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Original article:

Expression of prostacyclin-stimulating factor (PSF) in mononuclear cells of human peripheral blood and THP-1 derived macrophage-like cells, and effects of high glucose concentration

Takashi Etoh[1, *], Toshihiko Hashimoto[2], Naotaka Sekiguchi[3], Minako Imamura[3], Toyoshi Inoguchi[3], Hajime Nawata[3]

[1]Medical Co. LTA, Medical Co. LTA (PS Clinic), 6-18 Tenya-machi, Hakata-ku, Fukuoka-city, Fukuoka, 812-0025, Japan, Tel: +81 92 283 7777, Fax: +81 92 283 7770, E.mail: takashi-eto@lta-med.com (*corresponding author), [2]Fukuoka City Hospital, Fukuoka, Japan, [3]Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

ABSTRACT

Prostacyclin (PGI_2) synthesis by vascular endothelial cells (ECs) decreases in diabetic subjects, possibly leading to development of diabetic angiopathies including that in atherosclerosis. We identified a bioactive peptide, prostacyclin-stimulating factor (PSF), which stimulates PGI_2 synthesis in cultured aortic ECs. Our previous studies demonstrated that PSF was predominantly expressed by arterial smooth muscle cells (SMCs) and ECs. We immunohistochemically showed that PSF existed in SMCs of human coronary arteries, and PSF staining was markedly reduced in coronary arterial SMCs of patients with type 2 diabetes and/or myocardial infarction. In the present study, we investigated the existence of PSF in human serum, and effects of glucose on serum PSF levels in patients with type 2 diabetes. Immunoblot analysis revealed the presence of PSF in serum, and showed that serum PSF protein concentration was significantly decreased in type 2 diabetic patients. Moreover, there was a significant negative correlation between serum PSF and HbA1c levels in these patients. Using immunohistochemistry, we also showed that PSF was present in serum and in macrophages (Mϕs). PSF mRNA was found in Mϕs using reverse transcription-polymerase chain reaction (RT-PCR). In addition, effects of high glucose conditions on PSF production in Mϕs were examined by Western blotting, and we showed that PSF significantly decreased when Mϕs were cultured in high glucose conditions. These results strongly suggested that decreased PSF production might result in decreased production of PGI_2 in atherosclerotic lesions, thus leading to development of diabetic macroangiopathy and atherosclerosis.

Keywords: diabetes mellitus, prostacyclin stimulating factor (PSF), macroangiopathy, macrophage

INTRODUCTION

Prostacyclin (PGI_2) is mainly synthesized by vascular endothelial cells (ECs). It is a potent vasodilator, and inhibitor of platelet adhesion and aggregation, and also contributes to the maintenance of vessel wall homeostasis (Moncada et al., 1976; Moncada, 1982). It has been previously reported that vascular PGI_2 synthesis was reduced in patients with

diabetes (Colwell et al., 1983; Inoguchi et al., 1987) and atherosclerosis (Larrue, 1980). Decreased PGI_2 production could be associated with pathogenesis of vascular lesions such as those seen in diabetic angiopathy and atherosclerosis. In addition to results described by Maclntyre et al. (1978), we demonstrated that human serum stimulated PGI_2 production in cultured bovine aortic ECs (Inoguchi et al., 1987), and that activity of serum stimulating PGI_2 production decreased in patients with diabetes as well as in an animal model of diabetes (Inoguchi et al., 1986; Inoguchi et al., 1992).

Moreover, we found that cultured bovine aortic ECs similarly stimulated PGI_2 production in serum-free conditioned medium from cultured human fibroblast cells (Yamauchi et al., 1993). We also purified and cloned a novel bioactive peptide, prostacyclin-stimulating factor (PSF), which was responsible for stimulation of PGI_2 production (Yamauchi et al., 1994). PSF mRNA is particularly expressed in lungs and kidneys of humans and rats (Ono et al., 1994).

In vitro, PSF production by ECs and SMCs was reduced when these cells were cultured in high glucose conditions.

Decreased PSF production leads to decreasing PGI_2 concentration, and to vessel wall constriction, followed by platelet aggregation. In local lesions of vessel walls, ECs, SMCs, and Mϕs play important roles, because Mϕs synthesize tumor necrosis factor-α(TNF-α), endothelin-I (ET-I), and other factors associated with pathogenesis of atherosclerosis.

