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Expression of lipocalin-type prostaglandin D synthase (β-trace) in human heart and its accumulation in the coronary circulation of angina patients

(prostaglandin D_2/myocardial cells/endocardial cells/smooth muscle cells/atherosclerosis)

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ABSTRACT **Lipocalin-type prostaglandin D synthase (L-PGDS) is localized in the central nervous system and male genital organs of various mammals and is secreted as β-trace into the closed compartment of these tissues separated from the systemic circulation. In this study, we found that the mRNA for the human enzyme was expressed most intensely in the heart among various tissues examined. In human autopsy specimens, the enzyme was localized immunocytochemically in myocardial cells, atrial endocardial cells, and a synthetic phenotype of smooth muscle cells in the arteriosclerotic intima, and accumulated in the atherosclerotic plaque of coronary arteries with severe stenosis. In patients with stable angina (75–99% stenosis), the plasma level of L-PGDS was significantly ($P < 0.05$) higher in the great cardiac vein (0.694 ± 0.054 μg/ml, $n = 7$) than in the coronary artery (0.545 ± 0.034 μg/ml), as determined by a sandwich enzyme immunoassay. However, the veno-arterial difference in the plasma L-PGDS concentration was not observed in normal subjects without stenosis. After a percutaneous transluminal coronary angioplasty was performed to compress the stenotic atherosclerotic plaques, the L-PGDS concentration in the cardiac vein decreased significantly ($P < 0.05$) to 0.610 ± 0.051 μg/ml at 20 min and reached the arterial level within 1 h. These findings suggest that L-PGDS is present in both endocardium and myocardium of normal subjects and the stenotic site of patients with stable angina and is secreted into the coronary circulation.**

Prostaglandin (PG) D_2 is actively formed in a variety of tissues and cells (1), and is involved in many physiological events (2); PGD_2 regulates sleep (3, 4) and ocular pressure (5), prevents platelet aggregation (6), and induces vasodilation and bronchoconstriction (7).

Two distinct types of enzymes have been characterized as PGD synthase (PGDS), which catalyzes the isomerization of PGH_2, a common precursor of various prostanoids, to PGD_2 (8). One enzyme is glutathione independent, the lipocalin-type PGDS (L-PGDS) (9); the other is glutathione requiring, the hematopoietic PGDS (10–12). Human albumin also catalyzes the conversion of PGH_2, which is released from platelets, to PGD_2 and has thus been proposed to contribute PGD_2 to the anticoagulant system (13). Human platelets do not contain any type of PGDS, whereas their progenitor cells, the megakaryocytes, express the hematopoietic PGDS (14).

L-PGDS is localized in the central nervous system (1, 9), retina (15), and male and female genital organs (1, 16, 17) of various mammals. The enzyme was originally purified from rat brain as a 26-kDa glycoprotein (9). The cDNAs and genes for the rat and human enzymes have been isolated (18–21). A homology search in various databases of protein primary structure revealed that the enzyme is a member of the lipocalin superfamily (19–21), a group of secretory proteins, such as retinol-binding protein and β-lactoglobulin (22), that bind and transport a variety of lipophilic molecules.

In 1961, Clausen (23) discovered β-trace as a major protein in human cerebrospinal fluid; it was recently found to be identical to human L-PGDS (24, 25). Several studies by our group and others revealed that L-PGDS is actively secreted not only into the cerebrospinal fluid as β-trace but also into the interphotoreceptor matrix (15), aqueous and vitreous humor (26), seminal plasma (17, 27), and several other body fluids (28, 29). L-PGDS was also detected in human serum at concentrations of 0.2 to 0.4 μg/ml, which are 2 to 4% of those in the cerebrospinal fluid (11–15 μg/ml). However, the origin of this serum L-PGDS remains unclear.

In this study, we found that mRNA for human L-PGDS (β-trace) was most intensely expressed in the heart among various tissues examined and that the immunoreactivity of the enzyme was localized in myocardial cells, atrial endocardial cells, and the synthetic state of smooth muscle cells in the arteriosclerotic plaques. We also showed that the enzyme was secreted into and accumulated in the plasma of the coronary circulation of angina patients.

