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Original Article

Urinary leukotriene E_4 and 11-dehydro-thromboxane B_2 excretion in children with bronchial asthma

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Abstract

Background: Cysteinyl leukotrienes (CysLTs) and thromboxane (TX) A_2 have been implicated in the pathogenesis of bronchial asthma. Urinary leukotriene E_4 (LTE_4) and 11-dehydro-TXB_2 ($11DTXB_2$) levels are often used to assess the production of CysLTs and TXA_2. However, few studies have examined the products of these two mediators in the same asthmatic patients. To define the potential roles of CysLTs and TXA_2 in the pathogenesis of bronchial asthma in children, their urinary levels were measured in the present study.

Methods: Urinary LTE_4 and $11DTXB_2$ levels were measured by enzyme immunoassay (EIA) and radioimmunoassay (RIA), respectively. Urine samples from asthmatic children were measured during the stable condition and during an acute attack.

Results: Urinary LTE_4 levels during an acute attack (median 476 pg/mg creatinine; range 191–1100 pg/mg creatinine) and during the stable condition (median 332 pg/mg creatinine; range 128–965 pg/mg creatinine) were significantly higher ($P < 0.05$) than those of controls (median 233 pg/mg creatinine; range 103–389 pg/mg creatinine). Urinary $11DTXB_2$ levels during an acute attack and during the stable condition (median 1666 (range 110–5105) and 1009 (range 46–6070) pg/mg creatinine, respectively) were significantly higher ($P < 0.05$) than those of controls (median 252 pg/mg creatinine; range 41–716 pg/mg creatinine). Comparing different stages of asthma, LTE_4 levels during an acute attack were significantly higher ($P < 0.05$) than during the stable condition; however, there was no difference in urinary TXB_2 levels.

Conclusions: The present findings suggest that high levels of CysLTs and TXA_2 are associated with the pathogenesis of bronchial asthma. The measurement of urinary LTE_4 and $11DTXB_2$ would be useful in understanding the individual pathogenesis of asthmatic children.

Key words: bronchial asthma, cysteinyl leukotrienes, 11-dehydro-thromboxane B_2, leukotriene E_4, thromboxane A_2.

Introduction

Cysteinyl leukotrienes (CysLTs), namely leukotrienes C_4, D_4 and E_4, and thromboxane (TX) A_2 are considered to play important roles in bronchial asthma.[1–10] Cysteinyl leukotrienes are derived from arachidonic acid by the action of 5-lipoxygenase and increase vascular permeability, stimulate mucus secretion and induce bronchial hyperreactiveness and bronchoconstriction. Moreover, increased production of CysLTs in asthmatic patients *in vivo* has been observed in several studies.[1–5,11,12] A potent bronchoconstrictor, TXA_2 is generated from arachidonic acid by cyclooxygenase. Enhanced TXA_2 release has also been reported in asthmatic patients after allergen challenge.[7] Owing to the significant roles of CysLTs and TXA_2, their inhibitors or receptor antagonists have been developed extensively and recently some drugs have become available.[13]

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Although these mediators of asthma have been discussed previously, few studies have examined the products of these two mediators in the same asthmatic patients. In addition, few studies have compared the TXA_2 products of asthmatic patients with those of healthy control subjects. Leukotriene E_4 is a stable product of CysLTs and is considered an index of the systemic production of CysLTs production in humans.[14] 11-Dehydro-thromboxane B_2 ($11DTXB_2$) is the most abundant degradation product of TXB_2 and is also considered an index of systemic TXA_2 production.[15–17] The measurement of urinary LTE_4 and $11DTXB_2$ is a non-invasive method for assessing the production of CysLTs and TXA_2 *in vivo*, respectively. Oosaki *et al.*[18] previously established sensitive and selective methods of determination of urinary LTE_4 by enzyme immunoassay (EIA) and Ruitta *et al.*[16] established the method of determining urinary $11DTXB_2$ by radioimmunoassay (RIA). In the present study, using these methods, the products of CysLTs and TXA_2 were assessed *in vivo* in control subjects and in children with bronchial asthma during the stable condition and during an acute attack.

METHODS

Subjects

Twenty-nine children with bronchial asthma (19 males, 10 females) and nine control subjects (six males, three females) were enrolled in the study (Table 1). The mean age of asthmatic children and control subjects was 7 years (range 1–15 years) and 8 years (range 1–15 years), respectively. None of the patients had a history of aspirin sensitivity. Twenty-six of the asthmatic patients were receiving theophylline, all were using inhaled disodium cromoglycate (DSCG) and β_2-adrenergic receptor agonists, 10 were receiving inhaled corticosteroids and 17 were using a leukotriene receptor antagonist (LTRA). All patients were classified into one of four categories (intermittent, mild persistent, moderate persistent and severe persistent) according to Glolabal Initiative for Asthma (GINA) guidelines (http://www/ginasthma.com/ Table 1). None of the patients had been treated previously with oral prednisolone (PSL) prior to their enrollment in the study. In the present study, the urinary excretion of LTE_4 or $11DTXB_2$ was not influenced by the usage of LTRA or corticosteroids, because the patients kept the treatments unmodified throughout the duration of the study. Informed consent to participate in the study was obtained from all subjects or their parents.

Urine samples from children with bronchial asthma were measured during the stable condition and during an acute attack. Urine samples from nine asthmatic children selected at random (Table 1, patients 1–9) were also measured 2 days after treatment. The 'stable condition' refers to the condition in which the patients did not complain of any symptoms with or without receiving their usual medications. The 'acute attack' refers to a condition in which the patients complained of some active symptoms, cough and/or wheezing and/or chest tightness, which were occurring repeatedly on waking and/or disturbing sleep at night; therefore, they needed additional treatment to their usual treatments.[19] Patients were treated with steroid and/or theophylline by injection and/or inhaled β_2-adrenergic receptor agonists and, 2 days after treatment, they felt better but were still complaining slightly of some asthmatic symptoms, such as cough and/or wheezing and/or chest tightness.

Urine samples were collected when the asthmatic children visited our hospitals during the stable condition maintaining their usual treatments. Urine samples from asthmatic children were also collected on arrival at hospital when they had acute asthma attacks. Patients were treated with theophylline and/or β_2-adrenergic receptor agonists and/or corticosteroid drip infusion. Urine samples from nine asthmatic children were collected 2 days after treatment.

Measurement of LTE_4

Urine samples were stored at –80°C and analyzed within 1 month of collection. An aliquot of urine was removed to determine creatinine concentration. The urinary creatinine level was determined using a Creatinine test kit (Pure Auto S CRE-L; Daiichi-kagaku, Tokyo, Japan).

Approximately 3000 d.p.m. [^{3}H]-LTE_4 was added to each urine sample as an internal standard and the urine was applied to a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) that had been preconditioned by the serial addition of methanol and distilled water. Then, the cartridge was washed with distilled water, followed by 40% methanol; LTE_4 was eluted with 80% methanol. This elution was dried with nitrogen gas and was dissolved in the elution buffer used in high-performance liquid chromatography (HPLC; 486 Tunable Absorbance Detector; Waters) and the solution was injected onto a C18 reverse-phase column (CAPPCELL PAC UG 120; Shiseido, Tokyo, Japan). The fractions that contained peak [^{3}H]-LTE_4 radioactivity and also corresponded to the

Table 1 Characteristics of patients and urinary levels of leukotriene E_4 and 11-dehydro-thromboxane B_2

Patient no.	Gender	Age (years)	Serum IgE (U/mL)	HD score	Mite score	Severity (GINA)	Steroid treatment during attack	Theophylline	LTRA	DSCG	β_2-Adrenergic receptor agonist	Steroid inhalant	Stable condition LTE_4	Stable condition $11DTXB_2$	Acute attack LTE_4	Acute attack $11DTXB_2$	After treatment LTE_4	After treatment $11DTXB_2$
1	M	7	1784	5	6	Moderate	+	+	–	+	+	–	183	861	671	1285	156	2130
2	M	5	200	4	5	Mild	+	+	–	+	+	–	212	2166	611	1188	295	820
3	M	14	900	6	6	Moderate	+	–	–	+	+	+	482	745	1100	563	443	785
4	M	1	21	0	0	Severe	+	+	+	+	+	–	373	282	280	3122	524	2708
5	M	9	70	0	0	Moderate	+	+	+	+	+	–	480	1059	546	2096	771	2137
6	F	10	199	2	2	Moderate	+	+	+	+	+	–	253	1515	437	2247	231	842
7	F	5	82	6	6	Mild	+	+	–	+	+	–	188	2150	409	589	340	492
8	F	9	294	4	4	Severe	+	+	–	+	+	+	484	131	797	166	473	533
9	F	7	1281	4	4	Moderate	+	+	+	+	+	–	556	1009	483	2087	872	1515
10	M	10	653	2	3	Moderate	+	+	+	+	+	+	378	904	615	1847		
11	M	4	208	4	5	Moderate	+	+	+	+	+	–	341	1842	468	3254		
12	M	5	2892	4	4	Moderate	+	+	+	+	+	+	274	163	553	2461		
13	M	3	401	5	5	Moderate	+	+	+	+	+	–	519	2462	567	2352		
14	M	9	265	5	5	Mild	+	+	+	+	+	–	254	1476	369	2499		
15	M	3	180	5	6	Moderate	+	+	+	+	+	+	332	1392	424	480		
16	M	3	1381	3	4	Moderate	+	+	–	+	+	+	606	167	191	379		
17	M	9	890	2	1	Mild	+	+	+	+	+	–	165	873	269	1259		
18	M	9	703	6	5	Severe	+	+	+	+	+	+	278	804	334	939		
19	M	2	1417	6	6	Moderate	+	+	+	+	+	–	755	3535	743	4075		
20	M	9	578	5	6	Mild	+	+	+	+	+	–	286	793	284	1700		
21	M	8	62	2	0	Intermittent	+	–	–	+	+	–	365	1992	371	258		
22	M	4	334	3	3	Moderate	+	+	+	+	+	+	328	3483	394	2548		
23	M	2	394	5	6	Mild	+	+	+	+	+	–	610	6070	980	5104		
24	F	15	301	0	0	Moderate	+	+	–	+	+	+	284	46	1100	311		
25	F	5	866	6	6	Intermittent	–	–	–	+	+	–	727	196	245	110		
26	F	7	1300	5	6	Mild	+	+	–	+	+	–	259	1837	476	2413		
27	F	7	1000	2	1	Mild	–	+	–	+	+	–	128	903	289	1318		
28	F	13	597	5	5	Mild	–	+	–	+	+	–	332	1026	504	1666		
29	F	3	600	6	6	Moderate	+	+	+	+	+	+	965	261	528	429		
Median			577.5										332	1009	476	1666	443	842
Average		7	684.5	4	4								393.0	1384.2	518.6	1680.9	456.1	1329.2

GINA, Global Initiative for Asthma (GINA) guideline (http://www.ginasthma.com/).

