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FIGURE LEGENDS

Fig. 1

Suppression of *SOCS-1* gene expression by hepatitis C virus core protein.

A, B: RNA from mouse liver tissues (A) or HepG2 cells (B) with or without the core protein was subjected to RT-PCR for the determination of *SOCS-1* and *SOCS-3* gene expression. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. HepG2 cells (Hep396 and Hepswx) were treated with 10 ng/ml of IL-6 for 0, 30 or 60 min before RNA extraction. Bottom panels in Figures 1A and 1B show the expression level of the housekeeping gene GAPDH as an internal control.

C, D: Figures 1C and D represent means ± S.D. of five independent experiments on *SOCS-1* gene expression corresponding to the lanes in Figures 1A and 1B, respectively. *, $p<0.05$.

E, F: Figures 1E and F represent means ± S.D. of five independent experiments on *SOCS-3* gene expression corresponding to the lanes in Figures 1A and 1B, respectively.

Fig. 2

Effect of hepatitis C virus core protein on the expression of STAT-target genes.

RNA from mouse liver tissues with or without the core protein was subjected to RT-PCR for the determination of *IRF1* **(A)**, *c-myc* **(B)** and *bcl*-X_L**(C)** gene expressions. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g

BW of IL-6.

*, $p<0.05$.

Fig. 3

Methylation status of *SOCS-1* gene in liver from hepatitis C virus core gene transgenic mice.

DNA from the liver tissues of core gene transgenic mice at the age of 3 months (3M), 6 months (6M) or 13 months (13M) was subjected to methylation-specific PCR. Only PCR with unmethylation-specific primers yielded bands indicating that the *SOCS-1* gene was unmethylated in the liver tissues of core gene transgenic mice.

M, methylation-specific primers; U, unmethylation-specific primers

Fig. 4

Increase in the level of tyrosine phosphorylation of STAT3 and STAT1 by hepatitis C virus core protein.

Whole cell lysates from mouse liver tissues (**A**) and HepG2 cells (**B** & **C**) were subjected to SDS-PAGE followed by Western blotting with anti-STAT3 and anti-p-STAT3 (**A** & **B**) or with anti-STAT1 and anti-p-STAT1 (**C**). Liver tissues were obtained from the mice treated as described in the Fig. 1 legends. HepG2 cells were treated with 10 ng/ml IL-6 or vehicle for 1 hour.

p-STAT3, phosphorylated STAT3; STAT3, total STAT3; p-STAT1, phosphorylated STAT1; STAT1, total STAT1. The experiments were repeated three times.

Fig. 5

Hepatitis C virus core protein did not affect subcellular localization of STAT3 or STAT1.

Cytoplasmic and nuclear fractions from HepG2 cells with or without the core protein were subjected to Western blotting with the anti-STAT3 antibody (**A**) or anti-STAT1 antibody (**C**). HepG2 cells were fixed and an immunocytofluorescence study was performed using the anti-STAT3 antibody (**B**). Cells were processed before or 60 min after the treatment with 10 ng/ml of IL-6.

cyto, cytoplasmic fraction; nuc, nuclear fraction.

Fig. 6

Effect of the core protein on the interaction of STATs and PIASs.

Cell lysates were immunoprecipitated with anti-PIAS1 or anti-PIAS3 antibody, and immunoblotted with anti-STAT1 or STAT3 antibody, respectively. There was no difference in the amounts of STAT1 or STAT3 that were co-immunoprecipitated with anti-PIAS antibodies.

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Hepatitis C Virus Core Protein Exerts an Inhibitory Effect on Suppressor of Cytokine Signaling (SOCS)-1 Gene Expression

(Running Title: Suppression of SOCS-1 by HCV Core)

