et al. reported that albumin upregulated ROS production in proximal tubular cells and this was mediated by NADPH oxidase and PKC (34), indicating that albumin has the ability to increase the generation of ROS in PTC. In the present study, we used 1 - 5 mg/mL of HSA or AOPPs-HSA to investigate ROS production. The content of AOPPs in the plasma of hemodialysis patients is reported to be 267.5 μM (48) and the AOPPs content in 5 mg/mL AOPPs-HSA used in the present study is 911.5 μM, thus indicating that we used a 3.4-fold higher concentration of AOPPs protein than the pathophysiological concentration. We performed these experiments with short-term incubation in such a comparatively high concentration of AOPPs-HSA because AOPPs is continuously generated *in vivo*. The results of present study clearly showed that AOPPs-HSA also upregulated increases of intracellular ROS in a dose-dependent manner and, in particular, 5 mg/mL AOPPs-HSA induced increases in intracellular ROS about 1.8 times higher than did the control. In contrast, 5 mg/mL HSA slightly elevated intracellular ROS, about 1.3 times higher than did the control. A previous report showed that 30 mg/mL HSA significantly induced intracellular ROS about 1.8 times higher than did the control (34). These results demonstrated that AOPPs-HSA potentially activated the generation of ROS as well as HSA. Interestingly, the increases in ROS induced by AOPPs-HSA were significantly decreased by inhibitors of NADPH oxidase and PKC. It is highly possible that interaction of CD36 and AOPPs-HSA results in increase production of intracellular ROS that may take place via a mechanism that involves NADPH oxidase and PKC.

Since anti-CD36 antibody only partially suppresses the uptake of AOPPs-HSA by HK-2 cells, other receptors in PTC may also involve in the uptake of AOPPs-HSA. In

this study, SR-B1 and galectin-3 were also expressed in HK-2 cells (Fig. 3), thus suggesting that those are likely to be involved in the endocytosis of AOPPs-HSA in HK-2 cells. Recently, Marsche et al. reported that CHO cells overexpressing class B scavenger receptors such as CD36 and SR-B1 recognize HOCl-modified LDL (30). SR-B1 also has a multi-ligand specificity for various forms of native and modified (lipo)proteins (24) and the protein expression of SR-B1 has been observed in proximal tubular cells (51). Therefore, SR-B1 may also be involved in the uptake of AOPPs-HSA in the proximal tubular cells. In addition, Nishiyama et al. reported that galectin-3, a multi-functional beta-galactoside-binding lectin, is expressed by epithelial cells of the kidney and participates in development, oncogenesis, cell-to-cell attachment and inflammation, suggesting that galectin-3 may also play an important role in acute tubular injury (36). Furthermore, galectin-3 was identified as a component of the AGE receptor complex (28). These observations suggested that SR-B1 and galectin-3 might also be involved in proximal tubular cell injury. Although further studies using CHO cells overexpressing SR-B1 and galectin-3 and those neutralizing antibodies would be required to determine whether SR-B1 and galectin-3 are also involved in the proximal tubular injury by AOPPs, there are no reports using the optimal neutralizing antibodies against SR-B1 and galectin-3, thus suggesting that we have to conduct another approach such as the silencing strategies for SR-B1 and galectin-3. However, there is only commercial reagent for SR-B1 knock down, not for galectin-3. Therefore, for the time being, we performed the transfection of SR-B1 siRNA to HK-2 cells. As a result, although transfection by SR-B1 siRNA (80 pmol) resulted in more than 60% suppression of SR-B1 protein expression in HK-2 cells, the endocytic association and degradation of ^{125}I-AOPPs-HSA did not

decrease in these cells (data not shown). This result directly demonstrated that SR-B1 is not involved in the endocytosis of AOPPs-HSA in HK-2 cells. Further studies using siRNA knock-down against galectin-3 in HK-2 cells would therefore shed some light on the mechanisms of renal tubular injury as well as the extent of the contribution of each factor identified in this study.

