cDNA cloning, expression and characterization of human prostaglandin F synthase[1]

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Received 13 October 1999; received in revised form 7 November 1999

Edited by Shozo Yamamoto

Abstract A cDNA clone of prostaglandin F synthase (PGFS) was isolated from human lung by using cDNA of bovine lung-type PGFS as a probe and its protein expressed in *Escherichia coli* was purified to apparent homogeneity. The human PGFS catalyzed the reduction of prostaglandin (PG) D_2, PGH_2 and phenanthrenequinone (PQ), and the oxidation of 9α,11β-PGF_2 to PGD_2. The k_{cat}/K_m values for PGD_2 and 9α,11β-PGF_2 were 21 000 and 1800 min^{-1} mM^{-1}, respectively, indicating that the catalytic efficiency for PGD_2 and 9α,11β-PGF_2 was the highest among the various substrates, except for PQ. The PGFS activity in the cytosol of human lung was completely absorbed with anti-human PGFS antiserum. Moreover, mRNA of PGFS was expressed in peripheral blood lymphocytes and the expression in lymphocytes was markedly suppressed by the T cell mitogen concanavalin A. These results support the notion that human PGFS plays an important role in the pathogenesis of allergic diseases such as asthma.

Key words: Prostaglandin F synthase; Human lung; Aldo-keto reductase; Prostaglandin D_2; 9α,11β-Prostaglandin F_2; Peripheral blood lymphocyte

1. Introduction

Prostaglandin (PG) F_2 is widely distributed in various organs and exhibits various biological actions such as smooth muscle contraction of uterus, bronchus and trachea [1], the initiation of parturition [2] and pain transmission [3]. We discovered PGD_2 11-ketoreductase involved in the formation of PGF_2 from PGD_2 in rat tissues [4]. The enzyme purified from bovine lung is a dual function enzyme, which catalyzes the reduction of not only PGD_2 but also PGH_2 on the same molecule, and we named it PGF synthase (PGFS) (EC 1.1.1.188) [5]. Then, we characterized the enzymatic and molecular properties of bovine lung [5–9] and liver [10,11] PGFSs. Although bovine PGFSs had low reductase activity towards PGE_2 and various steroid compounds containing a keto group and a low oxidase activity for converting 9α,11β-PGF_2 to PGD_2, the enzymes exhibited reductase activities towards various carbonyl compounds, such as phenanthrenequinone (PQ), nitrobenzaldehyde (NB) and nitroacetophenone, in addition to PGD_2 and PGH_2. Bovine PGFSs belong to the aldo-keto reductase (AKR) superfamily based on substrate specificity, molecular weight and the amino acid sequence. In 1995, Nagase et al. [12] isolated full-length cDNA clones from size-fractionated cDNA libraries of human immature myeloid cell line KG-1. The amino acid sequence of one clone, KIAA0119, was similar to that of the AKR family. The nucleotide and deduced amino acid sequences of KIAA0119 were identical to those of AKR1C3 [13] except for six nucleotides and two amino acids. AKR1C3 was reported to be a 3α-hydroxysteroid dehydrogenase (HSD) [13,14], but the enzymatic properties of KIAA0119 were not known. Matsuura et al. [15] examined the enzymatic properties of AKR1C3. They constructed AKR1C3 by site-directed mutagenesis of KIAA0119 and reported that AKR1C3 acted as a PGD_2 11-ketoreductase. PGD_2 11-ketoreductase activity is associated with PGFS, but the PGFS activity of KIAA0119 and AKR1C3 has not been reported yet. Moreover, the physiological roles of AKR1C3 and KIAA0119 have not been determined yet. Bovine lung PGFS is abundant in the contractile interstitial cells in the alveolar septum, based on the results of immunohistochemical techniques at both light and electron microscopic levels [16,17], and this implies that PGFS may play an important physiological role in human lung tissue. In fact, Beasley et al. reported that 9α,11β-PGF_2 possibly contributes to the bronchoconstrictor effect of a mast cell-derived mediator in asthma [18]. In this paper, as an initial approach to clarify the physiological role of human PGFS, we isolated a cDNA clone of human PGFS from lung and expressed the protein in *Escherichia coli*. Furthermore, we examined the enzymatic properties and described the possible relationship between PGFS and allergy.

