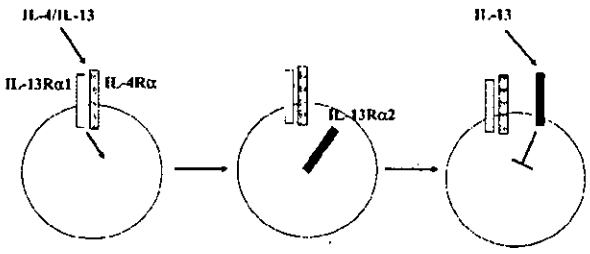

Figure 3 The negative feedback system of IL-13 signal in bronchial epithelial cells. Either IL-4 or IL-13 induces IL-13Rα2 in bronchial epithelial cells, which self-limits IL-13 signal.

Table 1 Identified IL-4/IL-13-inducing genes.

SCCA1*
SCCA2*
DD96 (MAP17)*
KAL1*
Periostin
Tenascin-C
Carboxypeptidase M
IL-13Rα2
Cathepsin C
CYP1B1
Carbonic anhydrase II
Endothelin-A receptor

*Up-regulated in bronchial tissues of asthma patients.

To verify induction of expression of SCCA1 and SCCA2, we used quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (36). While the fold change varied between individual cultures, increased expression of SCCA1 and SCCA2 was confirmed. Kinetic analysis showed that the expression profile of SCCA1 and SCCA2 was augmented within 6 h of stimulation by IL-13 and continued to be enhanced for up to 48 h.

Davies and co-workers (36) analyzed the expression profiles of cDNA libraries constructed from atopic normal or asthma-derived bronchial biopsies using LifeSeq™ Gold from Incyte Genomics Inc. (Palo Alto, CA, USA). Comparison of the 12 differentially identified genes induced by both IL-4 and IL-13 (Table 1) with sequence data from these cDNA libraries revealed that four of the genes, *SCCA1/2*, *KAL-1*, and *DD96* were represented in at least one of the two libraries. The number of SCCA-related clones derived from SCCA was significantly higher in bronchial tissues of asthma patients than those of normal donors (11 clones *vs.* 3 clones; $p < 0.005$). One clone derived from *KAL-1* and one from *DD96* existed in the atopic asthmatic library but none were detected in the atopic, non-asthmatic library.

5. High Serum Levels of SCCA in Asthma Patients

SCCA1 and SCCA2 are members of the ovalbumin-serpin (ova-serpin) proteinase inhibitor family (37). They are 92% identical at the amino acid level and have probably arisen by gene duplication. SCCA was originally isolated from human cervical squamous carcinoma cells and is produced by various squamous tumors, as well as normal bronchial epithelium (38). Thus far, no normal cell except BECs is known to produce SCCA1 and SCCA2. It has recently been revealed that SCCA1 and SCCA2 have distinct properties and substrates: SCCA1 inhibits cysteine proteinases such as cathepsins K, L, S, whereas SCCA2 inhibits serine proteinases including cathepsin G and mast cell chymase (37). At present, it remains to be determined

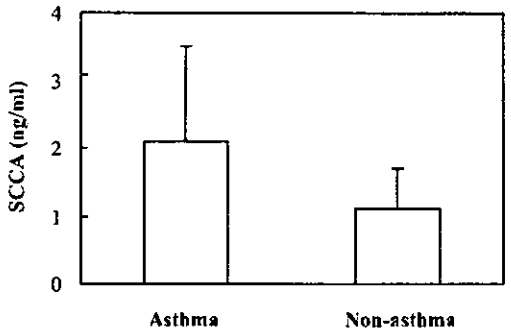

Figure 4 Serum levels of SCCA in asthma patients. Serum levels of SCCA in asthmatic and non-asthmatic children are shown. The medians are depicted as bars.

whether SCCA1 and SCCA2 are acting as protective or exacerbating molecules to modify the asthmatic phenotype by inhibiting these intrinsic proteinases. Alternatively, SCCA1 and SCCA2 may act on extrinsic proteinases derived from mites or parasites, because it is known that mite- or parasite-derived cysteine proteinases have an important role for their responsiveness or virulence (39–41). Further studies are now underway aiming to clarify this point.

While most serpins are secreted proteins that work extracellularly, the ov-serpins also function as intracellular proteinase inhibitors (37). We next analyzed the localization of SCCA proteins in human BECs. Immunocytochemical analysis using an antibody that recognizes both SCCA1 and SCCA2 (mAb27, Dainabot, Tokyo, Japan) showed positive staining only when cells were cultured in the presence of IL-4 or IL-13. Kinetic analysis of cellular extracts and supernatants by ELISA (IMx, Dainabot) showed that intracellular expression of SCCA protein increased within 24 h of IL-4 or IL-13 stimulation and was slightly augmented thereafter. SCCA secretion was not detected at 24 h but was observed 48 h after stimulation and reached a plateau thereafter. These results suggest that SCCA1 and SCCA2 could act both intracellularly and extracellularly in BECs.

It has been reported that secreted SCCA can be detected in serum of certain cancer patients (42) and that increased serum levels of SCCA are also observed in children suffering from atopic dermatitis (with or without asthma), with levels correlating with disease severity but not with serum IgE or eosinophils (43). Based on these observations, we explored the possibility that expression of SCCA in bronchial tissue of asthmatic patients was also reflected in the circulation. Assessment of serum concentrations of SCCA in asthmatic and non-asthmatic children by ELISA showed that levels were significantly higher in the asthmatic subjects (2.1 (1.4–3.3) ng/ml, median (interquartile range), n = 32) than in control, non-asthmatic subjects (1.1 (0.6–1.9) ng/ml, n = 23, $p < 0.005$; Figure 4). In a small group of patients (n = 8) who were studied during, and 4–5 days after, an excerbation, SCCA levels were found to be higher during the attack phase (2.4 (1.7–3.8) ng/ml) than during recovery (1.4 (0.9–1.7) ng/ml), although this failed to reach statistical significance ($p = 0.09$). These results suggest that measuring the serum level of SCCA may be relevant for diagnosing or monitoring the status of bronchial asthma.

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