MATERIALS AND METHODS

Northern Blotting. A multiple-tissue Northern blot containing 2 μg of poly(A)$^+$ RNA from human tissues (CLONTECH) was probed with ^{32}P-labeled cDNAs for human L-PGDS (19) and glyceraldehyde-3-phosphate dehydrogenase. Total RNAs were isolated from monkey (*Macaca mulatta*) heart and brain, which were kindly provided by T. Yamashima (Kanazawa University School of Medicine) and H. Onoe (Osaka Bioscience Institute). The total RNAs were denatured with 1 M of glyoxal in 50% dimethyl sulfoxide, electrophoresed on a 1.5% agarose gel, and transferred to a Biodyne transfer membrane (Pall Ultrafine Filtration, Glen Cove, NY).

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Abbreviations: PG, prostaglandin; PGD_2, prostaglandin D_2; PGDS, PGD synthase; L-PGDS, lipocalin-type PGDS; PTCA, percutaneous transluminal coronary angioplasty.

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Tissue Preparation. We obtained atria, ventricles, and coronary arteries of human heart from autopsy specimens. Samples obtained after extensive (>30 min) cardiopulmonary resuscitation were excluded from immunohistochemical studies. Immediately after removal, the tissues were fixed with 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) at 4°C for 6 h followed by the same fixative adjusted to pH 3.5 at 4°C for 4 h, and then were soaked in 20% (wt/vol) sucrose in PBS (pH 7.4) at 4°C overnight. The specimens were dehydrated, embedded in paraffin, and cut into 4-μm thick sections. The sections were mounted on poly-L-lysine-coated slides and subjected to immunohistochemical staining.

Immunohistochemistry. In this study, we used two different monoclonal antibodies against human L-PGDS, Mab 1B7 and 7F5, which recognized different antigenic epitopes of the enzyme, and a rabbit polyclonal antibody raised against recombinant human L-PGDS. These antibodies were prepared as described previously (28, 29). Deparaffinized sections were digested with 0.3% (wt/vol) pepsin (Sigma) in 0.01 M HCl for 5 min at room temperature to unmask the antigens and incubated at 4°C overnight with 4 μg/ml of monoclonal antibody or 10 μg/ml of polyclonal antibody in PBS containing 0.1% (vol/vol) goat normal serum and 0.05% (vol/vol) Triton X-100. Immunohistochemical staining was performed with a Histofine kit (Nichirei, Tokyo) according to the manufacturer's instruction. The immunoreactivity was visualized with a H_2O_2-supplemented aminoethylcarbazole chromogen. The sections were counterstained with hematoxylin. For the control experiments, pre-immune mouse or rabbit IgGs were used as the primary antibody. The absorbed antibody was prepared by incubation of the polyclonal antibody with excess amounts of the recombinant human L-PGDS (1 mg/ml) (30) at 4°C overnight and used as another control. The tissue sections were also immunostained with monoclonal antibodies against CD34 (Nichirei), von Willebrand factor, CD68, and alpha-smooth muscle actin (Dako) to identify myofibroblasts, endothelial cells, macrophages, and smooth muscle cells, respectively.

Blood Sampling and Percutaneous Transluminal Coronary Angioplasty (PTCA). Patients who complained of chest pain were diagnosed by coronary angiography. An angioplasty-guiding catheter was inserted into the femoral artery and advanced to the ascending aorta at a position of the orifice of the left coronary artery. Another catheter was inserted through the right brachial vein into the great cardiac vein. In patients with stable angina, a PTCA balloon was inserted at the stenotic sites and then inflated twice for 2 min each with a 3-min reperfusion interval. The stenotic sites were successfully dilated to <25% stenosis in all cases. Immediately before and 2 min, 5 min, 7 min, 20 min, 1 h, 2h, and 4 h after PTCA, blood was collected simultaneously through the inserted catheters from the great cardiac vein and the ascending aorta into tubes containing 3.8% trisodium citrate. Blood was also sampled from the peripheral vein 1, 2, and 7 days after operation. The blood samples were immediately centrifuged at 1,500 × *g* for 15 min at room temperature. The plasma samples were stored at −70°C until analyzed. The plasma concentration of L-PGDS was determined by the sandwich enzyme immunosorbent assay with Mab 1B7 and 7F5 as described previously (28). We obtained informed consent from all patients in this study. Blood sampling at the catheterization laboratory was approved by the Institutional Committee at Ishinkai Yao General Hospital. All manipulations of human tissues and plasma were approved by the Committee for the Protection of Human Subjects at Osaka Bioscience Institute.