In conclusion, a new functional role of CD36 was identified as an essential mediator of tubulointerstitial diseases induced by AOPPs in PTC. Furthermore, because AOPPs-HSA by itself stimulates CD36 expression in human PTC, it is speculated that uptake of AOPPs-HSA by PTC would be increased, causing further progression of renal disease. Therefore, CD36 appears to be closely associated with renal tubular dysfunction. Modulating the expression of CD36 or blocking this receptor may be a good approach in the treatment of renal tubular fibrosis.

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FIGURE LEGENDS

Fig. 1. Dose-dependent endocytic association (A and B) and degradation (C and D) by HK-2 cells of ^{125}I-HSA (A and C) and ^{125}I-AOPPs-HSA (B and D). HK-2 cells were incubated at 37°C for 24 hr with the indicated concentrations of ^{125}I-HSA and ^{125}I-AOPPs-HSA in the presence (nonspecific: □) or absence (total: ○) of 50-fold excess amounts of the unlabeled ligands. The specific cell-association or degradation (●) was plotted after subtracting the non-specific values from the total values. Data represent the means ± SD (n=6). * $P < 0.05$, ** $P < 0.01$ versus each specific cell-association or degradation of HSA.

Fig. 2. Effects of HSA on the endocytic association of ^{125}I-AOPPs-HSA with HK-2 cells and of megalin siRNA transfection on the expression of megalin transcript and on the endocytic association of ^{125}I-AOPPs-HSA with HK-2 cells. A: cells were incubated at 37°C for 24 hr with 0.5 mL of K-SFM medium containing 2.5 μg/mL of ^{125}I-AOPPs-HSA in the absence or presence of 125 μg/mL of unlabeled AOPPs-HSA or HSA, followed by determination of endocytic association. Data represent the means ± SD (n=5). * $P < 0.01$ versus control. B: immunoblot analyses of megalin in HK-2 cells transfected with 80 pmol megalin siRNA was performed using goat anti-human megalin

antibody (C19). The graphic representation of an immunoblot analysis demonstrated the effect of megalin siRNA on their ptotein expression in HK-2 cells. C: cells transfected with 80 pmol megalin siRNA were incubated at 37°C for 24 hr with 0.5 mL of K-SFM medium containing 2.5 μg/mL of ^{125}I-AOPPs-HSA, followed by the determination of any endocytic association. Data represent the means ± SD (n=4).

Fig. 3. Immunoblot analyses of A: CD36, B: SR-A, C: LOX-1, D: SR-B1, E: RAGE and F: Galectin-3 in HK-2 cells. Cellular proteins (10 μg) were subjected to an immunoblot analysis using anti-CD36 polyclonal antibody (1:100), anti-SR-A polyclonal antibody (1:200), LOX-1 antibody (1:1000), anti-SR-B1 polyclonal antibody (1:1000), anti-human RAGE polyclonal antibody (1:1400), anti-human galectin-3 polyclonal antibody (1:1000), or anti-β-actin polyclonal antibody (1:500). PC: Positive control (human macrophage: CD36, SR-A, LOX-1 and galectin-3; RAGE-CHO cells: RAGE; SR-B1-CHO-cells: SR-B1). All experiments were repeated three times with almost identical results.

Fig. 4. Dose-dependent endocytic association (A and B) and degradation (C and D) of ^{125}I-AOPPs-HSA by CD36-CHO cells (A and C) or mock-CHO cells (B and D). Cells were incubated for 37°C for 5 hr in 0.5 mL DMEM medium containing 3% BSA with concentrations of ^{125}I-AOPPs-HSA in the presence (nonspecific: □) or absence (total: ○) of 50-fold excess amounts of unlabeled ligands. The specific cell-association and degradation (●) were plotted after subtracting the non-specific values from the total values. Data represent the means ± SD (n=3). * $P < 0.01$ versus each specific cell-

association or degradation of AOPPs-HSA in mock-CHO cells.