HD, house dust; LTRA, leukotriene receptor antagonist; DSCG, disodium cromoglycate; LTE_4, leukotriene E_4; $11DTBX_2$, 11-dehydro-thromboxane B_2.

retention time of authentic LTE_4 were dried and resuspended in assay buffer, which was supplied in the Leukotriene C4/D4/E4 enzyme-immunoassay system (Amersham, Buckinghamshire, UK). Urinary LTE_4 concentrations determined by EIA were corrected for recovery of [^{3}H]-LTE_4. The urinary LTE_4 level was expressed as pg/mg creatinine.

Measurement of 11DTXB$_2$

The 11DTXB$_2$ was extracted from an acidified sample by adding an equal volume of octadecylsilyl silica powder (ODS) suspension (80 mg/mL in 40% ethanol) followed by mixing, centrifuging (at 2000 g for 3 min at room temperature) and either decanting or aspirating. The pellet was washed with an acidic alcohol solution and then with petroleum ether for deproteinizing and defatting. The 11DTXB$_2$ was eluted by ethyl acetate. The pooled ethyl acetate was evaporated to dryness with nitrogen gas. The dried residue, containing 11DTXB$_2$, was dissolved in the eluent (acetonitril : chloroform : acetic acid, 10 : 90 : 0.5, v/v/v) and applied to the open silica mini column (Bond Elute SI; VARIAN, Palo Alto, CA, USA). The column was washed with the eluent (acetonitril : chloroform : acetic acid, 20 : 80 : 0.5, v/v/v). The elution buffer, containing the 11DTXB$_2$, was dried with nitrogen gas and the amount of 11DTXB$_2$ was quantitated by RIA (11-Dehydrothromboxane B2 [^{125}I] RIA kit; Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). The urinary 11DTXB$_2$ level was also expressed as pg/mg creatinine.

Statistical analyses

The Mann–Whitney unpaired *U*-test was used to compare controls and asthmatic children during the stable condition and the Wilcoxon paired test was used to compare asthmatic children during the stable condition and during an acute attack. Correlation was analyzed by Pearson correlation analysis. The percentage of changes was calculated using the following equation: % change = (level during stable condition – level during attack) × 100/level during attack. Data are expressed as the median (range) and $P < 0.05$ was considered significant.

RESULTS

Urinary LTE_4 and 11DTXB$_2$ levels

Urinary levels of LTE_4 and 11DTXB$_2$ were measured to define the potential roles of CysLTs and TXA_2 in children with bronchial asthma.

Leukotriene E_4 was measured by EIA. Urinary LTE_4 levels are plotted in Fig. 1. Urinary LTE_4 levels in asthmatic children during the stable condition (332 (128–965) pg/mg creatinine) was significantly higher ($P < 0.05$) than that of control subjects (233 (103–389) pg/mg creatinine; Fig. 1a). Comparing the different conditions of asthma, LTE_4 levels during an acute attack (476 (191–1100) pg/mg creatinine) were significantly higher ($P < 0.05$) than those during the stable condition (Fig. 1b).

11-Dehydro-thromboxane $B_2$was measured by RIA and was detectable in all urine samples. Urinary 11DTXB$_2$ levels are shown in Fig. 2. Urinary 11DTXB$_2$ levels in asthmatic children during the stable condition (1009 (46–6070) pg/mg creatinine) were significantly higher ($P < 0.05$) than those of control subjects (252 (41–716) pg/mg creatinine; Fig. 2a). However, there was no significant difference in 11DTXB$_2$ levels during an acute attack (1666 (110–5105) pg/mg creatinine) and during the stable condition (Fig. 2b).

Urinary levels of LTE_4 and 11DTXB$_2$ were observed during the stable condition, an acute attack and 2 days after treatment in nine asthmatic children (Fig. 3). Urinary LTE_4 increased from 373 pg/mg creatinine (range 183–556 pg/mg creatinine) during the stable condition to 546 pg/mg creatinine (range 280–1100 pg/mg creatinine) during an acute asthma attack and then decreased to 443 pg/mg creatinine (range 156–872 pg/mg creatinine) 2 days after treatment (Fig. 3a). In contrast, urinary 11DTXB$_2$ levels exhibited different patterns after an attack. Urinary 11DTXB$_2$ levels increased from 1009 pg/mg creatinine (range 131–2106 pg/mg creatinine) during the stable condition to 1285 pg/mg creatinine (range 166–3122 pg/mg creatinine) during an acute asthma attack and gradually decreased to 842 pg/mg creatinine (range 492–2708 pg/mg creatinine) 2 days after treatment. However, each patient showed variable levels of urinary 11DTXB$_2$ 2 days after treatment (Fig. 3b).

Correlations between urinary LTE_4 and 11DTXB$_2$

We assessed the relationship between LTE_4 and 11DTXB$_2$ in children with bronchial asthma (Fig. 4). No relationship was noted between these prostanoids in children with bronchial asthma or in the controls. In plots of changes from levels observed during an attack to

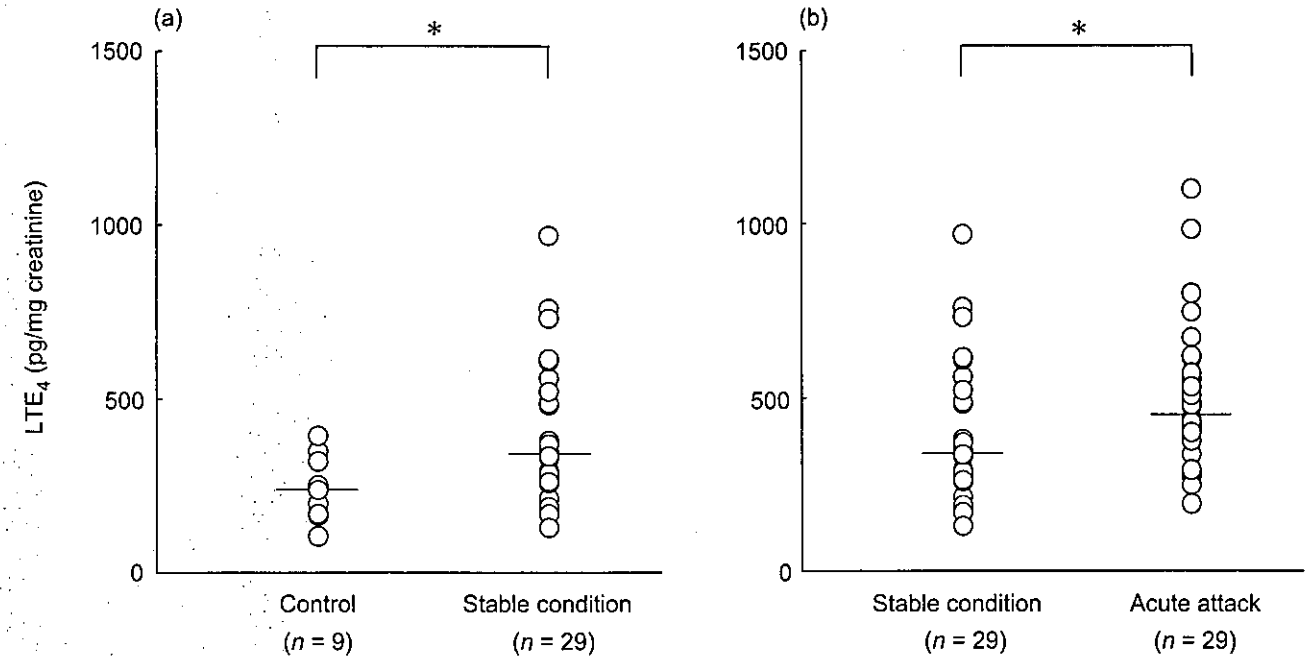

Fig. 1 (a) Urinary leukotriene E_4 (LTE_4) levels in asthmatic children during the stable condition (median 332 pg/mg creatinine; range 128–965 pg/mg creatinine) and in controls (median 233 pg/mg creatinine; range 103–389 pg/mg creatinine). (b) Urinary LTE_4 levels in asthmatic children during an acute asthma attack (median 476 pg/mg creatinine; range 191–1100 pg/mg creatinine) and during the stable condition (median 332 pg/mg creatinine; range 128–965 pg/mg creatinine). Horizontal bars indicate median values. *$P < 0.05$.