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[1] The amino acid sequence of human PGFS and the amplified genomic DNA with PGFS-F4 and R5 were registered in the DDBJ under accession no. AB018580 and no. AB028065, respectively.

Abbreviations: PG, prostaglandin; PGFS, prostaglandin F synthase; PQ, phenanthrenequinone; NB, nitrobenzaldehyde; AKR, aldo-keto reductase; HSD, hydroxysteroid dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PBL, peripheral blood lymphocytes; Con A, concanavalin A; DD, dihydrodiol dehydrogenase

0014-5793/99/$20.00
PII: S0014-5793(99)01551-3

2. Materials and methods

2.1. Materials

[5,6,8,9,12,14,15-^{3}H]PGD_2 (3.7 TBq/mmol) was obtained from Du Pont-New England Nuclear. [1-^{14}C]PGH_2 (2.07 GBq/mmol) was obtained from Daiichi Pure Chemicals (Japan). Authentic PGs were kindly donated by Ono Pharmaceutical Company.

2.2. cDNA cloning of human PGFS

A human lung 5′-stretch plus cDNA library in λgt 10 vector was purchased from Clontech Laboratories (USA). The library (4×10^6 plaques) was screened by plaque hybridization by using ^{32}P-labelled full-length cDNA for bovine lung PGFS as a probe [7]. Hybridization was conducted as described previously [11] and 20 positive clones were obtained and sequenced by TAKARA (Japan). One clone encoded the full-length cDNA of human PGFS and was designated as λ-hLuFS.

2.3. Expression of human PGFS in E. coli and purification of its expressed protein

Two oligonucleotides for N- and C-termini of the open reading frame amino acid sequences were synthesized, containing *Eco*RI (CCGGAATTCAATGGATTCCAAACAGCAGTG) and *Bam*HI (CGCGGATCCTTAATATTCATCTGAATATG) linkers, respectively. Using λ-hLuFS as a template and two primers, we inserted the DNA amplified by PCR with Vent DNA polymerase (BioLabs, USA) into a pUC8 vector. The resultant plasmid DNA (pUC-hLuFS) was sequenced with an ABI automated DNA sequencer 373A (Perkin Elmer, USA). *E. coli* HB101 cells transformed with the plasmid were cultured at 37°C overnight and the harvested cells were sonicated in three volumes of buffer A (10 mM potassium phosphate buffer, pH 7.0). The lysate was centrifuged at $100\,000\times g$ after $10\,000\times g$ centrifugation and was subjected to ammonium sulfate fractionation between 40 and 75% saturation. The precipitate formed was suspended in 10 mM Tris-Cl (pH 8.0) and applied onto an Ultrogel AcA 54 column (2.0×85 cm, BioSepra, France). The enzyme active fractions were applied to a DEAE-Toyopearl column (TOSO, Japan) and the enzyme was eluted with 10–100 mM Tris-Cl (pH 8.0). About 20-fold purification of the recombinant protein was achieved and the homogeneity was confirmed following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with 2D-SILVER STAIN·II 'DAIICHI' (Daiichi Pure Chemicals, Japan). The final purified enzyme was suspended in buffer A. The recombinant protein had no amino acid residues additional to those of the native protein.

A polyclonal antibody against human PGFS was raised in a rabbit with the purified recombinant enzyme used as the immunogen. Western blot analysis of a sample of each purification step of the recombinant enzyme was conducted, as described previously [11], by use of the anti-human PGFS antiserum.