In the present study, we investigated whether activated mononuclear cells produced PSF. We also examined effects of high glucose concentrations on PSF production in macrophage-like cells derived from the THP-1 cell line that was purified from human acute monocytic leukemia.

MATERIALS AND METHODS

Experi*mental subjects and treatment of blood samples*

Following an overnight fast, whole blood was randomly collected from 14 patients with type 2 diabetes (male/female, 4/10; mean age, 59 years old), and from 10 age-matched healthy controls (male/female, 5/5; mean age, 58 years old). Serum was immediately separated, and applied to a heparin affinity column (HiTrap Heparin, Pharmacia Biotech, Japan) which had been equilibrated with two column volumes of 0.01 M sodium phosphate buffer (start buffer) at pH 7.0. After washing with the start buffer until the effluent was clear, the heparin-bound fraction containing PSF proteins was eluted with two column volumes of start buffer and 1 mM Tris-HCl at 4 ^{o}C. Measurement of protein concentration and western blot analysis are described as follows.

Measurement of glycocylated hemoglobin A1c

Glycocylated hemoglobin A1c (HbA1c) was measured by high-performance liquid chromatography (HPLC).

Isolation of human mononuclear cells from cultured THP-1 cells and peripheral blood

The THP-1 cell line was purified from human acute monocytic leukemia, and was cultured in RPMI-1640 medium (Gibco Laboratories, NY) containing 5 % fetal bovine serum (FBS, Gibco), 5.5 mM glucose (Sigma-Aldrich, Japan), 100 μU/ml penicillin, and 50 μg/ml streptomycin (Gibco), under 95 % air and 5 % CO_2 at 37 ^{o}C. Cells were stimulated to transform to macrophage-like cells by addition of phorbol 12-myristoyl 13-acetate (PMA, Sigma-Aldrich) into the culture medium of an initial density of 1×10^6 cells/ml, followed by 48-h culture period (with 0.1 μM PMA). Then, cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich), cultured with 5.5 mM glucose for 72 h, and incubated with different concentrations of glucose for 72 h.

Human mononuclear cells were isolated from buffy coat preparations of whole blood taken from healthy volunteers. In brief, each buffy coat was mixed with Lymphocyte Separation Medium (ICN/Cappel R.O.) and physiological saline in a ratio of 3:2:2, and centrifuged for 30 min at 400 x g at room temperature. Then, the supernatant was removed, and the cell layer was transferred to a new tube. PBS (vol) was added, and the pellet was centrifuged for 10 min at 250 x g at 4 ^{o}C. The supernatant was removed, and resuspended in RPMI 1640 supplemented with 10 % FBS. For monocyte-macrophage differentiation, monocytes isolated as above were resuspended in culture medium at a density of 3×10^6/ml, seeded into 100-mm^2 tissue culture dishes (NUNC Denmark), incubated for 2 h at 37 ^{o}C, and washed four times with culture medium. Isolated cells were incubated at 37 ^{o}C for 7 days, and medium was changed every 48 h.

Reverse transcription-polymerase chain reaction (RT-PCR) of mononuclear cells and THP-1 derived macrophage-like cells

Total RNAs of human mononuclear cells and macrophage-like cells were isolated with ISOGEN (Nippon gene, Japan). Single-stranded cDNAs were prepared from total RNAs using random primers and Superscript II (Gibco BRL), by incubating for 60 min at 42 ^{o}C. Resulting single-stranded cDNAs were amplified using specific primers (Table 1) and the KOD plus polymerase (TOYOBO, Japan). PCR conditions involved 30 cycles of 1 min at 94 ^{o}C, 1 min at 50 ^{o}C, and 1 min at 72 ^{o}C. PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, C.A.). Following purification of PCR products, DNA sequences were determined.