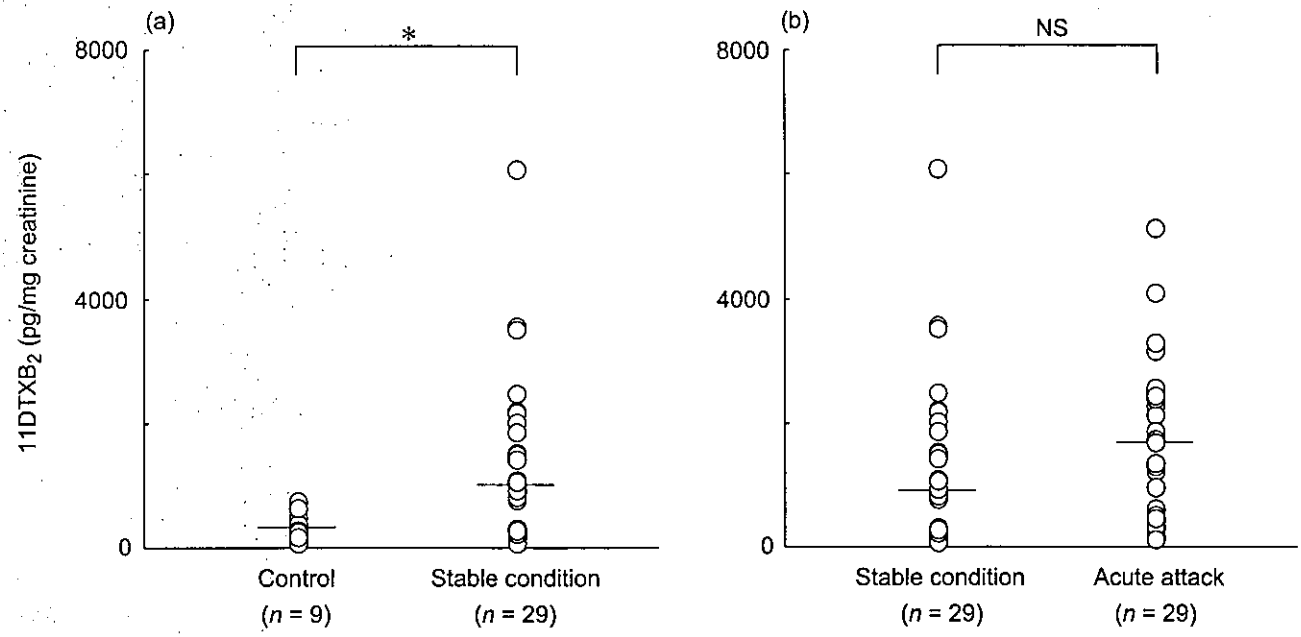

Fig. 2 (a) Urinary 11-dehydro-thromboxane B_2 (11DTXB$_2$) levels in asthmatic children during the stable condition (median 1009 pg/mg creatinine; range 46–6070 pg/mg creatinine) and in controls (median 252 pg/mg creatinine; range 41–716 pg/mg creatinine). (b) Urinary 11DTXB$_2$ levels in asthmatic children during an acute asthma attack (median 1666 pg/mg creatinine; range 110–5105 pg/mg creatinine) and during the stable condition (median 1009 pg/mg creatinine; range 46–6070 pg/mg creatinine). Horizontal bars indicate median values. *$P < 0.05$.

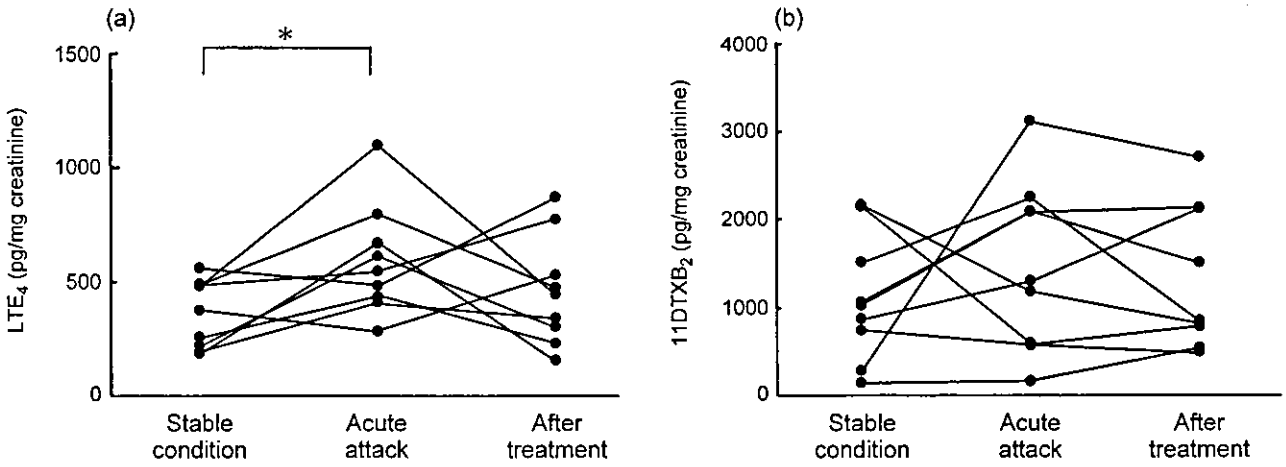

Fig. 3 Urinary leukotriene E_4 (LTE_4) and 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels in nine children with bronchial asthma during the stable condition, an acute asthma attack and 2 days after treatment. (a) Urinary LTE_4 levels increased from a median of 373 pg/mg creatinine (range 183–556 pg/mg creatinine) during the stable condition to 546 pg/mg creatinine (range 280–1100 pg/mg creatinine) during an acute asthma attack, decreasing again to 443 pg/mg creatinine (range 156–872 pg/mg creatinine) 2 days after treatment. (b) Urinary $11DTXB_2$ levels were apt to increase from a median of 1009 pg/mg creatinine (range 131–2166 pg/mg creatinine) during the stable condition to 1285 pg/mg creatinine (range 166–3122 pg/mg creatinine) during an acute asthma attack and then decrease slowly to 842 pg/mg creatinine (range 492–2708 pg/mg creatinine) 2 days after treatment. *$P < 0.05$.

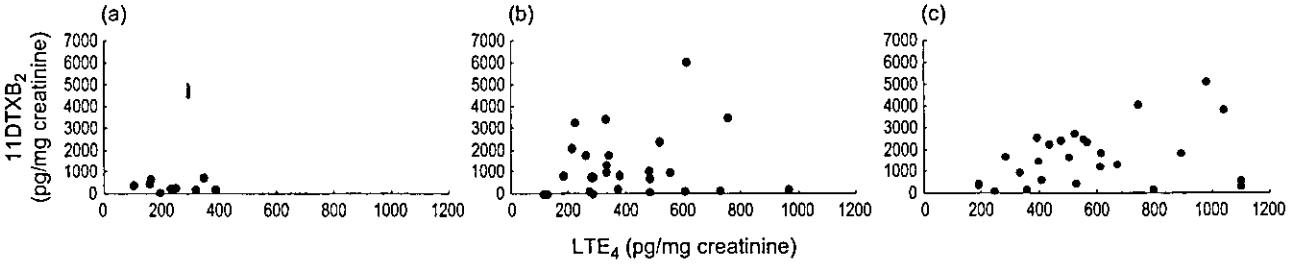

Fig. 4 Relationship between urinary leukotriene E_4 (LTE_4) and 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels. No relationship was noted between these prostanoids in (a) control subjects, (b) asthmatic children while in the stable condition and (c) asthmatic children during an acute asthma attack.

levels during the stable condition, the changes in LTE_4 were not related to changes in $11DTXB_2$ in children with bronchial asthma (Fig. 5). Neither gender, age, serum IgE nor eosinophil count had any relationship with urinary levels of LTE_4 or $11DTXB_2$ (data not shown). One patient (no. 5) had high eosinophil counts (1344/μL during the stable condition; 871/μL during an acute attack; and 2567/μL when he felt better 2 days after treatment). However, the eosinophil count did not correlate with urinary levels of LTE_4 or $11DTXB_2$. There was no significant correlation between urinary levels of LTE_4 and the severity of asthma; however, the severity of asthma in patients with high levels of urinary LTE_4 were classified as 'moderate persistent' or 'severe persistent'.

Discussion

Cysteinyl leukotrienes and TXA_2 are considered to play important roles in the pathogenesis of bronchial asthma. The relationship between urinary LTE_4 and $11DTXB_2$ in the pathogenesis of asthma has been reported by several investigators;[2–5,8,11,13,20] because of the instability of CysLTs and TXA_2, the end-products of the cascade were determined. However, most studies have been

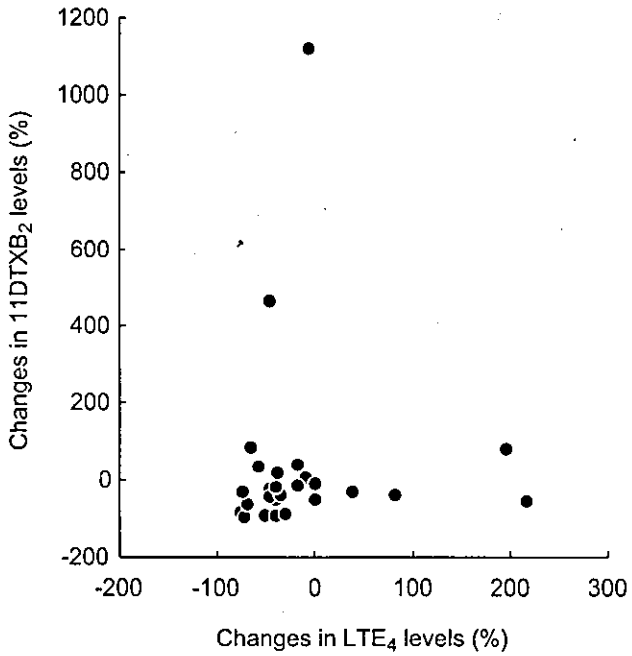

Fig. 5 Changes (%) in leukotriene E_4 (LTE_4) levels did not correlate with changes (%) in 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels in children with bronchial asthma. The percentage change was calculated as follows: (level during stable condition – level during attack) × 100/level during attack.

performed in adults. In the present study, we have demonstrated the relationship between urinary LTE_4 and $11DTXB_2$ in children with bronchial asthma.

In the present study, urinary LTE_4 levels in children with bronchial asthma during the stable condition were significantly higher than in control children. In addition, urinary LTE_4 levels in children during an acute asthma attack were higher than during the stable condition. Asano *et al.*[5] also demonstrated that patients with mild to moderate asthma excrete LTE_4 in the urine at a greater rate than control subjects. Taylor *et al.*[4] revealed that urinary LTE_4 was significantly higher in asthma patients after antigen challenge than in control subjects. The results of the present study are consistent with previous findings in adult asthmatic patients.[3,4,9,11,17,20]

In the present study, urinary $11DTXB_2$ levels were higher in children with bronchial asthma than in controls. Unlike LTE_4, urinary $11DTXB_2$ levels did not increase markedly during an acute attack.