Statistical Analysis. Data were expressed as the mean ± SE. Statistical analyses were carried out by Student's *t* test and chi-squared test contained in the STAT VIEW program. Differences at $P < 0.05$ were considered to be statistically significant. The characteristics of the patient groups were compared using the χ^2 test, and differences at two-sided *P* values below 0.05 were considered significant.

RESULTS

Expression of L-PGDS mRNA in Human and Monkey Hearts. When we examined the expression of mRNA for L-PGDS in various human tissues by Northern blot analysis (Fig. 1*A*), the signal was detected most intensely in the heart, moderately in the brain, and very weakly in the placenta, lung, liver, skeletal muscle, kidney, and pancreas, at a position corresponding to a polynucleotides of approximately 1.3 kb in length. The expression of the mRNA in the heart was unexpectedly much higher than that in any other tissues including the brain.

We then examined the expression of mRNA for L-PGDS among various cardiovascular tissues of monkeys (Fig. 1*B*). The mRNA was widely distributed in the right and left atria, both ventricles, interventricular septum, and the aorta. However, the intensity of the signal in those tissues was clearly weaker than that in the brain cortex, indicating that the tissue distribution profile of the expression of the transcript was distinct between humans and monkeys.

Immunohistochemical Localization of L-PGDS in Human Heart. Using immunostaining human autopsy specimens with monoclonal or polyclonal antibodies against human L-PGDS, we could see that immunoreactivity was localized in both atrial and ventricular myocardial cells (Fig. 2 *A* and *C*). The immunoreactivity for L-PGDS was also observed in the endocardium and diffusely found in the extracellular matrix of the endocardium (Fig. 2*D*). In high-magnification views, it was detected in the cytoplasm of endocardial cells (Fig. 2*E*), which were identified as such in an adjacent section stained with anti-von Willebrand factor antibody, a marker for endothelial cells (Fig. 2*F*).

The immunoreactivity for L-PGDS was hardly observed in the coronary arterial wall of normal subjects (Fig. 3*A*). Smooth muscle cells were stained in the media with anti-alpha-smooth muscle actin antibody in the adjacent section (Fig. 3*B*). Intense immunoreactivity for L-PGDS was detected in the early arteriosclerotic plaque in the intima of the coronary artery (Fig. 3*C*) and in the ventricular endocardium (Fig. 3*D*). The L-PGDS-positive cells were also immunostained in the adjacent sections with anti-alpha-smooth muscle actin antibody (Fig. 3*E*) but neither with anti-CD34 antibody, a marker for myofibroblasts, nor with anti-CD68 antibody, a marker for macrophages (data not shown). The L-PGDS-positive cells in the

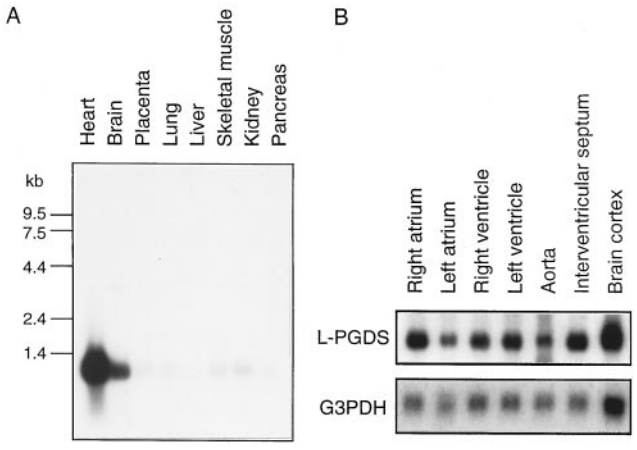

FIG. 1. Tissue specificity of gene expression of L-PGDS (β-trace). (*A*) Poly(A)$^+$ RNA of various human tissues (2 μg per lane) was analyzed by the Northern blot assay. (*B*) Total RNA of various monkey tissues (10 μg per lane) was probed with cDNAs for human L-PGDS (*Upper*) and glyceraldehyde-3-phosphate dehydrogenase (*Lower*).