Fig. 5. Effect of anti-CD36 antibody (FA6-152) on the endocytic uptake of ^{125}I-AOPPs-HSA by HK-2 cells. Cells were incubated at 37°C for 24 hr with 0.5 mL of K-SFM medium containing 2.5 μg/mL of ^{125}I-AOPPs-HSA in the presence or absence of unlabeled AOPPs-HSA (125 μg/mL), anti-CD36 antibody (FA6-152: 80 μg/mL), or control antibody (MOPC21: 80 μg/mL) or HSA(125 μg/mL). Results are the means ± SD (n=3). * $P < 0.05$, ** $P < 0.01$ versus control.

Fig. 6. Expression of CD36 induced by AOPPs-HSA in HK-2 cells. Cell proteins (10 μg) were subjected to immunoblot analysis using anti-CD36 polyclonal antibody (L-17) (1:100), or anti-ß-actin polyclonal antibody (1:500). Results are the means ± SD (n=3). * $P < 0.05$, ** $P < 0.01$ versus control cells.

Fig. 7. Assessment of reactive oxygen species (ROS) by fluorescence-activated cell sorter analysis. After CM-H_2DCFDA incubation, HK-2 cells were treated with HSA or AOPPs-HSA for 15 min. Accumulation of DCF was measured with a flow cytometer by monitoring fluorescence at 526 nm. Intracellular ROS formation is expressed as a ratio of the mean fluorescence intensity of control cells incubated in an albumin-free medium. Results are the means ± SD (n=5). [NAC]=20 mM. [DPI]=10 μM. [Staurosporine] =100 nM. * $P < 0.05$, ** $P < 0.01$ versus control cells. $^{\#}P < 0.01$ versus cells treated with 5 mg/mL HSA.

Fig. 8. Effect of anti-CD36 antibody (FA6-152) or ROS inhibitors on the TGF-β1 secretion induced by AOPPs-HSA. Cells were treated with HSA, AOPPs-HSA, or AOPPs-HSA in the presence of anti-CD36 antibody (FA6-152), NAC or DPI at 37°C for 24 hr. Results are the means ± SD (n=3). * $P < 0.01$ versus control cells.

Fig. 1

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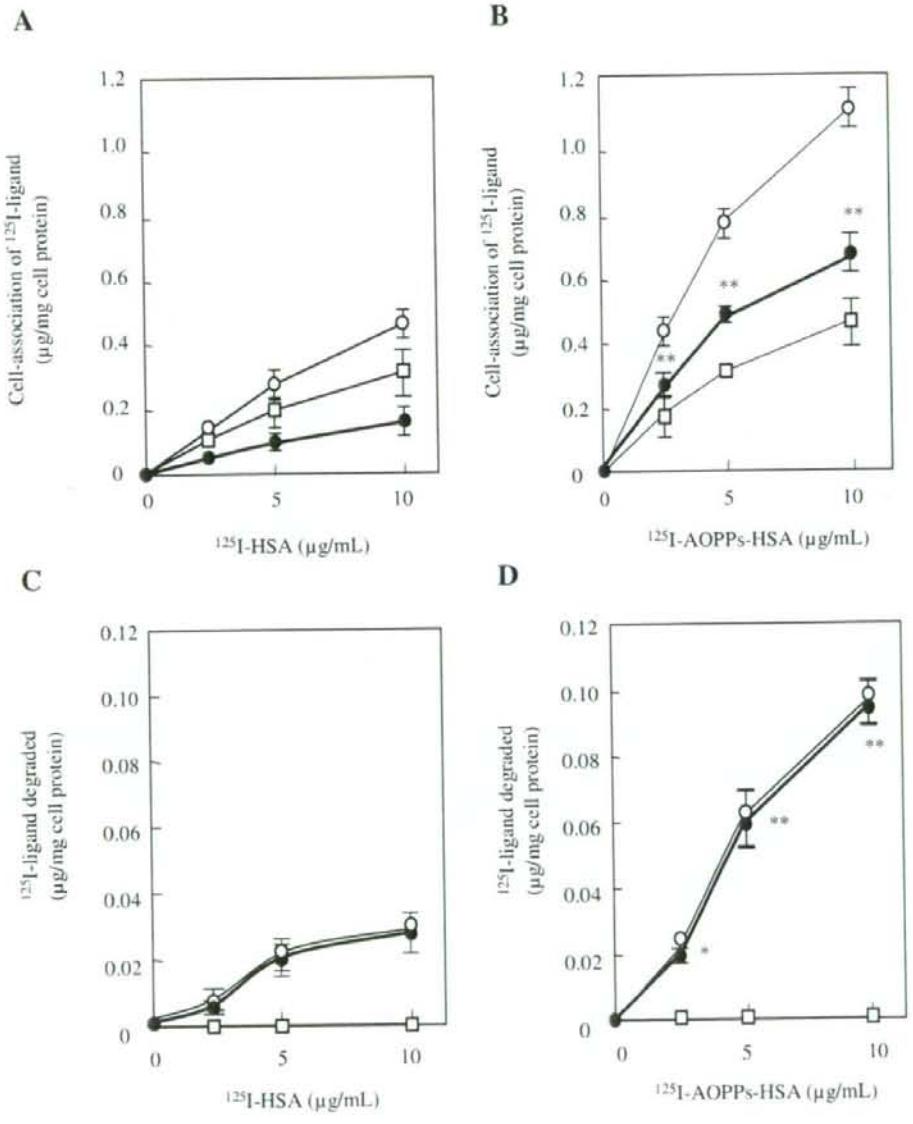