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Fig. 1

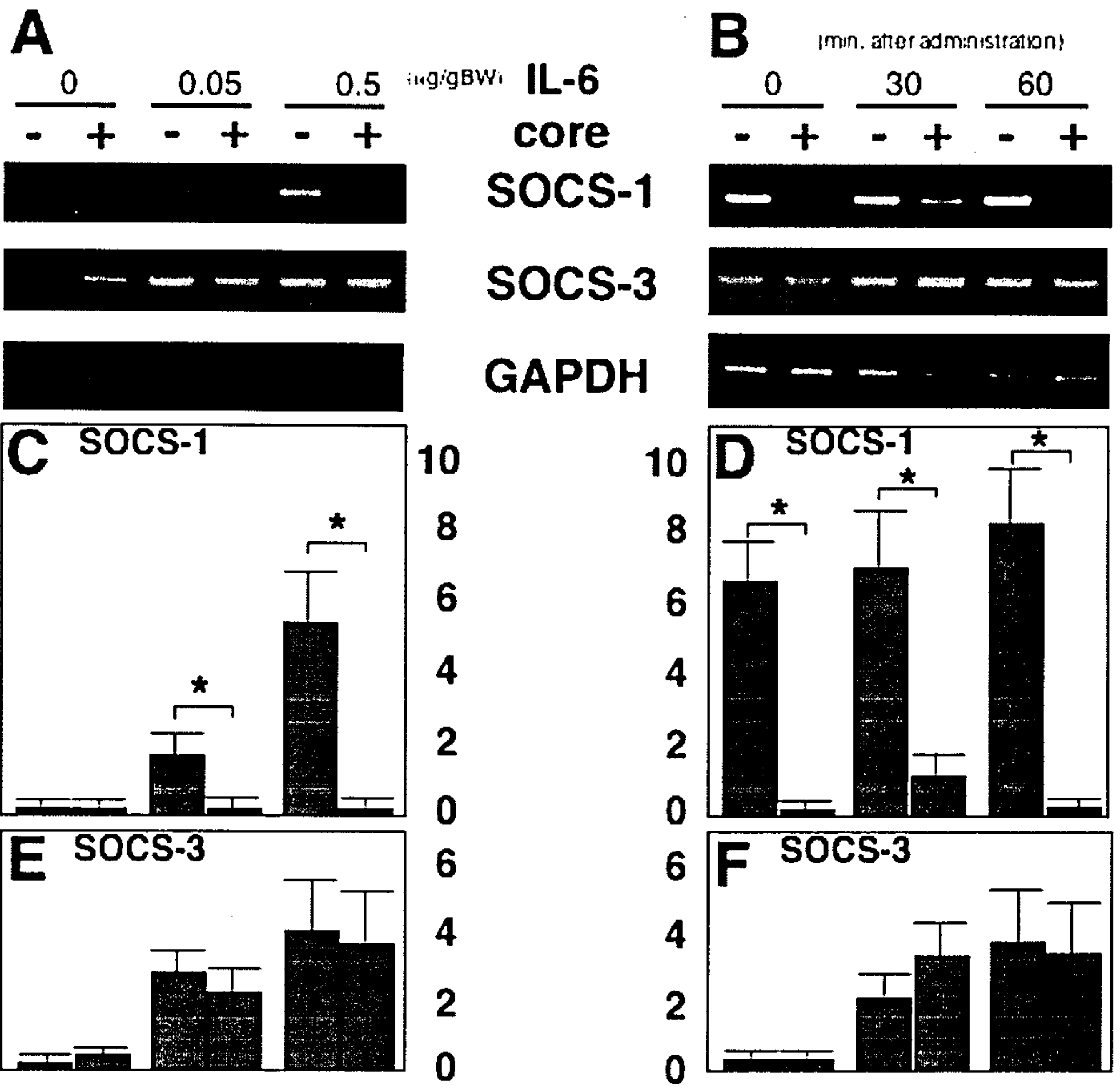
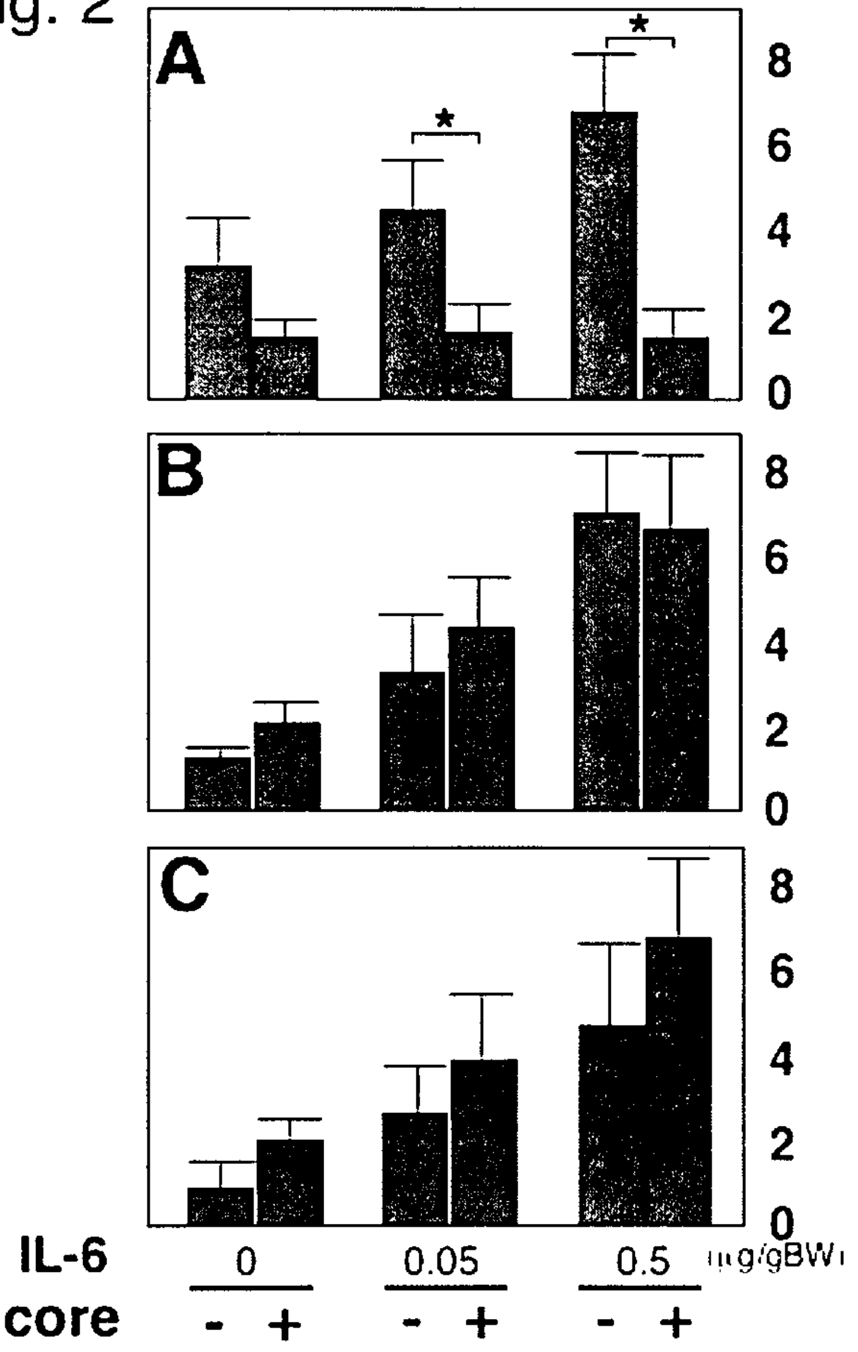