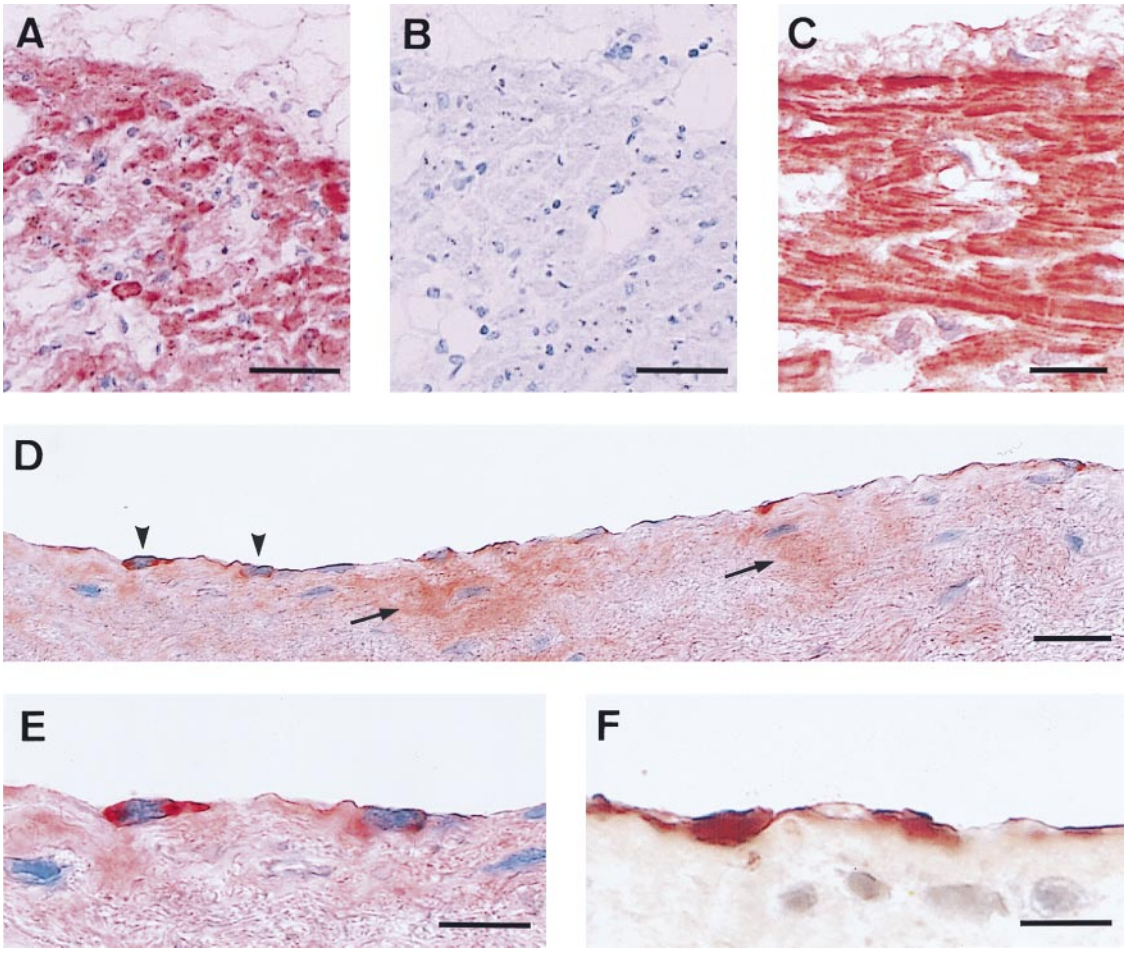

FIG. 2. Immunohistochemical demonstration of L-PGDS (β-trace) in the left atrium (*A, B, D, E,* and *F*) and right ventricle (*C*) of human heart. The tissue sections were immunostained with monoclonal antibodies against L-PGDS (*A, C, D,* and *E*) or von Willebrand factor (*F*), or with nonimmunized mouse IgG (*B*). (*D*) Arrowheads and arrows indicate the L-PGDS-immunoreactive endocardial cells and the immunoreactivity accumulated in the extracellular matrix, respectively. (Bars = 50 μm in *A* and *B*, 20 μm in *C* and *D*, and 10 μm in *E* and *F*.)

plaque were, therefore, identified as smooth muscle cells in the synthetic phenotype rather than as those in the contractile phenotype.

In the advanced atherosclerotic plaque (>75% stenosis) of a coronary artery with an area of intimal fibrosis containing a lipid core with calcification, the immunoreactivity indicated that L-PGDS had accumulated in the intima, but not in the media, and that the fibrous plaques had become enriched in the enzyme (Fig. 3*F*). A high-magnification view of the fibrous cap revealed that the L-PGDS-immunoreactivity was present along collagen fibers (Fig. 3*G*) in the vicinity of smooth muscle cells immunostained with anti-alpha-smooth muscle actin antibody (Fig. 3*H*).