Fig. 2

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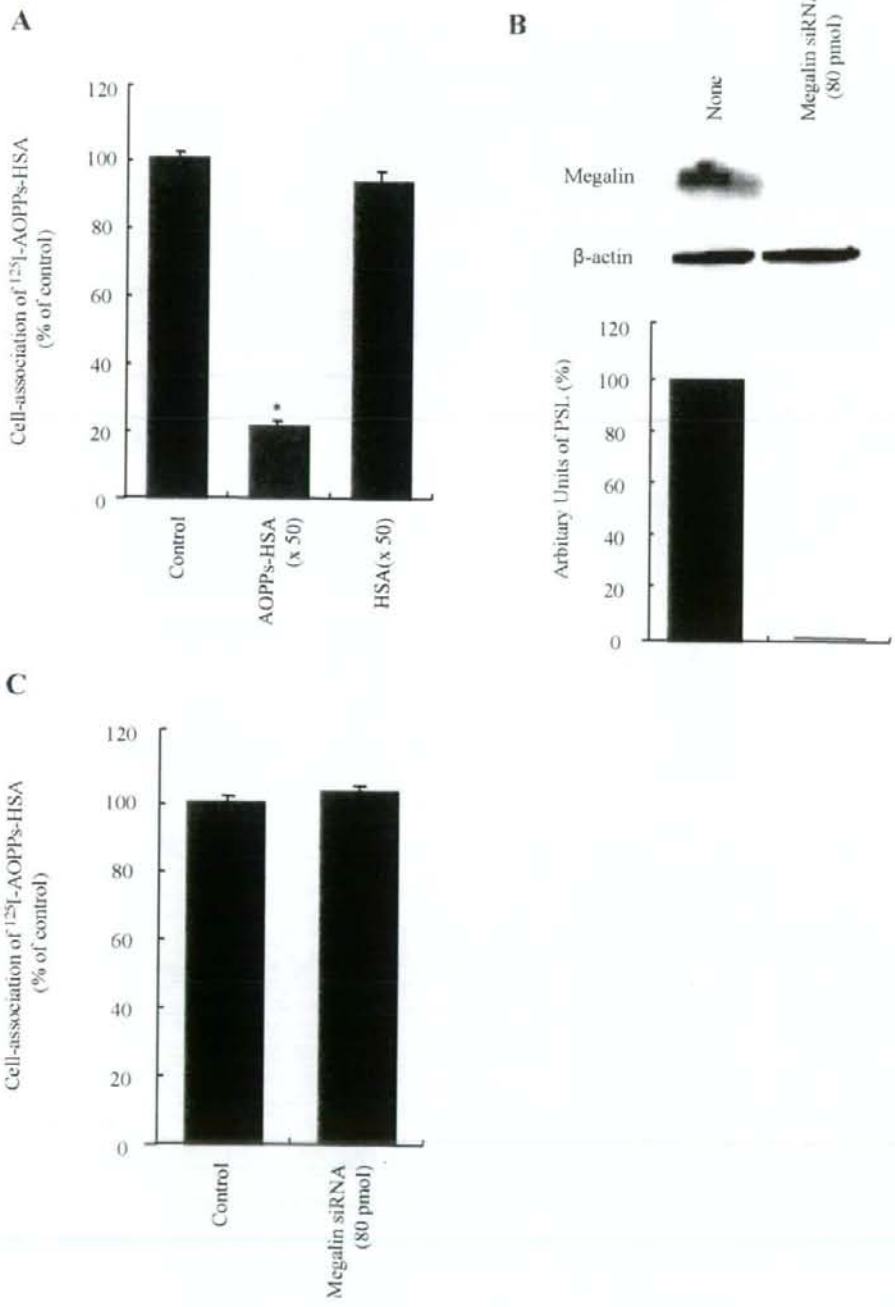