Oosaki *et al.*[3,20] reported on variations in urinary levels of these mediators in patients with spontaneous asthma attacks who were monitored for 3 days and whose state improved. The study of Oosaki *et al.*[3,20] showed that urinary levels of LTE_4 were significantly higher during the attack and returned to control levels once the patient's state had improved. In the present study, the urinary levels of these prostanoids were measured in asthmatic children during the stable condition, during an acute attacks and 2 days after treatment. In eight children, urinary LTE_4 levels increased during an acute attack and decreased 2 days after treatment. One patient (no. 8) exhibited a different pattern of urinary LTE_4 excretion: levels decreased during an acute attack and then increased when she felt better 2 days after treatment. However, the urinary $11DTXB_2$ levels in this patient increased during an acute attack and then decreased 2 days after treatment. This patient had atopic-type bronchial asthma and was treated with theophyline, steroid inhalant, DSCG and a β_2-adrenergic receptor agonist. Before she was enrolled in the study, she had been treated with an LTRA for 5 weeks. However, LTRA treatment had little effect on her asthma. Urinary $11DTXB_2$ levels tended to increase during an asthma attack and persisted 2 days after treatment. Similar to the findings of the present study, Oosaki *et al.* have shown that the median level of urinary $11DTXB_2$ was highest during the 3rd hospital day in atopic-type patients and during the 2nd hospital day in non-atopic-type patients.[3]

In the present study, urinary levels of LTE_4 and $11DTXB_2$ were slightly higher than those reported previously.[2–5,8,11,13,20] Osamura *et al.* had reported that urinary levels of $11DTXB_2$ were significantly high between 1 and 3 years after birth and that they tended to decrease gradually with age thereafter.[21] Because all our subjects were children (1–15 years of age), this may explain why the urinary levels of $11DTXB_2$ were slightly higher in the present study than those reported previously.

Suzuki *et al.*[2] reported that no significant relationship was observed between urinary LTE_4 and $11DTXB_2$ in asthmatic patients. Oosaki *et al.*[3] also examined the relationship in changes (%) between these two metabolites; however, they noted no significant difference. In the present study, consistent with results of previous studies, no relationship was observed between urinary LTE_4 and $11DTXB_2$ in children with bronchial asthma. In addition, changes (%) in LTE_4 levels were not associated with $11DTXB_2$ levels in children with bronchial asthma. This suggests that increases in the levels of these two metabolites are not correlated with one another.

Neither gender, age, serum IgE nor eosinophil count revealed any relationship with urinary levels of LTE_4 or $11DTXB_2$. Eosinophils play an important role in the

pathogenesis of bronchial asthma and the eosinophil count is correlated with the clinical severity of the disease.[22] However, there are few studies referring to the correlation between eosinophil count and urinary levels of LTE_4 or $11DTXB_2$. There was no significant correlation between urinary levels of LTE_4 and the severity of asthma; however, the severity of the asthma in patients with high levels of urinary LTE_4 tended to be classified as 'moderate persistent' or 'severe persistent'.

In conclusion, we have shown significantly higher levels of urinary LTE_4 and $11DTXB_2$ in asthmatic children during the stable condition. These findings strongly suggest that the arachidonate cascade metabolites CysLTs and thromboxanes play certain roles in the pathogenesis of bronchial asthma in children. According to the differential changes in urinary levels of these metabolites during an acute attack, we suppose that an imbalance in the metabolism arises between the 5-lipoxgenase pathway and the cyclooxygenase pathway. The measurement of LTE_4 and $11DTXB_2$ in urine samples, which is a safe and easily available method of estimating the synthesis and release of the mediator in children, would be useful in understanding the pathogenesis of bronchial asthma.

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Generation of highly stable IL-18 based on a ligand–receptor complex structure

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Abstract

Human interleukin-18 (hIL-18), initially cloned as an IFN-γ-inducing factor, has a key role in many inflammatory diseases. We have previously developed a high production system for correctly folded active hIL-18 protein, leading to the revelation of the 3D-structure and the receptor binding mode. These findings can strongly indicate the experimental and medical applications of IL-18; however, the recombinant protein is prone to be inactivated forming multimers. Recently, therapeutic approaches using recombinant IL-18 have shown the effectiveness for treatment of cancer; indicating the necessity of a more stable protein for therapy with intertrial reliability. Here we have generated a highly stable hIL-18 with replacement of cysteine by serine based on the tertiary structure and the binding mechanism, retaining the biological activity. Similar rational designs can be applied to develop new therapeutic molecules of other cytokines.

Keywords: IL-18; Mutant; Cysteine; Stability; 3D-structure

Human interleukin-18 (hIL-18), initially cloned as an IFN-γ-inducing factor secreted by macrophages or Kupffer cells, strongly augments the production of IFN-γ both in natural killer cells and T cells; having a key role in many inflammatory diseases including allergy and autoimmune diseases [1–3]. We previously developed a high production system of correctly folded active hIL-18 protein, and it enabled us to determine both the 3D-structure and the molecular mechanism of the receptor binding mode [4,5]. This production method and the molecular mechanisms can strongly assist in the experimental and medical applications of IL-18; however, one of the most common problems of recombinant protein usage for experiments and medicine is the inactivation of the protein, usually forming aggregates [6]. Recently, therapeutic approaches using recombinant IL-18 have been examined for treatment of cancer including a clinical trial in humans; indicating the necessity for a more stable form that will allow therapy to be undertaken with intertrial reliability [7–9].

Here, we describe the generation of highly stable hIL-18 based on the tertiary structure and the binding mechanism. The mutant protein with replacement of cysteine by serine showed marked antioxidative stability without formation of the oligomers, and there was no reduction in biological activity.

Materials and methods

Vector construction and protein expression. Construction of the expression vector, expression, and purification of wild type hIL-18 protein were carried out as described previously [4]. Briefly, the coding region for mature hIL-18 (157 residues) with FactorXa cleavage site just before the hIL-18 sequence was amplified by PCR and the amplified product was cloned into the pGEX-4T-1 vector (Pharmacia). BL21 (DE3) (Novagen) was transformed by the vector, and protein expression was performed as follows: the colony with the highest expression level was cultivated overnight in 200 ml of the LB medium with 100 μg/ml ampicillin. The culture was transferred into 2 L of the LB medium with 100 μg/ml ampicillin and then incubated at 37 °C until the OD600 = 0.45; it was then cooled to 25 °C. IPTG (final concentration 1 mM) was added to the medium when OD600 = 0.5. The culture was further incubated at 25 °C for 5 h.

A bacterial cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 10 mM of 2-mercaptoethanol (2ME), and

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1 mM EDTA) with 1 mM PefaBloc (Roche), lysed by sonication, and centrifuged. The clear lysate was applied onto a GST affinity column (Pharmacia) and the column was then washed. The captured fusion protein was eluted with elution buffer (50 mM Tris–HCl, pH 8.0, and 10 mM glutathione). The protein-containing fractions were concentrated and cleaved by bovine factor-Xa (Funakoshi) at a ratio of 1% (w/w) at 4 °C. The mature hIL-18 protein was isolated using Sephacryl S-100 26/60 (Pharmacia). The fractions were then stored at 4 °C until further experiments.

Oligomerization assay of the wild protein. Oligomerization assay of the protein using wild type protein was carried out in a sealed siliconized Eppendorf tube. The protein solution (400 ng/μl) in phosphate-buffered saline (NaCl 137 mM, Na_2HPO_4 8.10 mM, KCl 2.68 mM, and KH_2PO_4 1.47 mM, pH 7.0) was agitated at 150 rpm at 37 °C for 12 h in the incubator. The sample solution was mixed with 5* concentrated SDS–sample buffer (final concentration 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 62.5 mM Tris–HCl, pH 6.8, and with/without 5% (v/v) of 2ME) and boiled for 5 min. The samples were electrophoresed on SDS–PAGE (10–20% gradient gel) and visualized by Coomassie blue staining.

Structural analyses of hIL-18 and the receptor complex. Multiple sequence alignments of the IL-18 s were performed by ClustalW with BLOSUM matrix [10]. The structure of the hIL-18 (PDB code: 1J0S) was determined by us using nuclear magnetic resonance (NMR); and here we used the modeled structure of the hIL-18:hIL18R α complex for structural analyses [5]. Structural rendering was performed on RasMol software [11].

Production and analyses of the mutant proteins. Site-directed mutagenesis of the hIL-18 gene was performed using GeneEditor in vitro Site-Directed Mutagenesis System (Promega) according to the manufacturer's instructions. Four different primers were designed for mutations to serine at C38, C68, C76, and C127, respectively. The primer sequences were: C38S: 5′-GACTGATTCTGACTCTAGAGATAATG CACC-3′, C68S: 5′-CTATCTCTGTGAAGT CTGAGAAAATTTCA ACTC-3′, C76S: 5′-GAAAATTTCAACTCTCTCCTCTGA GAACA AAA TTATTTCC-3′, (C68S/C78S for IL-18-AS: 5′-GTAACTAT CTCTGTGAAGTCTGAGAAAATTTCAACTCTCTCCTCTGAG AACAAAATTATTTCC-3′), and C127S: 5′-GATACTTTCTAG CTTCTGAAAAAGAGAGAG-3′ All the plasmid sequences harboring respective mutations were confirmed bidirectionally. Expression, purification, and the oligomerization assays were performed the same as those for wild type protein.

Biological activity assay. A biological activity assay based on IFN-γ induction was carried out as previously described [12]. Briefly, human myelomonocytic KG-1 cells were grown in the culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL). KG-1 cells (3.0×10^5 cells) were cultured in the presence of 0.1–50.0 ng/mL of recombinant hIL-18 for 24 h in a volume of 0.2 ml at 37 °C in a humidified atmosphere containing 5% CO_2. The culture supernatant was centrifuged to remove cells and stored at −80 °C until assay was performed. IFN-γ concentration was measured by fluorometric microvolume assay technology using FMAT 8100HTS system (Applied Biosystems).