2.4. Enzyme assay

The PGD_2 11-ketoreductase, PGH_2 9,11-endoperoxide reductase and reductase activities towards various compounds containing a keto group, such as PQ, of the recombinant protein were measured as described previously [5], except that 0.1 M Tris-HCl (pH 8.5) was used instead of 0.1 M potassium phosphate buffer (pH 6.5). The oxidase activities towards 9α,11β-PGF_2 and various hydroxysteroids were measured spectrophotometrically at 37°C by following the increase in absorbance at 340 nm in the assay mixture consisting of 0.1 M Tris-HCl (pH 10.0), 80 μM $NADP^+$, various concentrations of each substrate and enzyme in a total volume of 0.5 ml. Protein was determined according to the method of Lowry et al. [19].

2.5. Immunoabsorption of PGD_2 11-ketoreductase activity in human lung

Human lung tissue was obtained from a patient at the Shiga Medical Center for Adults after informed consent was obtained as a part of routine diagnostic procedures. The cytosol fraction of the tissue (650 μg) was incubated overnight with various concentrations of the anti-human PGFS antiserum (0–200 μg) at 4°C. After addition of protein G Sepharose (20 μl), the mixture was incubated at 4°C for 1 h and centrifuged at $10\,000\times g$ for 15 min. PGD_2 11-ketoreductase activity of the supernatant was measured as described above.

2.6. RT-PCR of human PGFS mRNA

Peripheral blood was obtained from healthy volunteers and lymphocyte suspensions were prepared by use of Lymphoprep (Nycomed Pharma AS, Norway). Peripheral blood lymphocytes (PBL, 10^7 cells/well) were incubated for 24 h with or without 25 μg/ml concanavalin A (Con A) (Seikagakukogyo, Japan). Total RNA was prepared from PBL and human lung and RT-PCR of mRNAs was carried out with PGFS-specific primers: PGFS-F4, 5′-GGTGAGGAACTTTCACCAAC-3′ and PGFS-R5, 5′-GGTTGAAGTTTGACACCCCA-3′.

3. Results

3.1. cDNA cloning of human PGFS

Screening of 4×10^6 plaques of a human lung cDNA library with the cDNA probe for bovine lung PGFS gave 20 positive clones. DNA sequencing confirmed that one clone harbored a full-length cDNA for human PGFS, because the deduced amino acid sequence of human PGFS (Fig. 1) showed 76% and 78% identities with those sequences of bovine lung and liver PGFSs, respectively. As shown in Fig. 1, λ-hLuFS contained an initiation codon and a polyadenylation signal after the stop codon with an open reading frame of 969 bp encoding 323 amino acids. The calculated molecular weight of the human PGFS was 36 842 Da, a value similar to those values of bovine lung and liver PGFSs, which are about 36 kDa [7,11]. This enzyme showed a high identity in amino acid sequence with not only bovine lung and liver PGFSs but also human liver dihydrodiol dehydrogenase (DD) 1 (88%) [20], DD2 (87%) [20] and DD4 (84%) [21]. Moreover, its sequence was identical to that of KIAA0119 [12].

3.2. Characterization of recombinant human PGFS

The recombinant protein was expressed in *E. coli* (HB101) transformed with pUC-hLuFS and was purified to apparent homogeneity as described under Section 2. About 20-fold purification of the PGD_2 11-ketoreductase activity was achieved from the lysate of *E. coli* with a yield of 32%. The specific activity of the purified recombinant enzyme was 2 μmol/min/mg protein. A sample of each purification step was subjected to SDS-PAGE. Silver staining of the gel indicated that a 36.8 kDa protein was produced in the cells harboring pUC-hLuFS and that the enzyme was purified to apparent homogeneity (Fig. 2A). Western blot analysis of each sample revealed that the 36.8 kDa protein was recognized by anti-human PGFS antibody (Fig. 2B). The molecular weight of the expressed enzyme was almost the same as that of the recombinant bovine lung PGFS [8]. No protein from the control *E. coli* bearing pUC8 vector without the insert DNA interacted with this antibody (data not shown). The N-terminal amino acid sequence of the expressed recombinant protein was identical to that deduced from the λ-hLuFS cDNA without any additional amino acid residues.