Fig. 2



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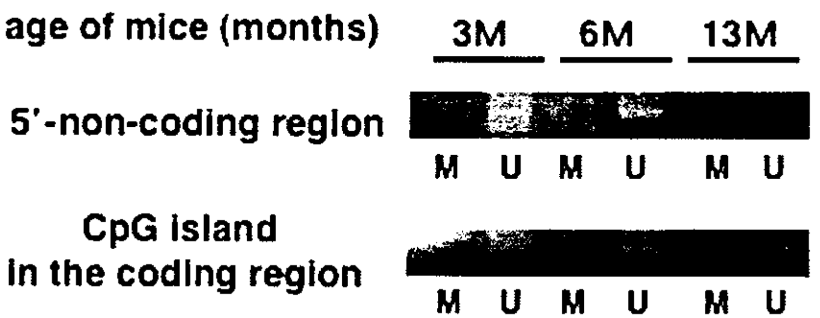
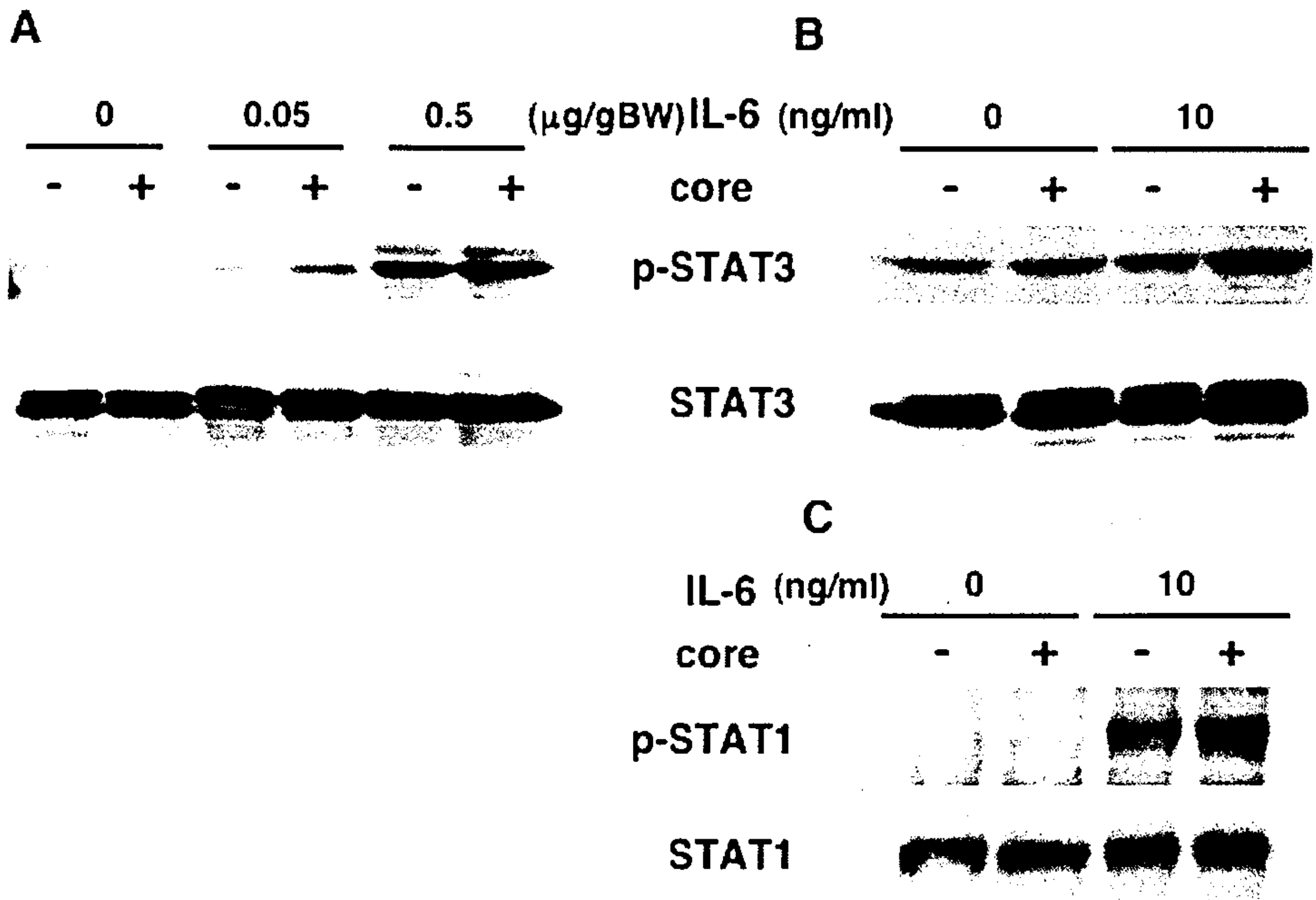