Two different monoclonal and polyclonal antibodies against human L-PGDS showed essentially identical immunostaining profiles. When IgGs obtained from non-immunized animals or the polyclonal antibodies preabsorbed with excess amounts of the purified enzyme were used instead of the primary antibody, no positive immunostaining was detected (Fig. 2*B*).

Accumulation of L-PGDS (β-Trace) in Plasma During Coronary Circulation of Patients with Stable Angina. L-PGDS is produced in the leptomeninges (31), retinal pigmented epithelium (15), and epididymal tubular epithelial and basal cells (16), and is secreted into the cerebrospinal fluid, interphotoreceptor matrix, and seminal plasma, respectively. Therefore, we predicted that the enzyme may also be secreted from the human heart into the plasma. To examine this possibility, we determined the L-PGDS concentration in the plasma collected from the orifice of the left coronary artery and great cardiac vein during coronary angiography for clinical diagnosis.

The patients were classified into two groups: patients with stable angina and normal subjects; clinical features are listed in Table 1. There were no statistical differences between these two groups in terms of age, serum levels of total cholesterol and triglyceride, and past history of hypertension, diabetes mellitus, and habit of smoking tobacco. The serum levels of glutamic-oxaloacetic transaminase, lactate dehydrogenase, and creatine kinase were also not different between the two groups.

In control subjects with normal coronary angiography, the level of plasma L-PGDS was 0.536 $\pm$ 0.027 μg/ml at the entrance of cardiac artery and 0.540 $\pm$ 0.021 μg/ml in the great cardiac vein, respectively (Table 1), showing no difference between these two sites. However, in patients with stable angina (75 to 99% stenosis), the L-PGDS concentration in the great cardiac vein (0.694 $\pm$ 0.054 μg/ml) was significantly ($P <$ 0.01) higher than that at the entrance of the cardiac artery (0.545 $\pm$ 0.034 μg/ml), indicating that L-PGDS accumulates in the plasma during coronary circulation in patients with stable angina.

Disappearance of Coronary Veno-Arterial Difference in Plasma L-PGDS (β-Trace) Level After PTCA of Atherosclerotic Plaque. After PTCA of the angina patients (Fig. 4), the plasma level of L-PGDS in the great cardiac vein decreased significantly ($P < 0.05$) to 0.650 $\pm$ 0.047 μg/ml at 5 min and to 0.610 $\pm$ 0.051 μg/ml at 20 min. Within 1 h, the level (0.561 $\pm$ 0.034 μg/ml) reached almost the same value as the arterial