Next, we examined the enzymatic properties of the purified recombinant protein. The optimal pH for the reduction of PGD_2 was pH 8–9, different from that (pH 6.5) for that of bovine lung and liver PGFSs, and the optimum for the oxidation of 9α,11β-PGF_2 was pH 9–10, similar to that of both bovine PGFSs. The substrate specificity of the purified recombinant PGFS was examined at pH 8.5 for the reductase activity and at pH 10 for the oxidase activity. As shown in Table 1, the K_m and k_{cat} values for PQ were 2 μM and 130 min^{-1}, respectively, giving the highest k_{cat}/K_m value (138 000 min^{-1} mM^{-1}). Although the k_{cat} value for PGD_2 was 1/4 of that for PQ, the K_m value for PGD_2 was 3 μM, almost the same as that for PQ, giving a high k_{cat}/K_m value (21 200).

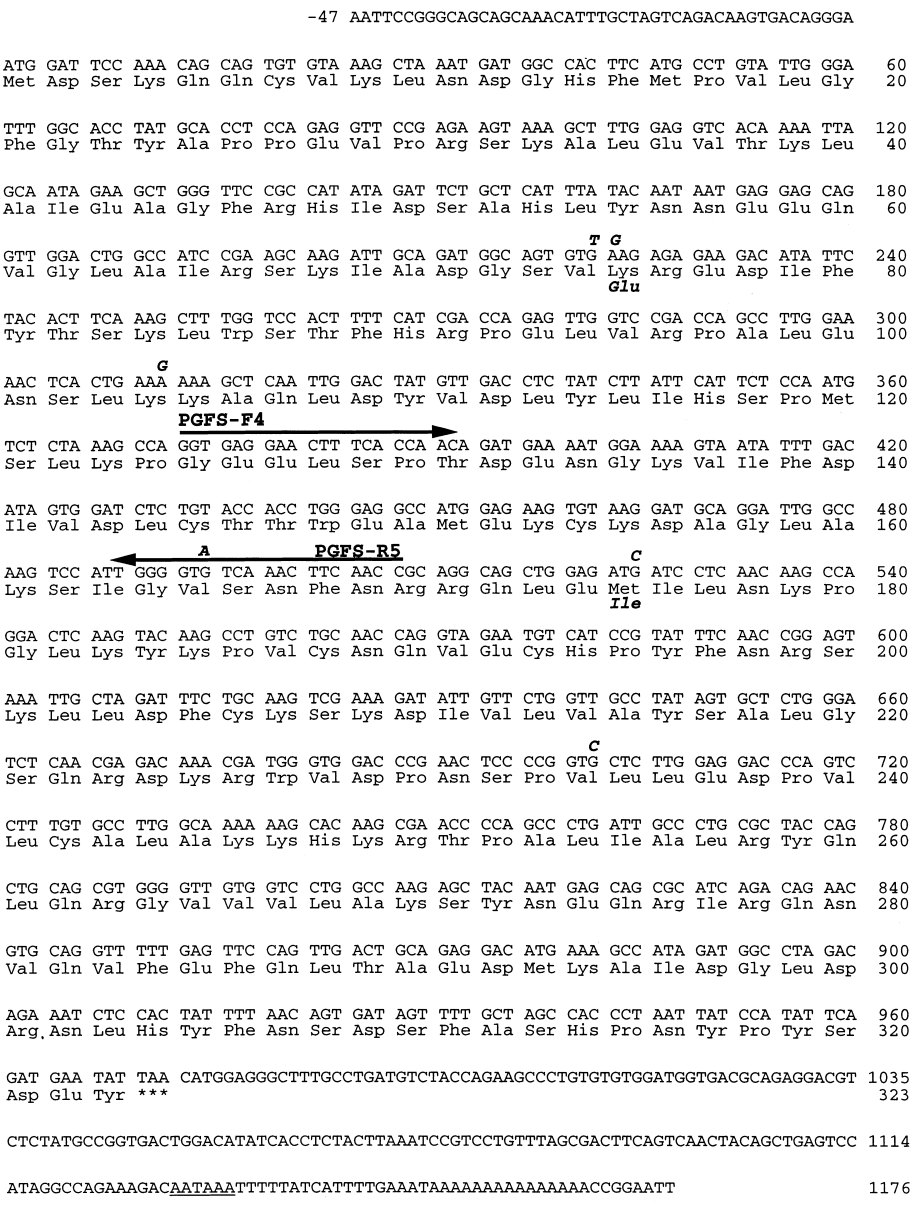

Fig. 1. Nucleotide sequence and deduced amino acid sequence of human PGFS. The asterisks indicate the stop codon and the underlined letters in the nucleotide sequence show the polyadenylation signal. The arrows indicate PGFS-specific primers used for RT-PCR of human PGFS mRNA. The bold letters are the nucleotides and the amino acid residues of AKR1C3 different from those of human PGFS.

However, the reduction of NB exhibited a high K_m value (100-fold of the K_m value for PQ) and low k_{cat} value (0.6-fold of the k_{cat} value for PQ), giving a low k_{cat}/K_m value. On the other hand, although the oxidation of 9α,11β-PGF_2 to PGD_2 showed a high K_m value (130 μM), the