Table 1: Primer sequences for RT-PCR

PSF
Sense, 5'-CATGGAGTGCGTGAAGAGCCGC-3'
Antisense, 5'-GAATTGGATGCATGGCACTCATATTC-3'

B-actin
Sense, 5'-AGAGATGGCCACGGCTGCTT-3'
Antisense, 5'-ATTTGCGGTGGACGATGGAG-3'

Immunohistochemistry

Monoclonal antibodies against PSF were generated from mice as described previously (Hata et al., 2000). Immunohistochemistry was performed using the avidin-biotin peroxidase complex method. Macrophage-like cells were fixed on slide glasses by the method of cytospin, centrifuged at 3,000 rpm for 5 min at room temperature. Slides were incubated with 0.2 % Triton X-100 (Sigma-Aldrich) for 2 min at room temperature, and blocked with 3 % H_2O_2 (Sigma-Aldrich) in methanol to eliminate endogeneous peroxidase for 30 min on ice. Then, slides were incubated with 10 % normal goat serum (Amersham Pharmacia Biotech, UK) for 60 min at room temperature to block nonspecific bindings, followed by incubation with anti-PSF monoclonal antibody (5 μg/ml) overnight at 4 ^{o}C. Control specimens of macrophage-like cells (negative control) were incubated with PBS and normal mouse IgG (5 μg/ml, Amersham Pharmacia Biotech). After washing with PBS, the specimens were further incubated with biotinylated goat anti-mouse IgGs (1:2,000, Amersham Pharmacia Biotech) for 30 min, followed by incubation with a solution of avidin-biotin peroxidase complex (Histofine System, Nichirei, Japan), for 30 min at room

temperature. Finally, a diaminobenzidine solution (DAB, Nichirei) was applied to visualize immunoreaction for PSF, and nuclei of the specimens were counterstained with hematoxylin.

Western blot analysis of blood samples and culture medium

Blood samples were separated as described previously. After incubation with different concentrations of glucose for 72 h, culture medium was dialyzed with dialysis buffer (5 mM Tris-HCl (Sigma-Aldrich), pH 7.4) for 48 h at 4 ^{o}C, and dialysis buffer was changed at 24 h. The dialyzed culture medium was treated with a SpeedVac Concentrator SVC 100H (SAVANT) in an attempt to increase its concentration. Protein concentration was determined using the BCA Protein Assay Kit. Thirty-five μg proteins of each sample were mixed with loading buffer (10 mM Tris-HCl, pH 6.8, 1 % sodium dodecyl sulfate (SDS (Sigma-Aldrich)), 20 % glycerol (Sigma-Aldrich), 0.02 % bromophenol blue (Sigma-Aldrich), 1 % 2-mercaptoethanol (Sigma-Aldrich)), and boiled for 2 min. Samples were separated using 12 % SDS/PAGE with a running buffer including 25 mM Tris, 192 mM glycine (Sigma-Aldrich), and 0.1 % SDS. Proteins were then electrophoretically transferred to a nitrocellulose membrane (Hybond-C super, Amersham LIFE SCIENCE) at 1 mA/cm^2 of paper for 90 min using transfer buffer (20 % vol/vol methanol (Sigma-Aldrich), 25 mM Tris, 192 mM glycine, 0.05 % SDS). The membrane was incubated in T-PBS buffer (100 mM phosphate buffer containing 100 mM NaCl (Sigma-Aldrich), 0.1 % Tween-20 (Sigma-Aldrich), 5 % (wt/vol) non fat milk powder) for 1 h at room temperature, and with monoclonal anti-PSF antibody overnight at 4 ^{o}C. PBS and anti-PSF antibody incubated with C-terminal PSF peptides (20 amino acids, concentration ratio = 1:5) were used as negative control. Then, the membrane was washed three times with T-PBS buffer, and incubated with horseradish peroxidase-labeled antibody against mouse IgGs (Amersham) for 45 min. Finally, the membrane was washed five times with T-PBS buffer, and the specific band corresponding to PSF was visualized by enhanced chemiluminescence (ECL plus system, Amersham).

Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed by analysis of variance (ANOVA), calculated with the Statview program. P values < 0.05 were considered to be statistically significant.

RESULTS

Western blot analysis of PSF in serum

Western blot analysis revealed a PSF band in human serum with an approximate molecular weight of 31 kDa. We compared density of the serum PSF band in type 2 diabetic patients with that in age-matched controls. Density ratio of the PSF band from each experimental subject was expressed relative to that of healthy subjects. Density ratio of the PSF band representing PSF protein concentration in serum, was significantly decreased in type 2 diabetic patients compared with that in controls (0.69 ± 0.03 vs 1.00 ± 0.08, $P < 0.01$) (Fig.1A).