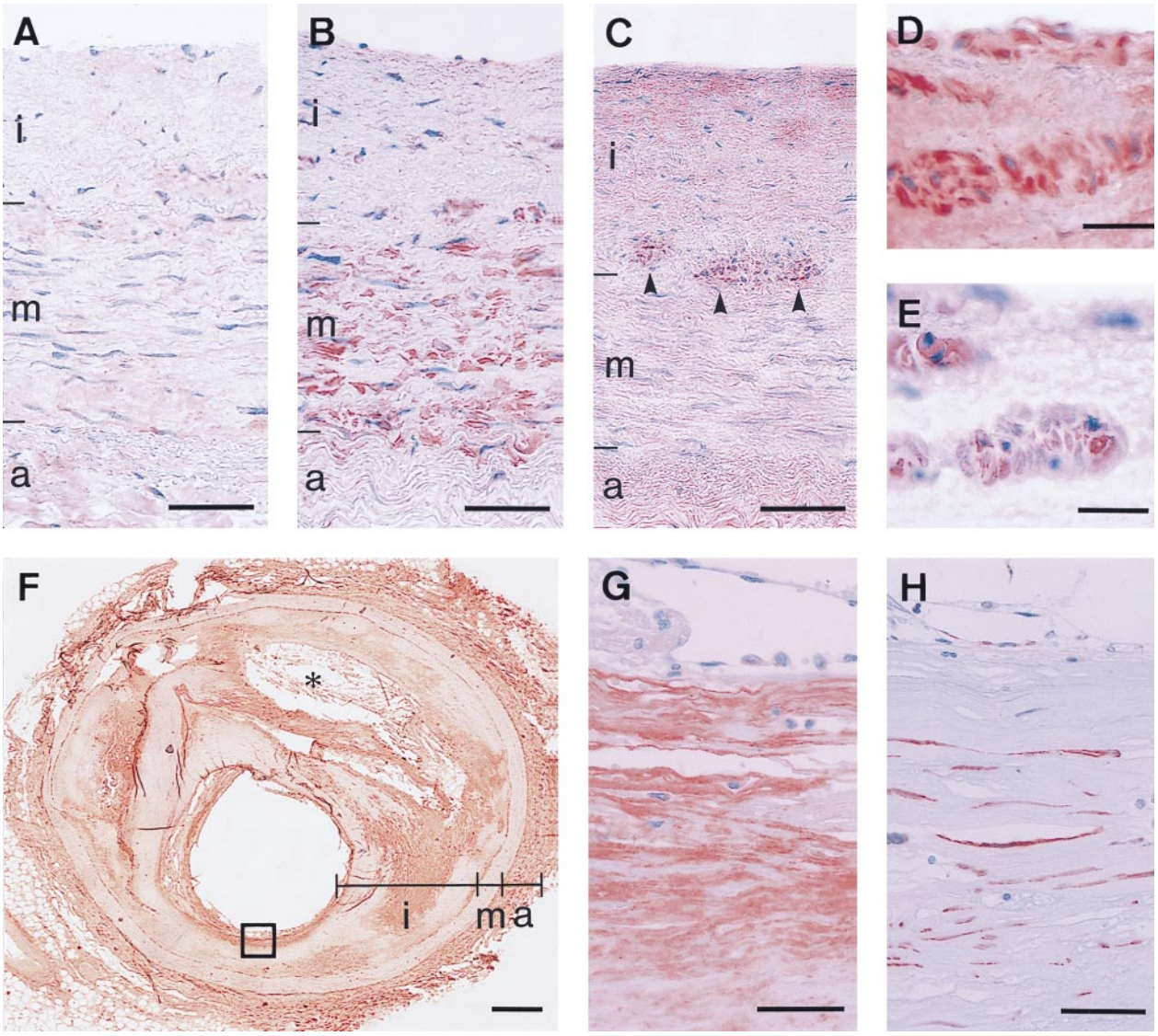

FIG. 3. Immunohistochemical demonstration of L-PGDS (β-trace) in the left (*A* and *B*) and right (*C, F, G,* and *H*) coronary arteries and the right ventricle (*D* and *E*) of human heart. The tissue sections were immunostained with monoclonal antibodies against L-PGDS (*A, C, D, F,* and *G*) and alpha-smooth muscle actin (*B, E,* and *H*). (*C*) Arrowheads indicate the early phase of an arteriosclerotic plaque in the intima. (*F*) In the advanced atherosclerotic coronary artery, calcification (*) and a lipid core including cholesterol clefts are observed. (*G*) A high-magnification view of a fibrous cap (squared in *F*). (Bars = 50 μm in *A, B, C, G,* and *H;* 20 μm in *D* and *E;* and 500 μm in *F*.) i, Intima; m, media; a, adventitia.

concentration, and maintained a plateau there up to 4 h (0.532 ± 0.040 μg/ml). In contrast, the serum levels of other clinical marker enzymes, such as glutamic-oxaloacetic transaminase, lactate dehydrogenase, and creatine kinase, remained unchanged after PTCA. The concentration of plasminogen activator inhibitor-1, a marker for perturbed endothelial cells, increased slightly after PTCA and returned to the pretreatment level within 2 h (data not shown).

The L-PGDS level in the peripheral vein was almost the same at 1 and 4 days after PTCA (0.533 ± 0.043 and 0.568 ± 0.041 μg/ml, respectively), which was identical to that in the great cardiac vein 4 h after PTCA, and significantly ($P < 0.05$) increased 7 days after the treatment (0.596 ± 0.042 μg/ml).