k_{cat} value was the highest among the substrates, giving the k_{cat}/K_m value of about 1800 min^{-1} mM^{-1}, following those of PQ and PGD_2. Moreover, the expressed human PGFS catalyzed the reduction of PGH_2 to $PGF_{2\alpha}$ and the reductase activity was 8% of that towards PGD_2, indicating that human PGFS is also a dual function enzyme. PGE_2 did not serve as a substrate for the enzyme. Various steroid compounds serve as the natural substrates for the enzymes of the AKR family [22]. However, the k_{cat}/K_m value for 5β-androstane-3,17-dione of human PGFS was only 0.4% of that for PQ and that of the reverse reaction with a substrate of 5β-androstan-3α-ol,17-one was 0.2%. These results suggest that PGD_2 and 9α,11β-PGF_2, but not steroids, are the best substrates among natural substances for human PGFS.

3.3. Expression of human PGFS in lung and PBL

To clarify the physiological roles of PGFS in humans, we used the anti-human PGFS antibody to examine the contribution of PGFS to PGD_2 11-ketoreductase activity in human lung. The cytosol fraction of human lung (lane 2 in Fig. 2C) gave a band at 36.8 kDa that cross-reacted with the anti-human PGFS antibody, the same position as the band of the purified recombinant PGFS (lane 1 in Fig. 2C). The PGD_2 11-ketoreductase activity in the cytosol of human lung was 0.04 nmol/min/mg protein. As shown in Fig. 3, whereas the control serum was almost ineffective, the activity was dose-dependently absorbed by anti-human PGFS antise-