Correlation between density ratio of the PSF band in serum and HbA1c level is shown in Fig. 1B. Interestingly, there was a significant negative correlation between density ratio of the PSF band and HbA1c level in patients with type 2 diabetes ($r = -0.747$, $P < 0.01$). This result indicated that serum PSF concentration decreased with deterioration of glycemic control in type 2 diabetic patients.

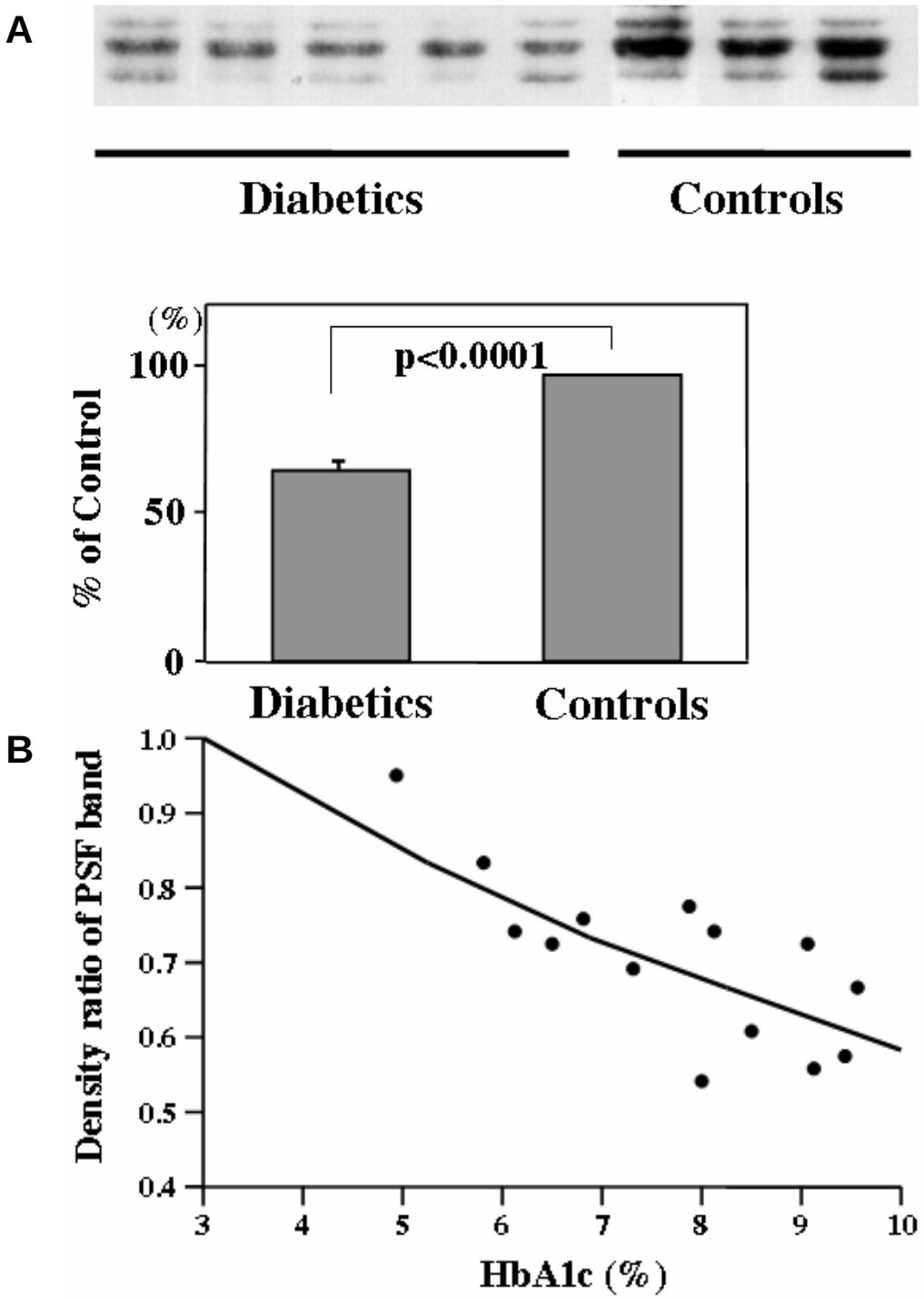

Figure 1: A. Western blot analysis of PSF contents in serum from type 2 diabetic and control subjects. Samples were collected from 14 patients with type 2 diabetes (male/female, 4/10; mean age, 59 years old) and from 10 age-matched healthy controls (male/female, 5/5; mean age, 58 years old). PSF contents were measured by western blot analysis using an anti-PSF monoclonal antibody. Data are expressed as means + S. E. M., $P < 0.05$ vs. healthy controls B. Correlation between density ratio of the PSF band and HbA1c level in patients with type 2 diabetes. Glycosylated hemoglobin A1c (HbA1c) was measured by high-performance liquid chromatography (HPLC). There was a significant negative correlation between density ratio of the PSF band and HbA1c level, indicating that serum PSF concentration decreased with deterioration of glycemic control ($r = -0.747$, $P < 0.01$).

Expression of PSF mRNA in cultured macrophage-like cells and human mononuclear cells