Fig. 3

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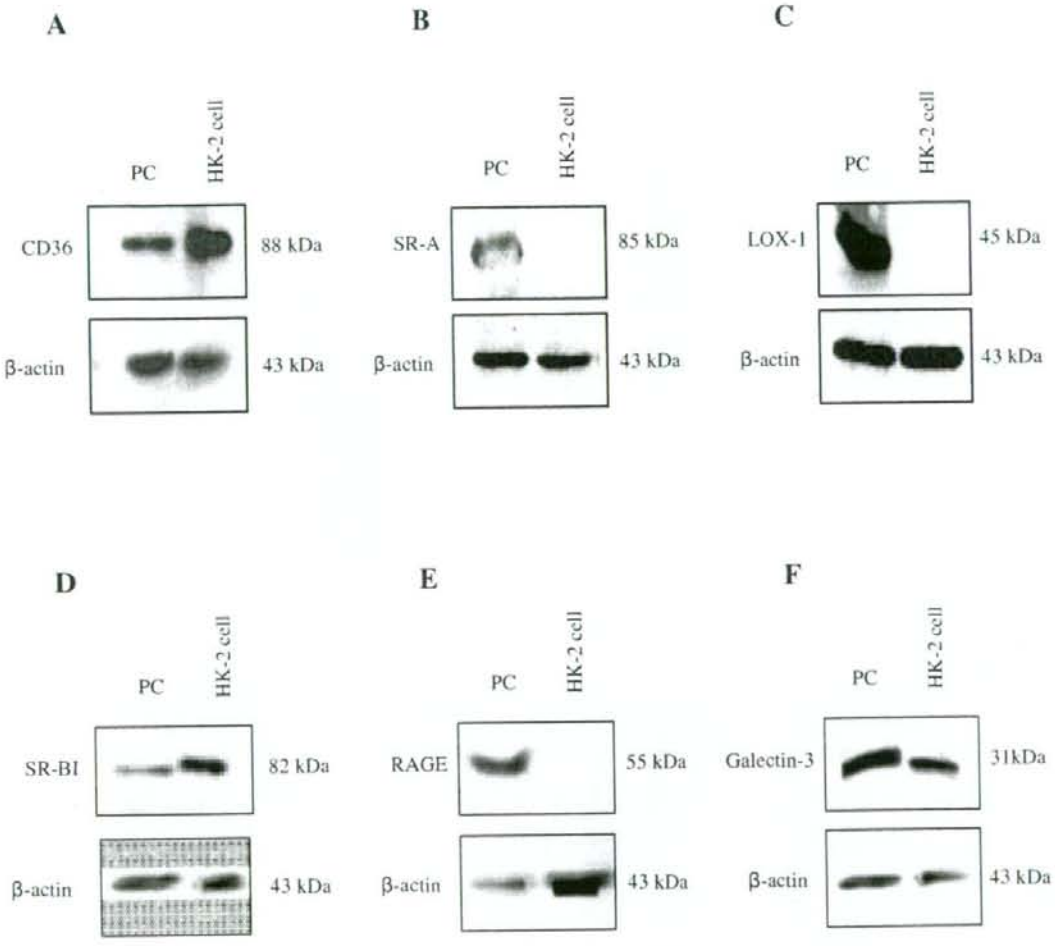