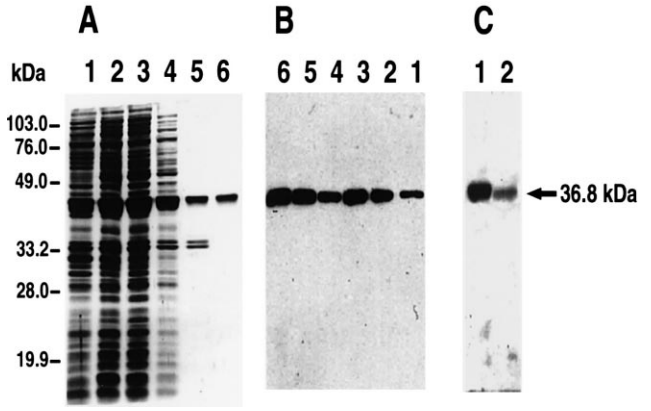

Fig. 2. SDS-PAGE (A) and Western blot analysis (B) of each purification step of the recombinant human PGFS and Western blot analysis (C) of the cytosol fraction of human lung. (A) SDS-PAGE (12%) silver stain and (B) Western blot analysis using the antiserum against the purified recombinant human PGFS: the homogenate (lane 1, 5 μg for A and 0.2 μg for B), the supernatant at $10\,000\times g$ (lane 2, 2 μg for A and 0.1 μg for B), the supernatant fraction at $100\,000\times g$ (lane 3, 2 μg for A and 0.1 μg for B), the ammonium sulfate fraction (lane 4, 2 μg for A and 0.1 μg for B), gel filtration fraction (lane 5, 0.2 μg for A and 0.1 μg for B) and DEAE-Toyopearl fraction (lane 6, 0.1 μg for A and 0.05 μg for B) were loaded into the indicated lanes. (C) Western blot analysis of the purified recombinant PGFS and of the cytosol fraction of human lung by use of the antiserum against the recombinant human PGFS. Shown are the results for the DEAE-Toyopearl fraction of the recombinant human PGFS (lane 1) and the supernatant fraction of human lung from the $100\,000\times g$ centrifugation (lane 2). The positions of the molecular mass standards are shown: phosphorylase b (103 000), bovine serum albumin (76 000), ovalbumin (49 000), carbonic anhydrase (33 200), soybean trypsin inhibitor (28 000) and lysozyme (19 900).

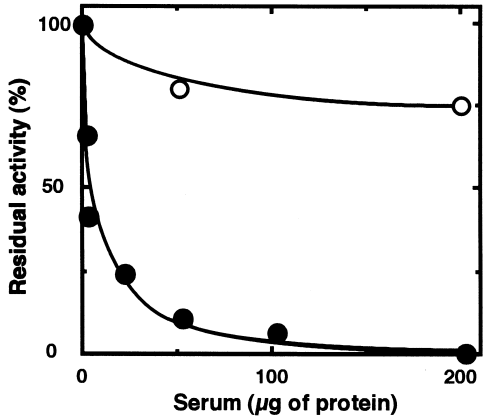

Fig. 3. Immunoabsorption of PGD_2 11-ketoreductase activity in human lung. Various concentrations of the anti-human PGFS antiserum (●) or normal rabbit serum (○) were incubated with the cytosol fraction of the human lung (650 μg), prepared as described in Section 2. The residual activity of PGD_2 11-ketoreductase was determined and is shown as 100% of the initial activity (0.04 nmol/min/mg protein).

rum and was completely lost at 100 μg of serum. These results suggest that λ-hLuFS is a clone of human PGFS and that PGD_2 11-ketoreductase activity in human lung is ascribed to the PGFS cloned here.

RT-PCR primers specific for human PGFS were designed on the basis of the cDNA sequence of PGFS shown in Fig. 1, because human DDs of the AKR superfamily show more than 80% homology between each other. As shown in Fig. 4A, the expected 136 bp band was specifically amplified from lung total RNA by RT-PCR with the PGFS-specific primers (PGFS-F4 and R5). Sequencing of the amplified DNA indicated it to be that of human PGFS and not that of other DDs. We also found that PGFS mRNA was expressed in PBL as well as lung. As PGD_2 is increased in alveolar lymphocytes as well as in macrophages, eosinophils and mast cells after a challenge with an inflammatory antigen [23–25], we examined the effect of Con A, which is considered to be a T lymphocyte-specific mitogen [26], on expression of PGFS mRNA in PBL. As shown in Fig. 4B, 24 h incubation of