PSF mRNAs in cultured human mononuclear cells derived from peripheral blood and in macrophage-like cells derived from the THP-1 cell line were amplified by RT-PCR. PCR products migrated as clear single bands on a 1 % agarose gel, and molecular size of the PSF band was 540 bp, which was in accordance with the expected DNA size from the human cDNA sequence. PSF mRNA was detected in cultured mononuclear cells and in macrophage-like cells (Fig. 2). PCR products purified using the PCR purification Kit, were used for DNA sequencing. The sequences obtained matched the human PSF cDNA sequence.

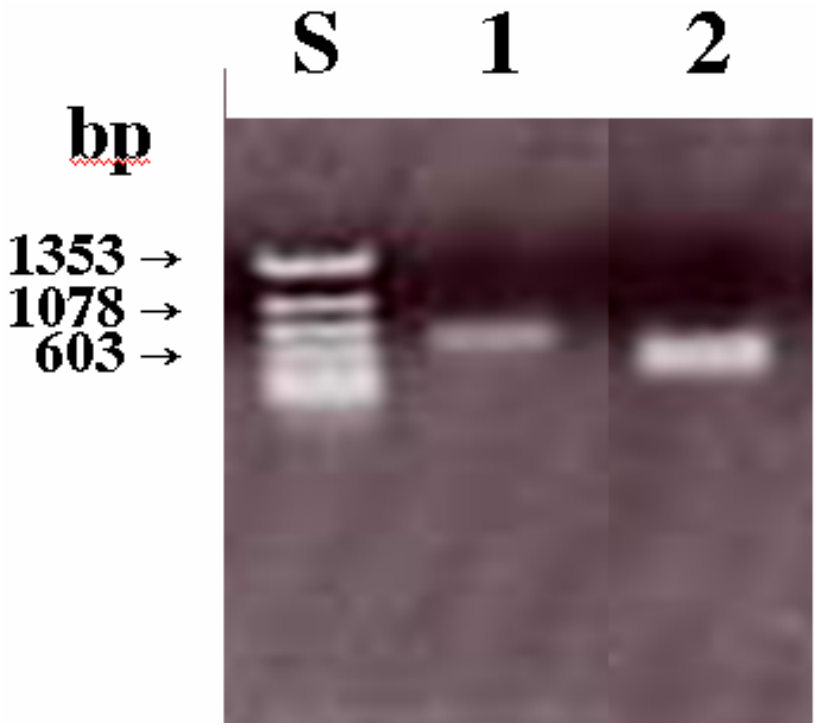

Figure 2: PSF mRNA expression in human peripheral mononuclear cells and THP-1 derived macrophage-like cells. Total RNAs of human mononuclear cells and macrophage-like cells were isolated using the ISOGEN method. Single-stranded cDNAs were prepared from total RNAs using random primers and Superscript II. The resulting single-stranded cDNAs were amplified using specific primers (Table 1) and the KOD plus polymerase. PCR products migrated as clear single bands on a 1 % agarose gel. Lane 1, PCR product of -actin; Lane 2, PCR product of PSF; Lane 3, size marker (Hinc-II).

Immunohistochemical study of PSF in macrophage-like cells

Fig. 3 shows immunostaining for PSF in macrophage-like cells. Positive immunostaining for PSF in macrophage-like cells incubated with anti-PSF antibody was found, but not in macrophage-like cells incubated with PBS and normal mouse IgGs.