Fig. 4



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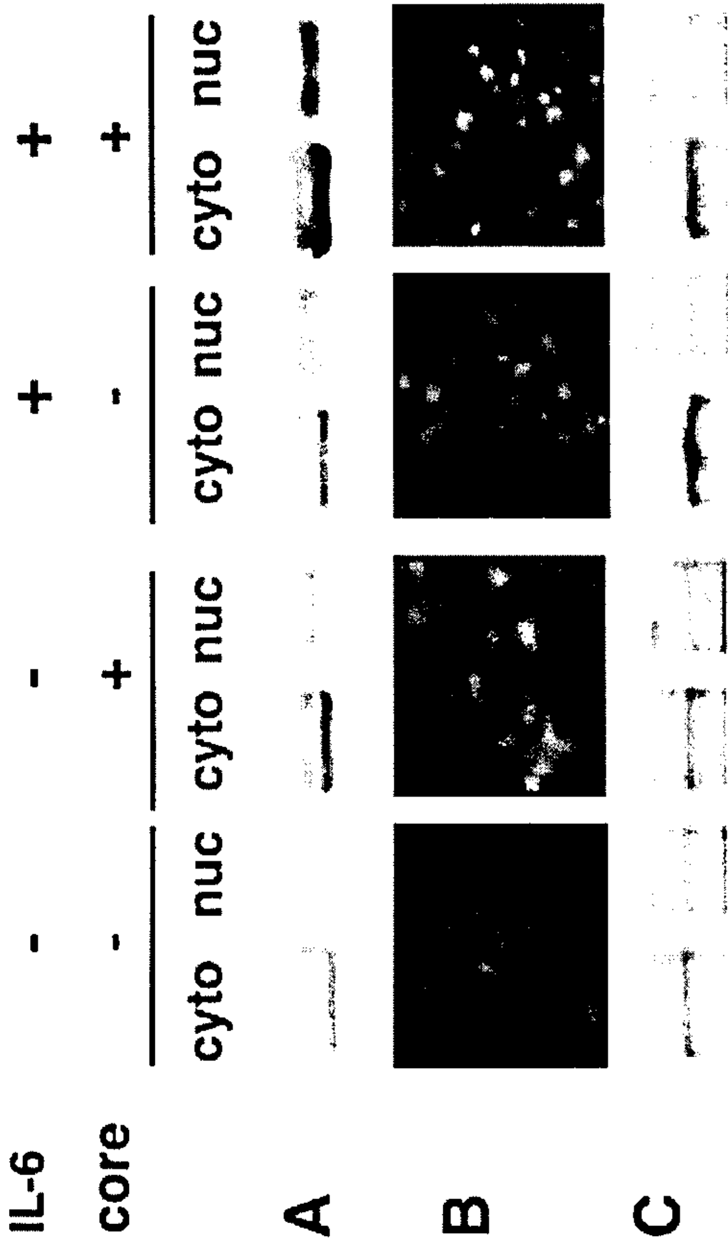
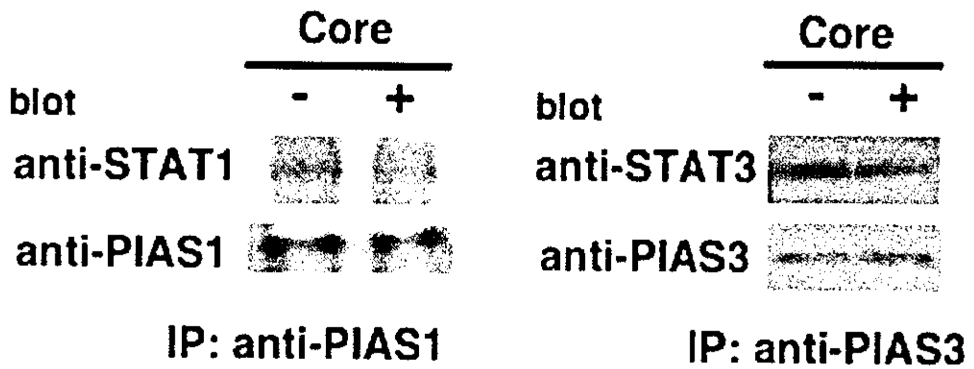


Fig. 6



Adenovirus-mediated gene transfer and lipoprotein-mediated protein delivery of plasma PAF-AH ameliorates proteinuria in rat model of glomerulosclerosis

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Abstract

Oxidative stress has been proposed to play a crucial role in glomerulosclerosis, although its in vivo demonstration has proved taxing given the difficulty of inducing gene expression in specific renal cells. In this study, we examined whether the liver-directed expression of plasma platelet-activating factor acetylhydrolase (PAF-AH) would affect the glomerular pathophysiology in Imai rats, an animal model for glomerulosclerosis. Adenovirus-mediated liver-directed gene delivery of human PAF-AH resulted in a significant increase in plasma PAF-AH activity, which was detected almost exclusively on HDL. Histological examination of rats over-expressing PAF-AH showed not only the deposition of PAF-AH in mesangial cells, but also a reduction in hydroxynonenal and matrix protein content in the glomeruli. In situ hybridization analysis was negative for human PAF-AH mRNA in the kidney, while injection of HDL abundant in PAF-AH resulted in the deposition of PAF-AH in mesangial cells. Urine protein levels did not increase in rats over-expressing PAF-AH, while those of control rats increased significantly with age. This study provides direct evidence of the in vivo role of an enzyme which degrades lipid peroxides during the progression of glomerulosclerosis. Adenovirus-mediated extra-renal gene expression and lipoprotein-mediated glomeruli-targeted protein delivery promise to be a novel therapeutic approach to glomerulosclerosis.