Table 1
Substrate specificity of the purified recombinant human PGFS

Substrates	Substrate concentration (μM)	%	K_m (μM)	V_{max} (μmol/min/mg protein)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Reduction						
5β-Androstane-3,17-dione	100	4	3.1	0.05	1.9	640
5α-Androstane-3,17-dione	100	1				
PQ	5	222	0.8	3.0	111	138 000
p-NB	100	100	77.4	1.8	65	840
PGD_2	900	154	3.4	2.0	72	21 200
PGH_2	40	20				
PGE_2	900	n.d.[a]				
Oxidation						
Acenaphtal	100	0.7				
Dihydrotestosterone	100	0.1				
cis-Androsterone	100	0.5				
5β-Androstan-3α-ol,17-one	50	1.1	1	0.01	0.3	310
5β-Pregnane-3α,20α-diol	10	1				
5α-Pregnane-3α,20α-diol	50	0.4				
9α,11β-PGF_2	150	307	134	6.56	242	1 810
$PGF_{2\alpha}$	150	1.2				

The enzyme activities with various substrates were measured under standard conditions including the enzymes (0.2–1.0 μg/0.5 ml of assay system) as described under Section 2. Enzyme assays were performed initially at substrate concentrations indicated by 'Substrate concentration'. When enzyme activities were sufficiently high, kinetic constants, K_m, V_{max} and k_{cat}, were determined. The NB reductase activity (1.8 U/mg protein) represents 100% activity.
[a]n.d., not detected.

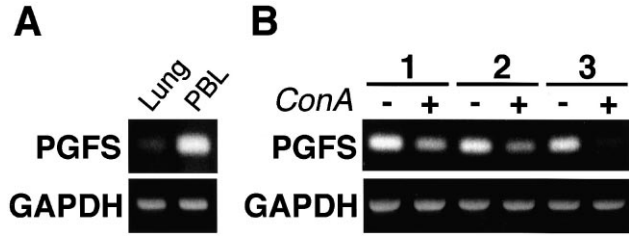

Fig. 4. (A) Detection of PGFS mRNA in human lung and PBL. Total RNA was prepared from human lung and PBL and subjected to RT-PCR with PGFS primers or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers as a control. RNA preparation and RT-PCR were performed as described under Section 2. Sizes of the amplified DNAs are 136 bp (PGFS) and 461 bp (GAPDH). (B) Decrease in PGFS mRNA level of PBL by treatment with 25 μg/ml Con A for 24 h. PBL were prepared from peripheral blood of three healthy volunteers (1, 2 and 3) and were incubated for 24 h in the absence (−) or presence (+) of Con A (25 μg/ml). Total RNA from PBL was subjected to RT-PCR with PGFS or GAPDH primers.

PBL with Con A almost completely suppressed the expression of PGFS mRNA in PBL from one volunteer (no. 3) and had smaller effects on that of the mRNA in the two other volunteers.

4. Discussion

We have reported here the cloning of human PGFS cDNA from human lung with bovine lung PGFS cDNA as a probe and the result of the enzymatic characterization of the protein expressed in *E. coli*. Moreover, we examined the expression of PGFS in human lung and PBL. The expressed protein showed PGFS activity and anti-human PGFS antiserum completely absorbed the PGD_2 11-ketoreductase activity associated with PGFS in the cytosol fraction of human lung.