Results

Oligomerization assay of wild type IL-18

On SDS–PAGE, wild type protein showed a marked oligomerization pattern after aeration by agitation (Fig. 1). However, the oligomerization pattern completely disappeared in the presence of 2ME; indicating that it was mainly due to the intermolecular disulfide bonds among the cysteine residues of hIL-18.

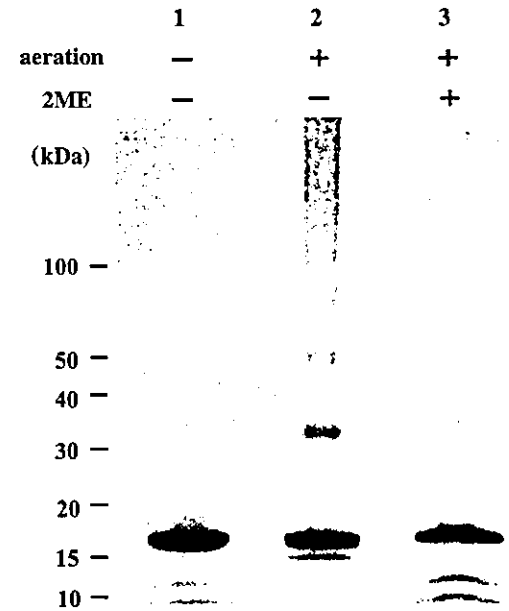

Fig. 1. Oligomerization assay of the wild type IL-18. Electrophoresis was carried on SDS–PAGE (10–20% gradient gel). (1) Before aeration without 2ME, (2) after aeration without 2ME, and (3) after aeration with 2ME. Four micrograms of each protein was loaded. The wild type protein shows a marked oligomerization pattern after aeration, but the pattern disappeared with 2ME.

Structural analyses of hIL-18 and the molecular mechanisms of receptor binding

Alignment of the amino acid sequences of the IL-18 proteins showed marked similarities among the different species. C76 and C127 are conserved among all the species, while C38 and C68 were replaced by the other residues in several species (Fig. 2). Conservation among different species usually indicates the importance of the conserved residues for the structure or activity; but conservation itself cannot show the positions of the residues in the 3D-structure associated with intra- or inter-molecular disulfide bonds.

The 3D-structure of hIL-18 determined by NMR clearly showed an absence of intramolecular disulfide bonds, and cysteine residues existing on the surface of the structure suggested a possible role for intermolecular disulfide bonds in the oligomerization (Fig. 3A). The atomic interactions among the cysteine residues and the other residues of hIL-18, and also the positions of the cysteine residues on the complex structure with hIL-18R α suggested that the replacement of the four cysteine residues by other types of amino acids, especially the conservative residue, serine, does not collapse the 3D-structure of hIL-18; retaining the capacity to bind to the receptor (Figs. 3A and B). According to structural analyses, five mutant proteins were designed (Table 1).

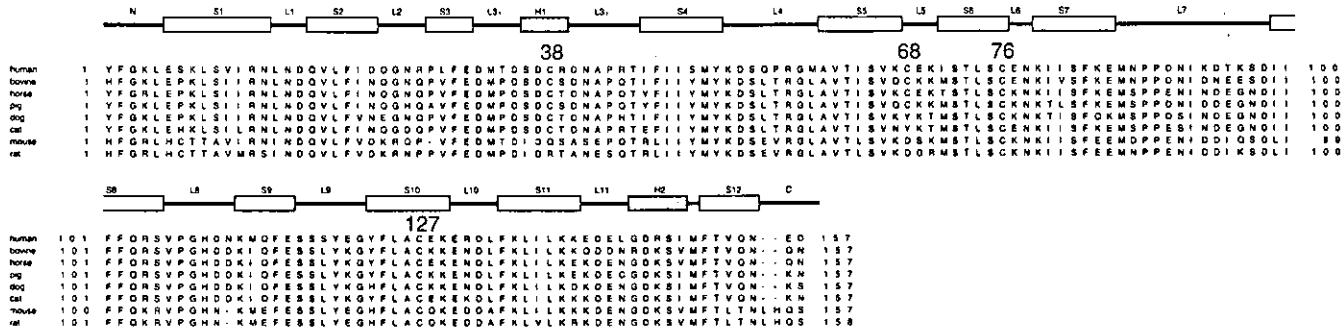

Fig. 2. Alignment of the amino acid sequences of IL-18 proteins. Completely conserved residues among the eight species are boxed in gray. The cysteine residues are boxed in yellow. The numbers of the cysteine residues are indicated as a human sequence. The secondary structure for human IL-18 previously determined by us is indicated over the sequences as the yellow (S; β-strand) and the blue (H; helix) boxes. The intermediate loop regions are indicated as L. C76 and C127 are conserved among all the species, while C38 and C68 were replaced by other residues in several species.

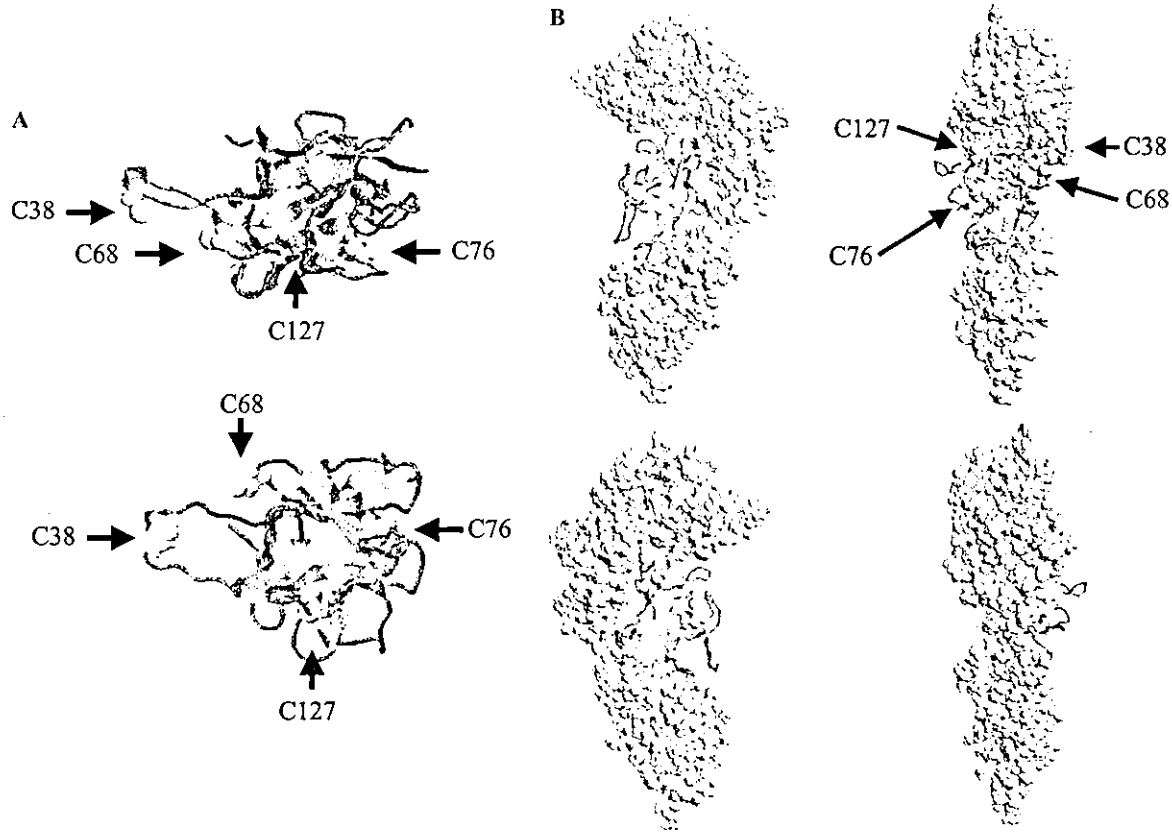

Fig. 3. Structure of the human IL-18 protein and the complex structure of human IL-18 and human IL-18 receptor α. (A) Structure of the human IL-18. The overall structure is shown as a ribbon model, and the four cysteine residues are shown as spacefill representation in yellow. The top figure and the bottom figure are tilted at 90° to each other. The four cysteine residues do not form any sulfide bonds and exist on the surface of the structure. (B) The complex structure of human IL-18 and human IL-18 receptor α. The overall structure of hIL-18 is shown as a ribbon model in cyan, and the four cysteine residues are shown as spacefill representations in yellow. hIL-18R α is shown as spacefill representation in white. The four cysteine residues of hIL-18 do not have any direct interaction with hIL-18R α; suggesting little influence on the binding.

Oligomerization assay of the IL-18 mutants

On SDS–PAGE, the mutants harboring one cysteine residue on the surface showed dimerization after the aeration procedure, but with different degrees (Figs. 4A and B). The findings indicate that all the cysteine residues of hIL-18 associated with the oligomerization phenomenon but to different extents. On the other hand, the IL-18-AS protein did not show any oligomerization pattern even after the aeration procedure (Fig. 5); indicating that the IL-18-AS protein can exist as a monomer in oxidative conditions.