Key words: platelet-activating factor acetylhydrolase, glomerulosclerosis, molecular therapy, lipoproteins, oxidative stress, adenovirus, Imai rat, lipid peroxide

Introduction

Glomerulosclerosis is an irreversible and terminal pathological change in glomeruli. Several diseases including glomerulonephritis, autoimmune diseases, hypertension and diabetes lead to the development of glomerulosclerosis, although the initiation of glomerular changes differs among these diseases. Nevertheless, one of the proposed common mechanisms among these diseases which render the glomeruli sclerotic is the succession of inflammatory reactions. Infiltration of immune cells, activation of inflammatory responses, and cross-communication of intrinsic glomerular cells with the invading immune cells result in the progression of glomerular sclerosis. Oxidative stress including the formation of free radicals and lipid hyperoxides supposedly plays a key role in this process [1-4].

Plasma platelet activating factor acetylhydrolase (PAF-AH) is an enzyme which hydrolyzes short chain acyl groups esterified to the sn-2 position of phospholipids such as platelet-activating factor and oxidatively fragmented phospholipids, thereby destroying biologically active lipids [5]. This enzyme is physiologically produced by immune cells and exists exclusively on lipoprotein particles in plasma. We have previously confirmed that PAF-AH possesses several anti-atherosclerotic properties in vitro [6]. Quarck et al demonstrated that over-expression of PAF-AH reduced oxidized LDL in atherosclerosis-prone mice, thereby inhibiting the progression of atherosclerosis in these mice [7]. In addition, other recent studies imply that PAF-AH polymorphism affects the progression of proteinuria in IgA nephropathy in humans [8, 9].

The Imai rat [10, 11] is an animal model which develops spontaneous glomerulosclerosis as well as hyperlipidemia through its nebulous genetic constellation [12, 13]. Male Imai rats, but not females, manifest progressive proteinuria 8 weeks after birth, and die from renal failure around 35 weeks of age. The characteristic glomerular histology of the Imai rat reveals the infiltration of immune cells and expansion of the mesangial matrix, which not only is most relevant to focal glomerular sclerosis in human diseases [12], but is also a common feature of glomerulosclerotic diseases.

Over-expression of any gene in the kidney, especially in specific cells such as mesangial cells, is difficult to achieve [14]. Intravenous administration of adenoviral vectors attains a highly efficient transgene expression, however, the main organ infected by adenoviruses after intravenous injection is the liver. We have previously demonstrated that apolipoprotein E (apoE), an apolipoprotein which binds to lipoproteins, was delivered to atherosclerotic lesions when over-expressed in apoE deficient mice [15]. In this study, we hypothesized that a lipoprotein-bound protein that is over-expressed in the liver would be delivered to the glomeruli, resulting in a local biological effect in vivo. Consequently, we over-expressed PAF-AH in Imai rats utilizing a second generation adenovirus vector encoding PAF-AH gene (AdPAFAH), and examined its effect on the progression of glomerular pathophysiology in Imai rats.

Results

Plasma PAF-AH activity and isoprostane 8,12-iso-iPF(2alpha)-VI levels after gene transfer

Injection of AdPAFAH in Imai rats resulted in around a 420-fold ($1.35 \times 10^5 \pm 1.34 \times 10^4$ IU/l) and 12-fold ($3.69 \times 10^3 \pm 8.97 \times 10^2$ IU/l) increase in plasma PAF-AH activity on days 3 and 7, respectively, compared to baseline (320 ± 8.5 IU/l); however, the activity declined quickly after Day 14 (Fig. 1A). A slight but significant increase in PAF-AH activity was also observed in the AdLacZ (a control second-generation adenoviral vector encoding β-galactosidase cDNA) group on Day 7 (Day 0: 346 ± 12 IU/l, Day 7:

1080 ± 60.6 IU/l) which would reflect the inflammation induced by adenoviral infection. No significant change in PAF-AH activity was observed in PBS group. After Day 14, no difference was observed in plasma PAF-AH activity among the three groups. Fig. 1B represents the distribution of plasma PAF-AH activity in the AdPAFAH group on Day 3. As shown in this figure, plasma PAF-AH activity was found almost exclusively in the HDL fractions.

In spite of the high plasma PAF-AH activity in rats injected with AdPAFAH, no change or difference was observed in plasma isoprostane 8,12-iso-iPF(2alpha)-VI levels for each group throughout the experiment (Fig. 1C).

Urinary protein excretion

Daily urinary protein excretion in the three groups before injection was as follows: AdPAFAH group, 168.5 ± 9.9 mg; AdLacZ group, 176.2 ± 11.0 mg; PBS group, 169.9 ± 6.8 mg. Daily urinary protein excretion of the two control groups increased gradually until Day 14, and rapidly thereafter (Fig. 2), consistent with a previous observation [12]. On the other hand, animals injected with AdPAFAH showed a significant decrease in urinary protein levels until Day 21, after which the levels shifted to a slight increase albeit still significantly lower than those of control groups.