We characterized the enzymatic properties of the recombinant PGFS in detail. The present study indicates that human PGFS had the highest catalytic efficiency for PQ, followed by PGD_2 and 9α,11β-PGF_2, but that steroids did not serve as substrates for this enzyme (Table 1). The k_{cat}/K_m value for PGD_2 was 11.7-fold higher than that for 9α,11β-PGF_2 and was 68.4-fold or more than those for various steroids. Human PGFS had a carbonyl reductase activity similar to that of bovine PGFS. However, PGD_2 and 9α,11β-PGF_2 were the natural substrates for human PGFS and the enzyme preferred the reduction of PGD_2 to the oxidation of 9α,11β-PGF_2. The K_m value for PGD_2 was about 3 μM, indicating that the enzyme has the high affinity for PGD_2. Arg^{91} and Arg^{223} were conserved between human lung PGFS and bovine liver PGFS, the latter of which also has a low K_m value (10 μM) for PGD_2 [11]. When Arg^{91} and Arg^{223} of bovine liver PGFS were changed to the Gln and Leu, respectively, of bovine lung PGFS, which has a high K_m value for PGD_2 (120 μM), the K_m values of these mutants were increased from 15 μM to 145 μM and 180 μM, respectively [11]. Our present finding of a low K_m value for human PGFS agrees with our previous finding [11] that Arg^{91} and Arg^{223} were the essential amino acid residues for a low K_m value.

PGFS belongs to the AKR superfamily classified by Jez et al. [22], based on the similarity of the amino acid sequence. AKR1C3 was reported to be human liver 3α-HSD type II [13,14]. The deduced amino acid sequences of human PGFS and the cDNA clone KIAA0119 [17] were identical and differed by only two residues (Lys^{75} and Met^{175}) from the sequence reported for AKR1C3 [13]. Matsuura et al. [15] reported that AKR1C3 prepared by site-directed mutagenesis of KIAA0119 showed the same enzymatic properties as the expressed protein of KIAA0119 and that AKR1C3 acted as a PGD_2 11-ketoreductase. This report suggests that Lys^{75} and Met^{175} have no effect on the enzyme activity. Although PGD_2 11-ketoreductase activity is associated with PGFS, they did not report whether KIAA0119 and AKR1C3 have the enzymatic properties of PGFS described previously [5]. The results on human PGFS in this paper indicate that AKR1C3 acts as PGFS and these results together with the reports described above suggest that PGFS is involved in metabolism of xenobiotics as well as PGD_2 and 9α,11β-PGF_2 in human liver.

PGD_2 is the major cyclooxygenase metabolite produced by mast cells in response to IgE-dependent stimulation and it is considered to be an important mediator in various allergic diseases such as allergic rhinitis, atopic asthma, allergic conjunctivitis and atopic dermatitis [27]. Although there are numerous reports on the contributions of PGD_2 to the pathogenesis of various allergic diseases on the basis of local production of PGD_2 and of increased urinary excretion of 9α,11β-PGF_2 after an allergen challenge [28], this study provides the first experimental evidence that PGFS is involved in the metabolism of PGD_2 in human tissues. More responsiveness to PGD_2 in asthmatic and atopic subjects than in normal subjects was previously ascribed to a cholinergic mechanism [24]. Here, we demonstrated that the expression of PGFS mRNA was markedly suppressed by a T cell mitogen, Con A, in three volunteers. Since PGFS mRNA is highly expressed in lymphocytes and the human mast cell is capable of releasing cytokines that have the capacity to initiate and maintain a chronic inflammatory response, the present study raises the possibility that PGFS expressed in lymphocytes may modulate the metabolism of PGD_2 in an inflammatory site and contribute to the pathogenesis of patients with allergic diseases. It is interesting that the PGFS gene has been assigned to chromosome 10 p14-p15, close to the α-chain genes of IL-2 and IL-15 receptors [29] (data not shown), in agreement with the placement of KIAA0119 cDNA at the sequence-tagged site SGC31479 between WI-5839 and WI-598 at the top of human chromosome 10 [12]. Cloning of human PGFS cDNA and the development of RT-PCR specific for PGFS may provide the tools required for in vivo and in vitro studies to further understand the gene regulation of PGFS in human tissues and pathophysiological roles of this enzyme in allergic diseases.

Acknowledgements: We are grateful to Fuso Pharmaceutical Industries for oligonucleotides. This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from Sankyo Foundation of Life Science.

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