Table 1
Amino acid composition of wild and mutant proteins

Position	38	68	76	127
Wild	Cys	Cys	Cys	Cys
C38	Cys	Ser	Ser	Ser
C68	Ser	Cys	Ser	Ser
C76	Ser	Ser	Cys	Ser
C127	Ser	Ser	Ser	Cys
AS	Ser	Ser	Ser	Ser

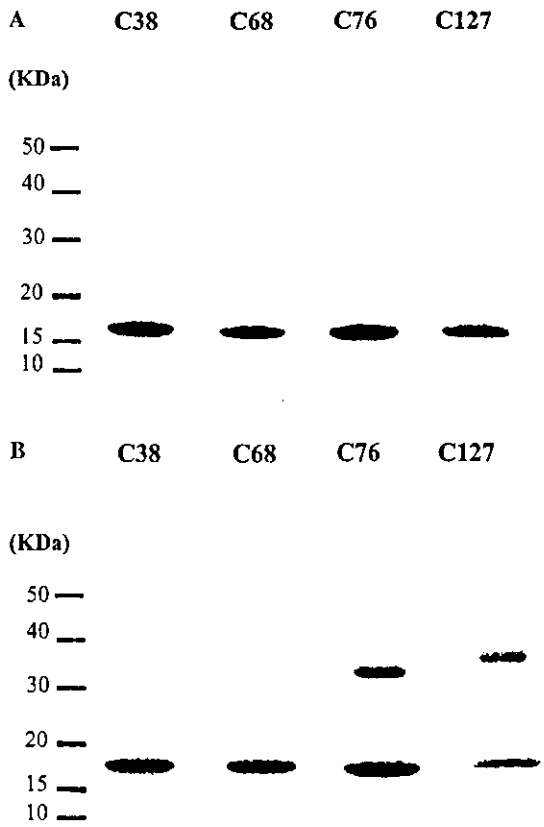

Fig. 4. Dimerization assay of the IL-18 mutants harboring one cysteine residue on the surface. Electrophoresis was carried out on SDS-PAGE (10–20% gradient gel). (A) Before aeration without 2ME, (B) and after aeration without 2ME. Four micrograms of each protein was loaded. All the mutants showed dimerization after aeration, indicating that all the cysteine residues of IL-18 associated with the oligomerization phenomenon.

Biological activity of wild and mutant protein, IL-18-AS

The biological activities of the wild type and IL-18-AS before aeration were compared. IFN-γ induction by different concentrations of the two IL-18 proteins showed no significant differences before oxidation (Fig. 6). The biological activities after aeration showed a marked reduction in the wild type protein; resulting in about five to ten times lower IFN-γ induction than that before oxidation between 1 and 10 ng/ml. However, IL-18-AS did not show any significant reduction even after extensive aeration (Fig. 6). These findings indicate

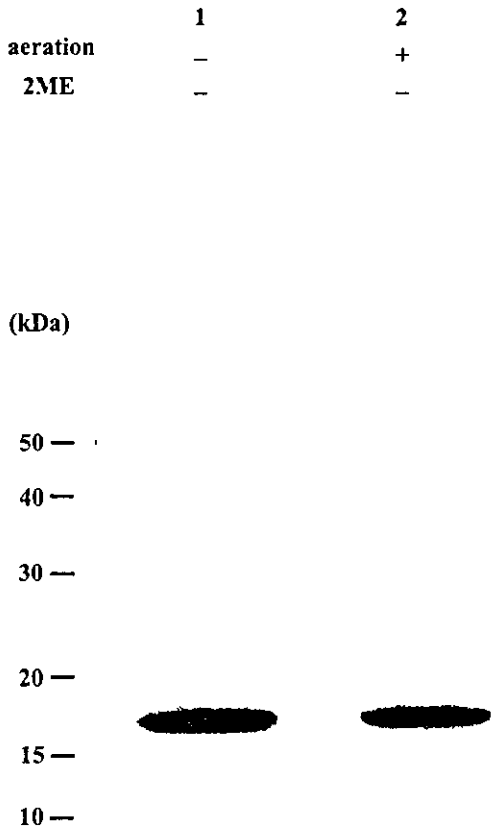

Fig. 5. Oligomerization assay of IL-18-AS. Electrophoresis was carried on SDS-PAGE (10–20% gradient gel). (1) Before aeration without 2ME, (2) after aeration without 2ME. Four micrograms of each protein was loaded. The IL-18-AS protein did not show any oligomerization pattern.

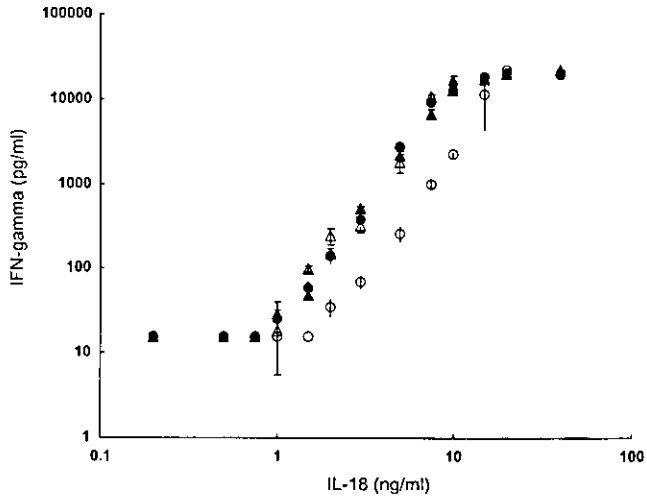

Fig. 6. IFN-γ induction (pg/ml) by the wild type and the IL-18-AS before and after oxidation. Mean values of triplicate IFN-γ induction assays are shown with standard deviation. Solid circle, hIL-18-wild before oxidation; open circle, hIL-18-wild after oxidation; solid triangle, hIL-18-AS before oxidation; and open triangle, hIL-18-AS after oxidation. IL-18-AS showed the same activity as that of the wild type even after oxidation, while wild type protein showed significant reduction in the activity after oxidation.

that the newly generated mutant, IL-18-AS, is highly stable against oxidative conditions; retaining the equivalent biological activity.

Discussion

Our method for the purification of hIL-18 shows that recombinant hIL-18 exist almost as a monomer in solution [4]. However, oligomerization and inactivation of IL-18 have been reported [13–15] and another of our studies also indicated that a small fraction of the purified protein existed as dimer or trimer even in an intensively reduced condition; suggesting that the IL-18 produced should be partially inactive [Kato et al. unpublished data]. However, the precise mechanisms of the inactivation have not yet been clarified.

Kikkawa et al. [14] speculated that one of the mechanisms of the inactivation should be misfolding; by the loss of the specific covalent intramolecular required for potent IFN-γ inducing function of IL-18, but the 3D-structure determined by us revealed that there are no intramolecular disulfide bonds in hIL-18 (Fig. 3). Further, the side chains of all the cysteine residues exist on the surface of the protein making it possible to be accessible by each other. From these observations, we can speculate that the oligomerization of IL-18 can be done using these free sulfatides on the molecular surface, and that the activity should be lost.

We have demonstrated here that wild type IL-18 forms extensive oligomers in oxidative conditions, and that the IFN-γ inducing activity was significantly reduced. The oligomerization mechanism is mediated by the intermolecular disulfide bonds among the four cysteine residues in IL-18 polypeptide, namely C38, C68, C76, and C127. To obtain an antioxidative stable hIL-18, we conservatively mutated all the cysteine residues to serine; and as the 3D-structure analyses predicted (Fig. 3), the resulting mutant protein, IL-18-AS, showed high stability retaining biological activity even after oxidation (Fig. 6).

In human fibroblast growth factor (hFGF), replacement of two of the four cysteine residues by serine (C70S and C88S) could improve instability; retaining the biological activities, but replaced the other two cysteine residues (C26S and C93S) resulting in a marked reduction in activity [16,17]. Biochemical analyses suggested that two cysteine residues (C26 and C93) form an intramolecular disulfide bond [17]. However, the structural determination of hFGF has revealed that C26 and C93 have no intramolecular sulfide bond with their side chains buried inside the molecule; indicating that the atomic interactions among the other residues will be important for the activity, while the side chains of C70 and C88 are exposed to the solvent without any disulfide bonds [18]. Moreover, the recent structure determination of the complex involving hFGF:FGF receptors has also revealed that C26 and C93 exist adjacent to the interface between ligand and receptor, while C70 and C88 are completely free from the interface of the receptor, similar to those observed in hIL-18 and the receptor [5,18].

Using a protein as a therapeutic agent requires not only production of a sufficient quantity of homogeneous protein, but also a stable formulation suitable for storage and delivery. The IL-18-AS protein that we have developed here showed significant stability against oxidation; retaining the same activity with wild type protein. Although recombinant hIL-18 has been examined in the treatment of cancer [7–9], the structure-based design of the mutants should be emphasized and the stable form of cytokine can be the first step for development of a more potent cytokine with additional site-specific modifications including glycosylation or pegylation [19–22].

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Systematic optimization of active protein expression using GFP as a folding reporter

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Abstract

Many recombinant proteins have been used as drugs; however, human proteins expressed using heterologous hosts are often insoluble. To obtain correctly folded active proteins, many optimizations of expression have been attempted but usually are found to be applicable only for specific targets. Interleukin-18 (IL-18) has a key role in many severe disorders including autoimmune diseases, and therapeutic approaches using IL-18 have been reported. However, production of IL-18 in *Escherichia coli* resulted in extensive inclusion body formation and previous conventional screenings of expression conditions could obtain only a condition with a low yield. To address the problem, we applied a folding reporter system using green fluorescent protein (GFP) for screening of the expression conditions for hIL-18. The established system efficiently screened many conditions, and optimized conditions for the expression of hIL-18 significantly enhanced the final yield of the active protein. Systematic screening using a GFP reporter system could be applied for the production of other proteins and in other organisms.

Production of recombinant proteins such as cytokines has been an important method for developing new therapeutic molecules. However, expression using heterologous target genes has production problems, especially the inactivation of the expressed proteins [1]. Recent advances in the proteomics require the availability of an efficient protein production system, but most of the target proteins cannot be properly produced, being an inactive aggregation [2]. To obtain correctly folded active proteins, many approaches such as expression condition optimization have been tried [3–5]. However, the methods reported have been relatively specific for their targets and are actually applicable only to specific or related proteins. Laborious and time-consuming screening steps for optimization of the expression conditions specific for the protein must be performed on an individual basis.