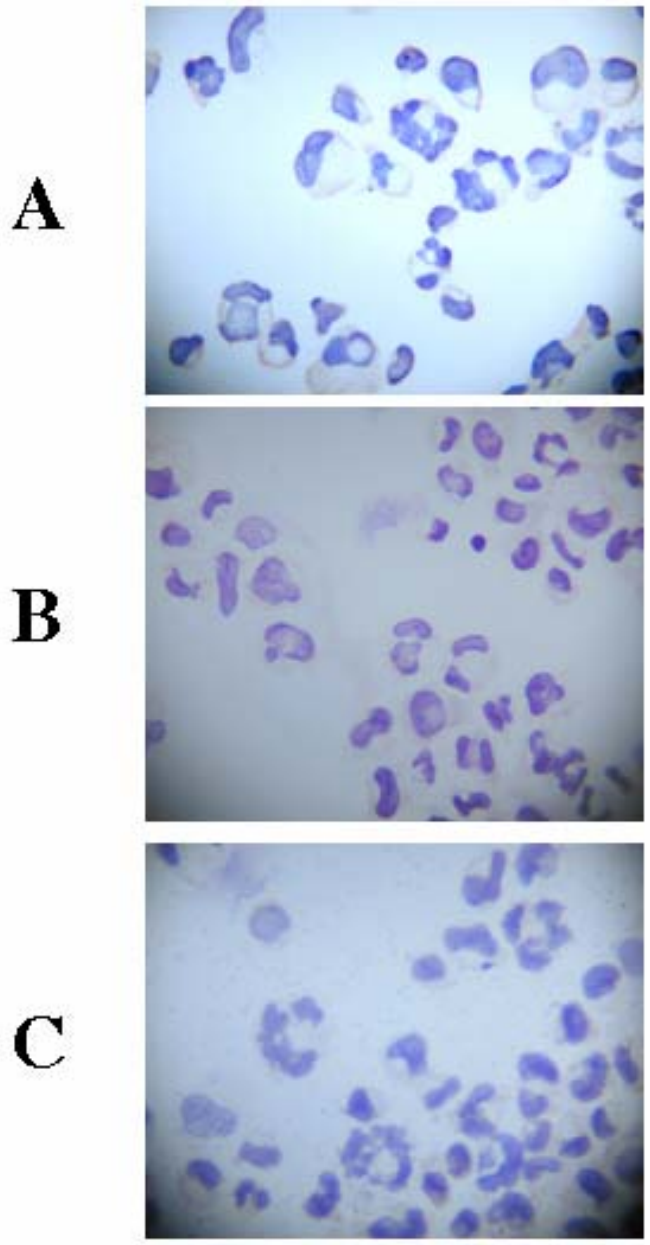

Figure 3: Immunocytochemical staining in THP-1 derived macrophage-like cells. Immunocytochemical staining was performed using the avidin-biotin peroxidase complex method. Cultured macrophage-like cells were fixed on slide glasses by the method of cytospin. We used anti-PSF monoclonal antibody as primary antibody (5 µg/ml), and PBS and normal mouse IgGs (5 µg/ml) were used as negative control. After washing with PBS, the specimens were further incubated with biotinylated goat anti-mouse IgGs as secondary antibody. Finally, a diaminobenzidine solution (DAB) was applied to visualize PSF, while cell nuclei of specimens were counterstained with hematoxylin. A. Anti-PSF monoclonal antibody, B. PBS, C. Normal mouse IgGs.

Western blot analysis of culture medium

In addition to immunocytochemical staining, we performed Western blot analysis to confirm the existence of PSF in cultured macrophage-like cells, and to examine effects of high glucose on PSF levels in these cells. Positive staining for PSF occurred when macrophage-like cells were reacted with anti-PSF antibody, but not found when cells were reacted with normal mouse IgGs

or with anti-PSF antibody incubated with PSF peptides. The same amount of culture medium obtained from macrophage-like cells cultured with high glucose concentration (500 mg/dl) was examined by immunoblotting for PSF, whether there were differences in PSF content among cultured macrophage-like cells. As shown in Fig. 4, western blot analysis indicated that PSF level was decreased by 85.4 % ($P < 0.05$) with increasing glucose concentration in the culture medium, in parallel to a decrease in PSF staining intensity.