Immunostaining of PAF-AH in the liver, kidney and aorta

Fig. 3 represents the time course changes in immunoreactive human PAF-AH protein (hPAFAH) in the liver and kidney samples of animals injected with AdPAFAH. The highest signal intensity for hPAFAH in liver sections were observed on Day 3 (Fig. 3B), after which the intensity dropped until almost no staining was observed on Day 28 (Fig. 3D). The signals were observed exclusively in the parenchymal hepatocytes. This time course change and the pattern of staining were identical with the results of β-galactosidase staining of liver sections from the AdLacZ group (data not shown), and consistent with the changes in plasma PAF-AH activity in the AdPAFAH group (Fig. 1A). On the other hand, immunostaining of the kidney samples for hPAFAH tested positive on Day 3 (Fig. 3F), however, the signal intensity became prominent on Day 7 (Fig. 3G), after which it waned in such slight decrements that the signal was detectable even on Day 28 (Fig. 3H). The hPAFAH signals in the kidney were detected exclusively in the glomeruli and arterioles (Fig. 4B), and were also positive in the wall of the thoracic aorta (Fig. 4E). Furthermore, the dual staining of hPAFAH and rat endothelial cell antigen (RECA-1) of the kidney sections confirmed the lack of co-localization of these proteins (Fig. 4F), while the staining of Thy-1 demonstrated its co-localization with hPAFAH (Fig. 4G); this co-localization of Thy-1 and hPAFAH was confirmed by immunostaining utilizing the fluorescent method (Figs. 4H-J). These results suggested that hPAFAH subsisted exclusively in mesangial cells but not in endothelial cells.

In situ hybridization analysis

To assess whether AdPAFAH infected kidney cells, we performed in situ hybridization of hPAFAH utilizing the liver and kidney specimens of rats injected with AdPAFAH. The liver sections on Day 3 tested positive for hPAFAH mRNA in the parenchymal hepatocytes (Fig. 5B). Even though immunostaining of hPAFAH on Day 7 in the glomeruli showed almost the same intensity as that observed in the parenchymal hepatocytes on Day 3, kidney sections on days 3 (Fig. 5C) and 7 (Fig. 5D) tested negative for hPAFAH mRNA.

Injection of PAFAH-rich HDL into Imai rat

In order to confirm that the hPAFAH protein was transferred through lipoproteins to the kidney, we administered male Imai rats with HDL particles abundant in hPAFAH protein for 4 consecutive days, extracted the kidney on Day 5, and performed hPAFAH staining. As shown in Fig. 4C, the hPAFAH protein was subsequently detected in the glomeruli and arterioles, as seen in the samples of Imai rats injected with AdPAFAH (Fig. 4B).

Hydroxynonenal (HNE) and PAS staining of the kidney

To assess the consequence of the deposition of PAF-AH protein in the kidney, we performed HNE staining and PAS staining on kidney samples from days 14 and 28, respectively. Samples from control groups showed stronger staining for HNE in renal tubules and interstitial tissues than those from the AdPAFAH group. Of note is that the glomerular regions were even more intensely stained than adjacent regions in samples from control groups, while the glomeruli in those from the AdPAFAH group were not, relative to adjacent regions (Figs. 6A and 6B). Figs. 6C and 6D are representative morphological analyses examined with PAS staining. Consistent with the result of urinary protein excretion, accumulation of extracellular matrix was prominent in samples from the control groups than in the AdPAFAH group.

Discussion

Gene transfer and protein expression in kidney cells, especially in specific cells such as mesangial cells, is difficult to achieve [14]. Several investigators have reported on their progress utilizing non-viral or viral vectors [16-18] as well as ex vivo techniques to directly transduce genes in the kidney. However, their results are mixed and their access methods are likewise not always practical both clinically and experimentally.