Interleukin-18 (IL-18) is functionally similar to IL-12 in terms of IFN-γ production; and the aberrant expression of IL-18 has been inferred to be associated with severe inflammatory conditions, such as autoimmune diseases, allergies, or neurological disorders [6–8]. Therapeutic approaches using recombinant IL-18 have been investigated for treatment of cancers, including in a clinical trial in humans [9–11]. Recently, we have successfully determined the 3D-structure of human IL-18 (hIL-18) and the structural basis of its receptor activation mechanisms using mutant proteins and receptor binding assays [12,13]. However, production of IL-18 in *Escherichia coli* resulted in extensive inclusion body formation and previous conventional screenings of expression conditions could obtain only a condition with a low yield ([12], Kato et al., unpublished data).

To address these problems, we have applied a folding reporter system using green fluorescent protein (GFP) for screening of the expression conditions of hIL-18 [14]. We constructed the expression vector encoding the hIL-18 with GFP protein in the C-terminus and evaluated the expression conditions by measuring fluorescence from the hIL-18-GFP fusion protein. The established system efficiently screened many conditions, and the optimized condition for the expression of hIL-18

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significantly enhanced the final yield of the active protein. This GFP reporter system could be applied to the production of other proteins and in other organisms.

Materials and methods

Construction of plasmid of GST-hIL-18wild-GFP fusion protein

mRNA was extracted from a blood sample obtained from a healthy volunteer, and cDNA was synthesized at 72 °C for 60 min using reverse transcriptase, MMLV, and oligo(dT) primers. The coding region for mature hIL-18wild (157 residues) was amplified by polymerase chain reaction (PCR). The coding region for GFP was also amplified from pEGFP-N2 vector (Clontech). Between the amplified fragments, the DNA fragment, 5′-GGATCC GCTGGCTCCGCTGCTGGTTCTGGCGAGTTC-3′, coding for amino acid linker GSAGSAAGSGEF, was placed [14]. The hIL-18wild-linker-GFP was cloned into a T-vector (Invitrogen). The primers were designed to insert the *Eco*RI and Factor Xa-cleavage sites immediately before the mature hIL-18wild sequence and the *Eco*RI site after the stop codon. The T-vector was digested with *Eco*RI and purified by electrophoresis. The purified product was then subcloned into the pGEX-4 T-1 vector (Pharmacia) and the DNA sequence of the clone was confirmed by bi-directional sequencing. The clone was named GST-hIL-18wild-GFP (Fig. 1).

Screening of induction temperatures

Escherichia coli BL21(DE3) (Novagen) was transformed by GST-hIL-18wild-GFP according to the manufacturer's instructions. The expression of the protein was performed as follows: each of the transformed colony was grown in 5 ml Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter of medium) with 100 μg/ml ampicillin and 2% glucose, and the cultures were shaken at 37 °C until an optical density at 600 nm (OD_{600}) = 0.4. *E. coli* cultures were transferred to each temperature (15, 25, and 37 °C) and continuously shaken until OD_{600} = 0.5. Protein production was induced by addition of isopropylthio-β-D-galactoside (IPTG) at a final concentration of 1 mM. After a 5 h induction of the protein, 1.5 ml of each culture was used as a sample for expression analysis by GFP. Fluorescences were measured using VersaFluor (Bio-Rad) (excitation, 490 nm; emission, 510 nm, averaging time, 4 s). For more precise examinations, induction temperatures from 23 to 37 °C were tested by the same procedures.

For SDS–PAGE analysis, 1.5 ml of each culture was centrifuged at 10,000 rpm for 2 min and the pellet was resuspended in 1.5 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 10 mM 2-mercaptoethanol (2-ME), and 1 mM EDTA) with 1 mM Pefabloc (Roche). The cells were completely lysed by sonication of 0.5-s active sonication with a 0.5-s interval on an ice-water bath for 10 min and then centrifuged for 10 min at 12,000 rpm, 4 °C. The pellet was then resuspended in 1.5 ml of lysis buffer; then 10 μl of the supernatant and the resuspended pellet were used for SDS–PAGE analysis.

Screening of concentrations of IPTG and induction periods

The *E. coli* culture incubated until OD_{600} = 0.4 at 37 °C was transferred into conditions at 26 °C and incubated until the cell density became OD_{600} = 0.5, which

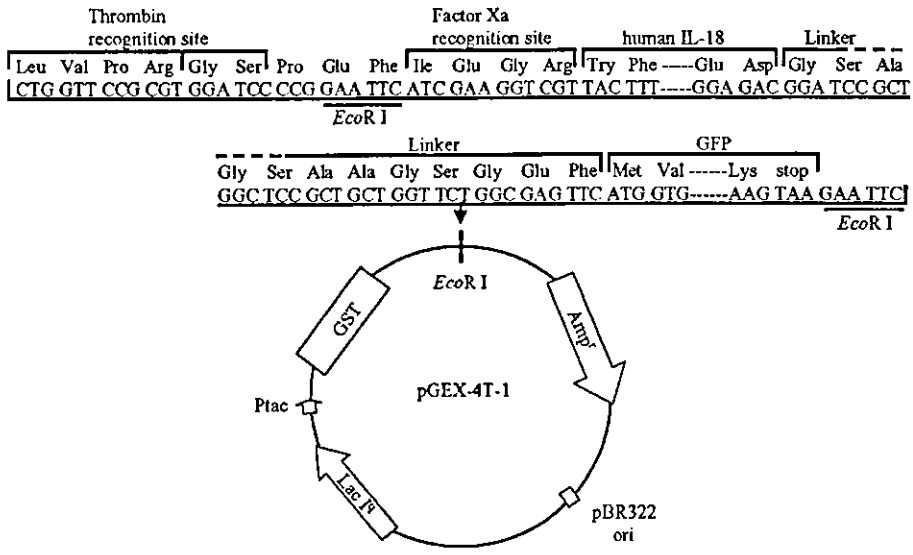

Fig. 1. Schematic representation of the expression vector for GST-hIL-18 fusion protein (pGEX-4T-Xa-hIL-18wild-GFP). The sequence encoding hIL-18wild and GFP was inserted into *Eco*RI site of pGEX-4T-1.

was usually after 5 h incubation. Then protein expression was induced by IPTG at final concentrations of 0.1, 0.5, 1, and 2 mM. OD_{600} and GFP expression of each culture was monitored for 30 h.

Large scale expression and purification

We used the expression vectors GST-hIL-18wild and GST-hIL-18opti for the examination to compare large scale production yields under different conditions. The vector, GST-hIL-18wild, had been constructed by subcloning wild-type hIL-18 into pGEX-4 T-1, and the vector, GST-hIL-18opti, contains the newly synthetic gene with optimized codons but without amino acid changes for expression in *E. coli* (BL21) [12].

Large scale expression and purification of the wild-type hIL-18 protein were carried out as described previously with minor modifications [12]. Briefly, BL21 was transformed by the vectors, GST-hIL-18wild or GST-hIL-18opti, and protein expression was performed as follows: the colony with the high expression level was cultivated overnight in 200 ml of the LB medium with 100 μg/ml ampicillin shaken at 160 rpm. The culture was transferred into 2 L of the LB medium with 100 μg/ml ampicillin. The culture was incubated at 37 °C until $OD_{600} = 0.45$ and then cooled to 26 °C. IPTG (final concentration 1 mM) was added to the medium when $OD_{600} = 0.5$. The culture was incubated at 26 °C with shaking at 160 rpm for 15 h. The culture was centrifuged at 5000*g* for 10 min and the *E. coli* pellet was stored at −80 °C until purification.

The bacterial cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA) with 1 mM Pefabloc, lysed by sonication, and then centrifuged. The clear lysate was applied onto a GST affinity column (Pharmacia) and the column was then washed by lysis buffer. The captured fusion protein was eluted with elution buffer (50 mM Tris–HCl, pH 8.0, and 10 mM glutathione). The protein-containing fractions were concentrated and cleaved by bovine Factor Xa (Funakoshi) at a ratio of 1% (w/w) at 4 °C. Mature hIL-18 protein was isolated using Sephacryl S-100 26/60 (Pharmacia). The fractions were then stored at 4 °C until further experiments. The concentration of the purified hIL-18 protein was estimated using the absorbance constant (6160) for hIL-18.

Biological activity assay

Human myelomonocytic KG-1 cells were grown in the culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/ml), and streptomycin (100 μg/ml). KG-1 cells (3.0×10^5 cells) were cultured in the presence of 0.1–50.0 ng/ml of recombinant hIL-18 for 24 h in a volume of 0.2 ml at 37 °C in a humidified atmosphere containing 5% CO_2. Culture supernatants in the microtest plates were spun to remove cells after the cultures and were stored frozen at −80 °C. The IFN-γ concentration was measured as previously described with minor modification by fluorometric microvolume assay technology using FMAT 8100 HTS system (ABI) [15].

Results

Screening of induction temperature

With expression at 37 °C, the fusion protein mostly precipitated as an inclusion body, while it was not observed in the pellet in expressions at 15 and 25 °C (Fig. 2A). Further, the expression level at 15 °C was lower than the level of expression at 25 °C. As examined on SDS–PAGE, the induction temperature at 25 °C showed the best yield. In addition, the fluorescence of the fusion protein at 25 °C was the highest among the three temperatures (Fig. 2B). To determine the optimized induction temperature, examinations measuring fluorescence were carried out at 15–37 °C. The fluorescence of *E. coli* culture expressing GST-hIL-18wild-GFP at 26 °C was the highest; at a level that was 4.3 times higher than that at 37 °C (Fig. 3).