DISCUSSION

In this study, we demonstrated that the mRNA for L-PGDS was expressed most intensively in the heart among various human tissues (Fig. 1*A*) and that the immunoreactivity of L-PGDS was localized in myocardial cells of normal subjects (Fig. 2 *A* and *C*). Our finding that L-PGDS is localized in myocardial cells is consistent with the report that it is highly expressed in a rhabdomyosarcoma cell line (32). The L-PGDS-immunoreactivity decreased markedly in myocardial cells after extensive cardiopulmonary resuscitation (Y.E., unpublished results). These results suggest that L-PGDS is actively produced in beating myocardial cells and that its intracellular concentration decreases after a decrease in contraction. The immunoreactivity was also detected in atrial endocardial cells (Fig. 2 *D* and *E*) but not in endothelial cells of the coronary artery (Fig. 3*A*), although both types of cells were immunoreactive with anti-von Willebrand factor antibody (Fig. 2*F*). In the early arteriosclerotic specimens, L-PGDS was localized in the synthetic state of smooth muscle cells in the intimal and endocardial plaques (Fig. 3 *C* and *D*) which cells could be differentiated from those with the contractile phenotype. Smooth muscle cells in the contractile state were negative for L-PGDS, as seen in the media of the coronary artery of a normal subject (Fig. 3 *A* and *B*). Therefore, L-PGDS is considered to be a useful marker for identification of the functional or differentiation stages of myocardial, endocardial, and smooth muscle cells. Moreover, we considered plasma L-PGDS to be, in part, secreted from these cells.

Table 1. Clinical features, laboratory data, and plasma levels of L-PGDS of the study population

Case	Age	Sex	Site of narrowing in LAD	% stenosis	Serum T-cho	Serum TG	Past history HT	Past history DM	Past history Tobacco	L-PGDS content, μg/ml A	L-PGDS content, μg/ml V0	L-PGDS content, μg/ml V4
Patients with angina												
1	62	M	Prox	90	127	56	+	+	+	0.677	0.837	0.579
2	47	M	Prox	75–90	207	35	+	+	+	0.477	0.592	0.473
3	54	M	Prox	90	232	155	−	+	−	0.601	0.871	0.609
4	70	M	Prox	50–75	169	213	−	+	+	0.442	0.600	0.415
5	79	M	Mid	99	183	76	−	−	+	0.542	0.727	0.650
6	71	M	Prox	90	186	52	−	−	−	0.621	0.749	0.617
7	65	M	Prox	90	144	33	−	−	+	0.457	0.480	0.384
										0.545 ± 0.034	0.694 ± 0.054	0.532 ± 0.040
Normal subjects												
1	68	M			207	66	+	−	−	0.473	0.455	
2	59	M			214	292	+	−	+	0.488	0.525	
3	63	F			268	223	−	+	−	0.638	0.629	
4	50	F			179	61	−	−	−	0.530	0.513	
5	40	M			178	145	+	+	+	0.466	0.513	
6	55	M			166	147	−	−	+	0.533	0.568	
7	49	M			177	83	−	−	−	0.626	0.581	
										0.536 ± 0.027	0.540 ± 0.021	

The plasma levels of L-PGDS were determined by sandwich enzyme immunoassay of samples taken at the entrance of the coronary artery (A) and from the great cardiac vein simultaneously before PTCA (V0), and 4 h after PTCA (V4). Data are presented as the mean ± SE. M, male; F, female; LAD, left anterior descending branch of coronary artery; Prox, proximal; Mid, middle; T-cho, total cholesterol; TG, triglycerol; HT, hypertension; DM, diabetes mellitus.

The mRNA for L-PGDS was found to be expressed in the heart of humans (Fig. 1*A*), monkeys (Fig. 1*B*), and mice (N.E. and Y.U., unpublished results). However, in the rat heart, the expression was not detected by Northern blot analysis or reverse transcriptase-PCR analysis with poly(A)$^+$ RNA (N.E. and Y.U., unpublished results). Therefore, the gene expression of L-PGDS is considered to be regulated in a highly species-specific manner, similar to the case of the DP receptor, a prostanoid receptor for PGD_2 (33, 34). Such diversity of tissue-distribution profiles of the production and receptor systems for PGD_2 seems to reflect the species-specific functions of PGD_2 (35). For example, the antiaggregatory activity of PGD_2 is observed with human and monkey platelets, but not with rat platelets (6).