Lipoproteins are good vehicles in the delivery of proteins that possess lipophilic properties and preference for lipoprotein particles in their amino acid construct. These proteins include apolipoproteins and PAF-AH. A previous study has demonstrated that adenovirus-mediated over-expression of apolipoprotein E in the liver results in the delivery of this protein to atherosclerotic lesions via lipoproteins [15]. In our present study, liver-directed gene transfer and expression of human PAF-AH in Imai rats demonstrated not only an association of this protein with HDL particles in plasma (Fig. 1B), but also its accumulation in mesangial cells as well as the arteriolar and aortic walls (Figs. 4B and 4E). This accumulation of PAF-AH in glomeruli is not due to the transgene expression in kidney cells (Fig. 5). Furthermore, infusion of HDL particles rich in PAF-AH into Imai rats resulted in its accumulation in the glomeruli and arterioles, a finding that was also observed in kidney samples of rats injected with AdPAFAH. This finding confirms the role of HDL in the delivery of PAF-AH. A previous report which clarified the anti-atherogenic property of PAF-AH in apoE deficient mice utilizing adenovirus-mediated gene transfer demonstrated neither the distribution of PAF-AH among lipoproteins nor its delivery to the arterial wall [7]. However, we speculate that even in that study, PAF-AH must have been delivered to the arterial wall and that it had contributed partly to the inhibition of atherosclerosis progression.

PAF-AH is a protein residing almost exclusively on HDL particles in mice [19, 20] and rats (unpublished observation) under normal physiological conditions; however, this same protein resides on both

LDL and HDL particles in humans [21]. Further experiments are needed to clarify whether all lipoprotein classes deliver PAF-AH to mesangial cells in the same manner as was observed in our study.

Oxidative stress induces peroxidation of lipids, and lipid hyperoxides or oxidized LDL (oxLDL) in turn provoke oxidative stress in the cell. They constitute a vicious cycle and supposedly play a pivotal role in the pathogenesis of glomerular diseases. Oxidative stress and oxidized lipids induce mitogenic activity in mesangial cells through the activation of several proteins associated with signal transduction [22-25] and increase production of the extracellular matrix (ECM) [26-28]. In addition, oxidative stress and oxidized lipids promote the expression of several cytokines, chemokines and adhesion molecules which play a crucial role in glomerular inflammation and recruitment of inflammatory cells to the glomeruli [29-31]. Furthermore, oxidative modification of ECM not only disrupts the integrin-mediated adhesion of mesangial cells [32], but also modulates the susceptibility of ECM to degradation by matrix metalloproteinase-2, resulting in the accumulation of ECM [33]. Finally, intrinsic glomerular composing cells oxidize LDL [34], and oxidative stress and oxLDL induce apoptosis of mesangial cells and facilitate glomerulosclerosis [35-37].

The present study demonstrated that accumulation of PAF-AH in mesangial cells not only inhibited the progression of proteinuria (Fig. 2) but also demonstrated amelioration of the glomeruli histologically (Fig. 6). In addition, the content of HNE, an end product of oxidative stress, is reduced in the glomeruli of Imai rats expressing PAF-AH. Between rats expressing PAF-AH and control animals, there was no significant difference in plasma levels of the specific isoprostane 8,12-iso-iPF(2alpha)-VI (Fig. 1C), which is a good indicator of systemic oxidative stress. These results suggest that PAF-AH disrupted the vicious cycle mentioned earlier through the inactivation of peroxidized phospholipids and reduced oxidative stress locally in the glomeruli. This amelioration in oxidative stress culminated in the reduced production of ECM from mesangial cells as well as reduced accumulation of ECM in the glomeruli (Fig. 6).

Glomerular injury provokes up-regulation of several antioxidant enzymes in mesangial cells [38, 39]. These enzymes include catalase, hemeoxygenase-1 and Mn-superoxide dimustase (SOD), which belong to endoenzymes. Over-expression of these endoenzymes in mesangial cells may prove to be an attractive means of inhibiting glomerulosclerosis should efficient methods for gene delivery to mesangial cells be developed. However, circulating lipoprotein-associated enzymes possessing antioxidative properties are at present the best candidates for molecular therapy in glomerulosclerotic diseases.

In summary, we demonstrated that extra-glomerular expression of the PAF-AH gene by means of an adenovirus-mediated gene transfer and lipoprotein-mediated delivery of PAF-AH to mesangial cells ameliorates proteinuria in Imai rats, an animal model for spontaneous glomerulosclerosis. This is the first direct in vivo demonstration of the deleterious role of oxidative stress and oxidized lipids on the progression of glomerulosclerosis, and the method utilized in this study, namely adenovirus-mediated extra-renal gene expression and lipoprotein-mediated glomeruli-targeted protein delivery, promise to be a novel therapeutic approach to glomerular diseases.

Materials and Methods

Materials and general methods

Urine protein levels were measured by Bradford Protein Assay (Bio-Rad, Hercules, CA, USA), and total cholesterol (TC) levels were determined by enzymatic methods utilizing Cholesterol C-test Wako