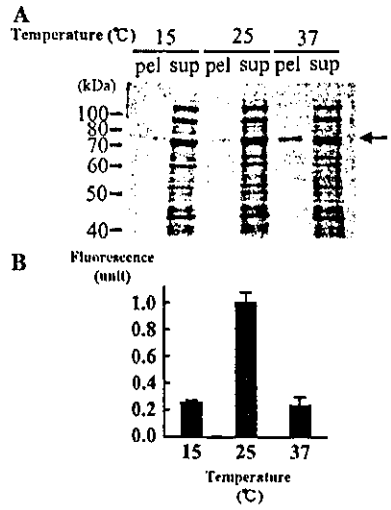

Fig. 2. Expression of GST-hIL-18wild-GFP. (A) SDS–PAGE (7.5% gel) of the expression of the GST-hIL-18wild-GFP fusion protein after 5 h of induction by IPTG (1 mM). The fusion protein migrated as a 71 kDa band. The band in the pellet at 37 °C indicated an extensive inclusion body formation. Mw, BenchMark Protein Ladder (Invitrogen); sup, supernatant of the cell lysate; pel, pellet of the lysate. Arrow indicates the GST-hIL-18wild-GFP protein. (B) Fluorescence of GFP at three different induction temperatures with 1 mM IPTG and 5 h of induction. The fluorescence was normalized by dividing by the highest fluorescence. Mean values of triplicate assays are shown with standard deviation. The fluorescence at 25 °C showed the highest in the three temperatures.

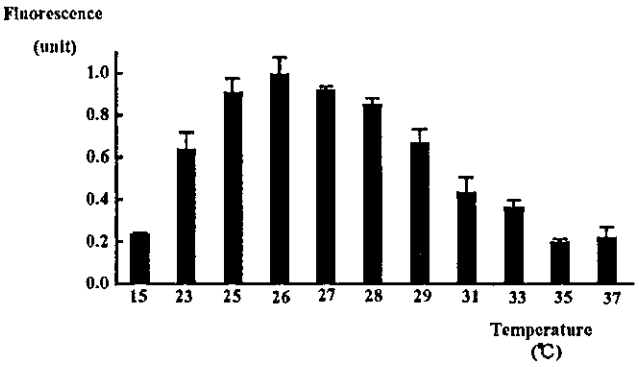

Fig. 3. Fluorescence at different induction temperatures with 1 mM IPTG and 5 h of induction. The fluorescence was normalized by dividing by the highest fluorescence. Mean values of triplicate assays are shown with standard deviation. The fluorescence at 26 °C showed the highest in the three temperatures.

Screening of IPTG concentration and induction period

The same approach could also be used to screen IPTG concentrations and induction periods (Fig. 4). Four IPTG concentrations were examined and fluorescence increased IPTG dose-dependently, but the fluorescence was saturated at 1 mM IPTG. The maximum intensity of fluorescence at 0.1 mM was obtained at 9 h, while the maximum intensity of fluorescence at 0.5, 1, and 2 mM was obtained at around 15 h. After that the fluorescence decreased gradually.

Large scale expression and purification

The target proteins, GST-hIL-18wild and GST-hIL-18opti, were expressed at 26 °C in *E. coli*. The culture medium of *E. coli* expressed GST-hIL-18wild showed $OD_{600} = 0.9$ after 15 h of induction, and 7.9 g of *E. coli* pellet was obtained from a 2-liter culture. On the other hand, culture medium expressed GST-hIL-18opti showed $OD_{600} > 3.0$ after 15 h induction and 13.0 g of *E. coli* pellet was obtained from a 2-liter culture. A GST affinity column was used to capture the GST fusion protein, which was concentrated and cleaved by Factor Xa. The cleavage was completed at 4 °C after about 16 h. Following gel filtration chromatography of the cleaved protein, highly purified hIL-18wild and hIL-18opti were obtained.

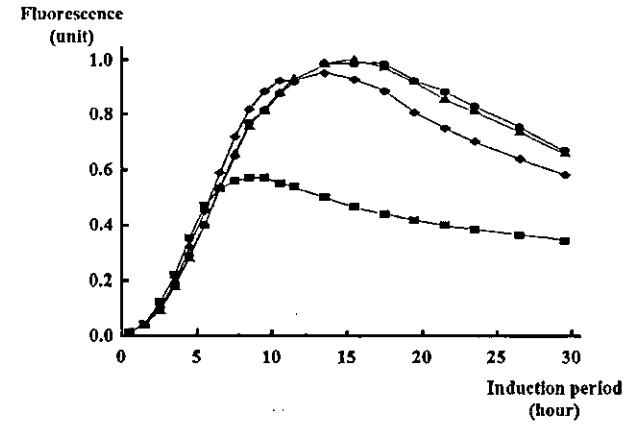

Fig. 4. Fluorescence at different concentrations of IPTG and induction periods. IPTG concentration: 0.1 mM (■), 0.5 mM (◆), 1 mM (●), and 2 mM (▲). Induction temperature was 26 °C. The fluorescence was normalized by dividing by the highest fluorescence at the optimal condition of 1 mM IPTG and 15 h. Mean values of triplicate assays are shown. The fluorescence induced with 1 and 2 mM IPTG showed the highest values at 15 h.

Actual fluorescence intensity by GST-IL-18-GFP fusion protein expressed in optimized conditions (11,191 at 26 °C, IPTG 1 mM, 15 h) showed a level about three times higher than that in the previous condition (4207 at 25 °C, IPTG 1 mM, 5 h). The yield of hIL-18wild protein was 3.0 mg from 2 L of the culture medium (1.5 mg/L; 0.38 mg/g wet weight cell), while the yield of the hIL-18opti protein was 23.1 mg from 2 L of the culture medium (11.5 mg/L; 1.78 mg/g wet weight cell) (Table 1). These yields obtained under the newly optimized conditions showed about three times higher levels than those previously used conditions indicating the correlation between fluorescence intensity and actual protein yield.

Biological activities of hIL-18 by different production methods

The biological activities of hIL-18 produced by different methods, commercially available hIL-18 (MBL),

Table 1
Comparison of final yields on large scale expression

Genes	Previous condition		Optimized condition	
	mg/L	mg/g cell	mg/L	mg/g cell
IL-18wild	0.5	0.21	1.5	0.38
IL-18opti	4.4	1.17	11.5	1.78

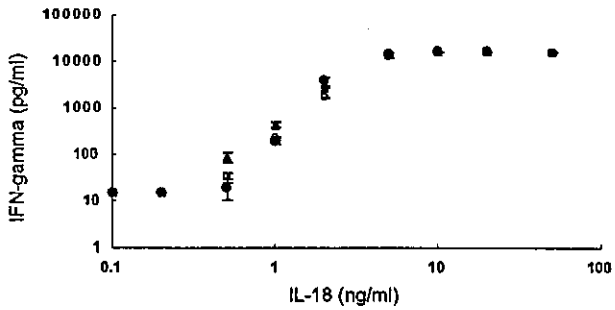

Fig. 5. IFN-γ induction by differently produced hIL-18s. Solid circle, commercially available hIL-18 (MBL); open circle, hIL-18wild produced by the previous protocol; and solid triangle, hIL-18opti produced by the new protocol. Mean values of triplicate IFN-γ induction assays are shown with standard deviation. IFN-γ production by different concentrations of the three IL-18 proteins showed no significant differences among them showing almost the same sigmoid curves. These findings indicate that the new expression conditions can produce high amounts of protein retaining the same biological activity as those made by conventional methods and conditions.

hIL-18wild or hIL-18opti, were estimated. IFN-γ production by different concentrations of the three IL-18 proteins showed no significant differences among them showing almost the same sigmoid curves (Fig. 5). These findings indicate that the new expression condition can produce high amount of protein retaining the same biological activity as those by the conventional methods. Conservation between the 3D-structures of the purified proteins using the hIL-18wild and hIL-18opti genes was previously confirmed by multidimensional-, multinuclear-, magnetic resonance techniques [12,13].

Discussion

Expression and purification of hIL-18 in *E. coli* have been described in two reports [7,16]; one a direct expression of mature IL-18 protein, which had methionine before the mature sequence; the other an expression of the GST-hIL-18 fusion protein, because direct expression of the mature hIL-18 polypeptide like the previous one by the authors did not yield any active protein [16]. The two studies used similar expression conditions (Ushio et al. 37 °C, 18 h, 1 mM IPTG; Liu et al. 37 °C, 3 h, 0.1 mM IPTG), but no findings concerning expression condition optimization were described. Neither were the final yields of the protein described, but the yields should have been quite low if in accord with our results.

Our previously reported method using a codon optimized synthetic gene could yield five times higher amounts of protein than that using a non-optimized wild-type gene [12]. The expression condition (25 °C, 5 h, 1 mM IPTG) used for the study was according to the results of a preliminary study; that showed that extensive inclusion body formation occurred and that the yield was very low at higher temperatures, such as 37 °C ([12], Kato et al., unpublished). However, this condition was not fully optimized and remained in a kind of empirical state.

To establish a systematic screening system for expression conditions, we have introduced GFP as the "folding reporter." The original usage of GFP as a folding reporter was described in an evaluation of mutagenesis to obtain a target protein as a soluble protein; but not to obtain the optimized expression conditions [14]. This strategy utilized the rationale that the chromophore formation of GFP depends on the correct folding of the protein. A "folding reporter" vector was constructed, in which the target protein was expressed as an N-terminal fusion protein before GFP. The fluorescence from *E. coli* cells expressing GFP was correlated to the correct folding of the upstream protein; and this system led them to achieve structural determination of a soluble mutant protein [17]. Recently, another application using GFP for screening of randomly cloned cDNAs has been reported to search for a soluble domain [18].

In our study, a systematic search of the expression conditions of temperature, inducer concentration, and induction period has been successfully performed (Figs. 3 and 4). Preliminary trials concerning condition optimization have been reported previously, but the actual effect for yields using these conditions remained unknown [19]. In contrast, we have here confirmed that the conditions obtained in small scale examinations could also be applied to large scale production with predicted yields of active protein using two different genes (Table 1). Moreover, this study also has indicated that the time required for screening can be significantly reduced. For example, large scale examination for one condition requires about 1 week in our system, and actual yields can be obtained only at the final steps. If we screen the conditions examined in this study using conventional methods, it would take more than 1 year; this reporter system requires only 1 week to carry out the same screening.

In conclusion, more efficient and cost-effective screening procedures could be performed using our GFP reporter system that should be applicable for the optimization of many expression conditions; not only of temperature, inducer, and periods, but also of other factors, such as host strains, additives, cell free systems in the production of many other proteins and using other organisms [5].

Acknowledgments

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