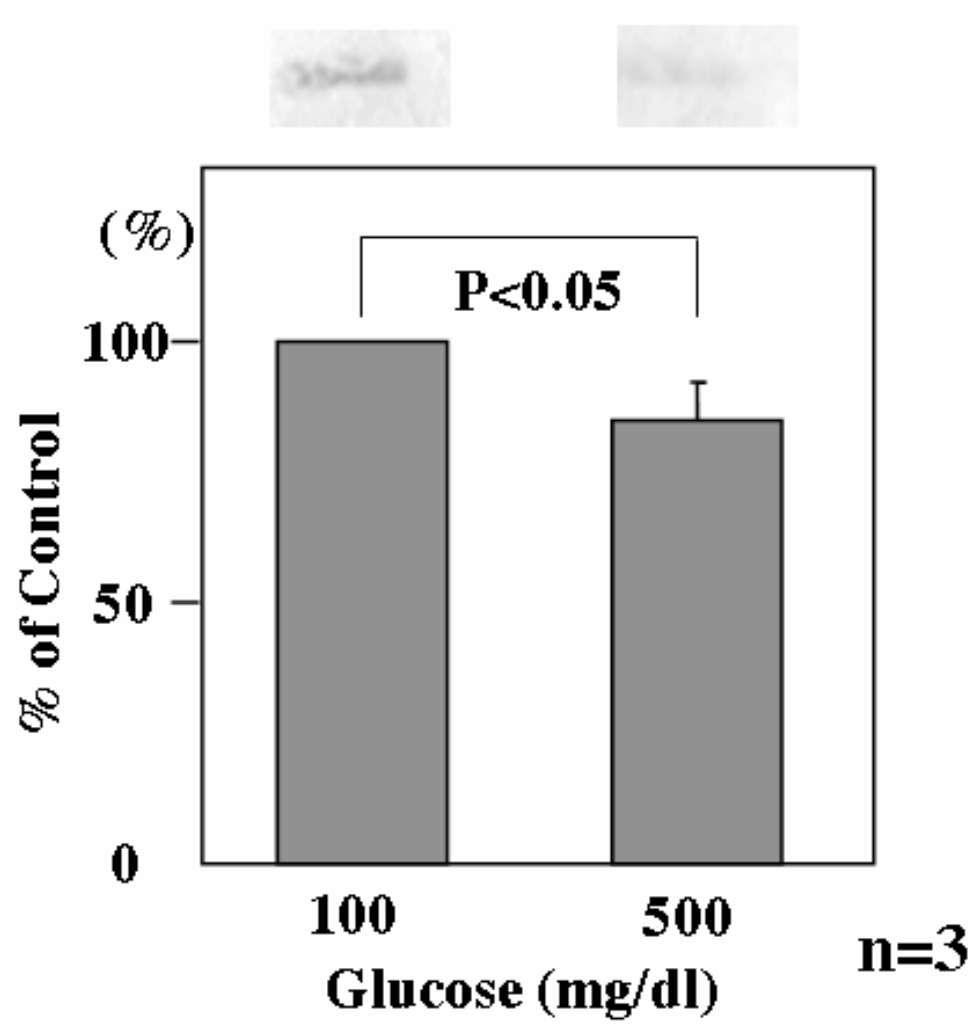

Figure 4: PSF contents in THP-1 derived macrophage-like cells cultured with normal and high concentrations of glucose. THP-1 derived macrophage-like cells were cultured with RPMI-1640 medium containing normal or high concentrations of glucose for 72 h. Cultured medium was dialyzed with dialysis buffer for 48 h. Thirty-five µg proteins of each sample were mixed with loading buffer, and samples were loaded for 12 % SDS/PAGE. Proteins were then electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with either monoclonal anti-PSF antibody, or PBS and anti-PSF antibody incubated with C-terminal portions of PSF peptides (negative control). Then, the membrane was washed three times with T-PBS buffer, and incubated with horseradish peroxidase-labeled antibodies against mouse IgGs. Following a final wash with T-PBS buffer, the specific band corresponding to PSF was visualized by enhanced chemiluminescence. Data are expressed as means + S. E. M. from two different experiments. $P < 0.05$ vs. normal glucose (100 mg/dl), n = 3, respectively