Fig. 4

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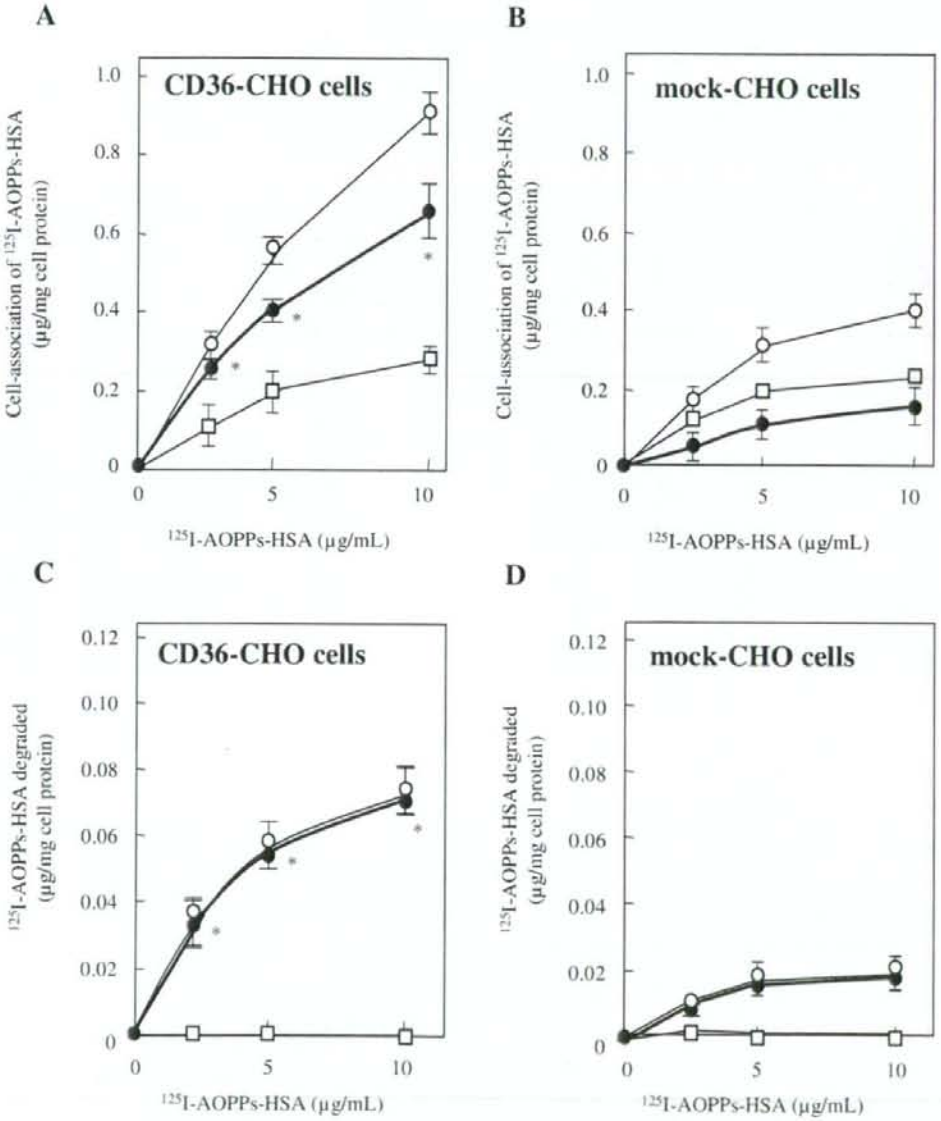

Fig. 5

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