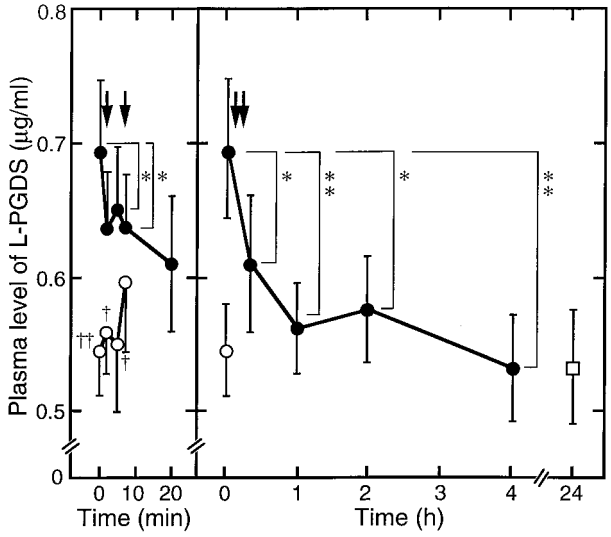

FIG. 4. Changes in the L-PGDS (β-trace) concentration in plasma obtained from the great cardiac vein (solid circles), the entrance of the coronary artery (open circles), and the peripheral vein (open square) before and after PTCA of patients with stable angina. Arrows indicate the inflation of a PTCA balloon. The data in the figure represent the average values ± SE. *, $P < 0.05$; **, $P < 0.01$ as compared with the L-PGDS level before PTCA. †, $P < 0.05$; ††, $P < 0.01$ as compared with the respective venous level.

In patients with stable angina, the plasma level of L-PGDS was significantly higher in the cardiac vein than in the coronary artery (Table 1). The L-PGDS concentration in the cardiac vein decreased immediately after compression of the plaque by PTCA (Table 1, Fig. 4). The apparent cancellation of the veno-arterial difference in plasma L-PGDS may be due to a dilution effect of the increased coronary blood flow after removal of stenosis by PTCA (36). Alternatively, release of L-PGDS from the myocardium and/or the synthetic type of smooth muscle cells may be up-regulated in regional ischemia due to coronary stenosis and downregulated after reperfusion. On the other hand, L-PGDS was found to accumulate within the fibrous plaque in the atherosclerotic stenotic lesions (Fig. 3 *F* and *G*). Therefore, the veno-arterial difference in plasma L-PGDS may also, in part, be due to secretion of L-PGDS from the atherosclerotic plaque into the coronary circulation. If any, mechanical damage to the L-PGDS-producing cells in the plaque by PTCA may explain the rapid decrease in the plasma level of L-PGDS in the coronary vein after balloon inflation.

Clinically, the acute occlusion by thrombosis often happens within several hours after PTCA, during which time the plasma L-PGDS concentration is decreasing in the cardiac vein (Table 1, Fig. 4). The occlusion hardly occurs 7 days after PTCA, when the plasma L-PGDS concentration has increased significantly as compared with that at 1 and 2 days after PTCA. Re-proliferation of smooth muscle cells in a cracked atherosclerotic plaque may contribute to an increase in the L-PGDS level in the peripheral vein. These results suggest that the disappearance of the homeostatic synthesis and secretion of L-PGDS in the atherosclerotic plaque after PTCA may result in stimulation of platelet aggregation mediated by loss of the antiaggregatory function of PGD_2. PGD_2 may function to protect against platelet aggregation in atherosclerotic blood vessels as does PGI_2, although its antiaggregatory potency is 3- to 10-fold weaker than that of PGI_2 (6). We recently found that in human seminal plasma increases in the L-PGDS level are correlated with increases in the PGD_2 level (37). Thus, the secretion of L-PGDS into plasma and its presence in myocar-

dial and other cells may be functionally correlated with levels of PGD_2 in plasma and the heart.

The immunoreactivity profile indicated that L-PGDS had accumulated in the lipid core of the advanced atherosclerotic plaque (Fig. 3*F*) and in the extracellular space of the fibrous cap (Fig. 3*G*), probably by secretion from neighboring smooth muscle cells in the synthetic state or by infiltration from the plasma. Alternatively, L-PGDS is likely upregulated in smooth muscle cells during dedifferentiation from the contractile phenotype to the synthetic state (Fig. 3). Although L-PGDS was originally purified as the enzyme responsible for biosynthesis of PGD_2 in the central nervous system (9), it has recently been characterized as an extracellular transporter of retinoids (38). Therefore, in the human heart and systemic circulation, L-PGDS may also act as a bifunctional protein, i.e., as a PGD_2-producing enzyme as well as an extracellular transporter of retinoids and/or several other possible lipophilic ligands.

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