DISCUSSION

Diabetic angiopathy is a well-known complication influencing the prognosis of diabetes. Many pathogenic factors have been shown to promote development of diabetic angiopathy. Recently, it has also been demonstrated that one of the most important causes of diabetic angiopathy are dysfunctions of vascular ECs and SMCs, and activation of Mϕs due to hyperglycemia. ECs generate and secrete many vasoactive substances regulating vascular tonus such as PGI_2 (Moncada et al., 1976), endothelium-derived relaxing factor/nitric oxide (Furchgott et al., 1980), and endothelin. Mϕs also generate and secrete many mediators, and are involved in the progression of atherosclerosis.

In particular, PGI_2 was first discovered by Moncada et al. (1976), and was shown to be a potent prostanoid acting as a vasodilator and an inhibitor of platelet aggregation. A decrease in PGI_2 production is thought to contribute to the pathogenesis of diabetic angiopathy (Johnson et al., 1988). Circulating PGI_2 concentrations are decreased in serum from both human and animal models with diabetes (Colwell et al., 1983; Karpen et al., 982), thus resulting in platelet hyperfunction, and vascular constriction (Udvardy et al., 1987; Ziboh et al., 1979; Aanderud et al., 1985; Umeda et al., 1989).

Relationships between PGI_2 alteration and atherosclerotic lesions have also been reported (Umeda et al., 1989; Inoguchi et al., 1991; Sekiguchi et al., 1997). Dembinska-Kiec et al. (1977) first demonstrated that generation of PGI_2 by arteries such as the coronary artery is reduced in experimentally-induced atherosclerosis of rabbits that had been overfed with cholesterol. Sinzinger et al. (1979) also demonstrated that such a decreased PGI_2 production might injure the protective function of vessel walls, and thus induce progression of atherosclerosis. It has also been reported that PGI_2 inhibited secretion of cytokines and growth factors by Mϕs (Willis et al., 1986). Hajjar et al. (1982) further demonstrated that PGI_2 modulated cholesteryl ester hydrolytic activity by affecting cAMP synthesis in rabbit aortic SMCs. Moreover, Orekhov et al. (1983) reported that PGI_2 analogs reduced the content of intracellular cholesterol in SMCs obtained from human atherosclerotic lesions. Satya et al. (1989) reported that cholesterol-rich macrophages decreased synthesis of PGI_2 and prostaglandin E_2 (PGE_2). Hashimoto et al. (2002) reported that lysophosphatidylcholine, which is a main component of oxidized low density lipoprotein, decreased PSF mRNA expression. PSF stimulates PGI_2 production, therefore low levels of PSF may reduce PGI_2 production. Decreased PSF level may initiate, and develop atherosclerotic lesions. Such evidence suggests that PGI_2 abnormalities may participate in the pathogenesis of atherosclerosis.

We hypothesize that PSF of Mϕs is mainly secreted, and does not accumulate in the cytosol. We also hypothesize that secreted PSF is more important than PSF in the cytosol, because PSF stimulates PGI_2 production from ECs, which is a protective function of vessel walls, and reduces progression of atherosclerosis.

We demonstrated that PSF was present in human mononuclear cells and THP-1 derived macrophage-like cells using RT-PCR, immunohistochemistry, and western blotting. PSF production by macrophage-like cells decreased when cells were cultured with high glucose concentrations. We have previously demonstrated that high glucose also decreased PSF production on ECs and SMCs. In the present study, we demonstrated that high glucose concentrations decreased PSF production on macrophage-like cells. We hypothesize that decreased PSF production by ECs and SMCs plays an important role in atherosclerosis. We also believe that PSF production by Mϕs is lower than that by ECs and SMCs, but decreased

PSF production by Mϕs also plays a role in atherosclerosis.

In conclusion, PSF was found to be produced by human peripheral mononuclear cells. PSF staining was observed in THP-1 derived macrophage-like cells. Furthermore, PSF expression was decreased in macrophage-like cells cultured in the presence of high glucose concentration. These results suggest that decreased PSF production may result in decreased PGI_2 production in vessel walls, thus leading to development of diabetic macroangiopathy and atherosclerosis.

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