RNA interference of megalin to HK-2 cells. Human megalin siRNA consisted of three target-specific 20- to 25-nt siRNAs, and the siRNA negative control that contained a scrambled sequence was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HK-2 cells, 1×10^5, in log phase were plated into 6-well tissue culture plates with normal growth medium 24 hr before transfection or when the cells reached 60–80% confluence. For each transfection well, the siRNA-transfection reagent complexes were overlaid onto the washed cells. Cells were incubated in normal cell culture conditions for 7 hr, and then fresh normal growth medium was added to each well to maximize cell growth and prevent potential cytotoxicity. After 24 hr of transfection, assays for target gene expression by Western blot were performed using goat anti-human megalin antibody (C19, Santa Cruz Biotechnology).

Cellular assays. Cellular endocytosis experiments were performed at 37°C in a humidified atmosphere of 5% CO_2 in air. HK-2 cells were cultured in supplemented K-SFM. Cells (1×10^6) were seeded in a 24-well plate and cultured for 1 day in 1.0 mL of this medium, which was then replaced by unsupplemented K-SFM (medium A). After culture for 1hr (cells cultured to 90 - 100 % confluence), each well received 0.5 mL of medium A containing various concentrations of ^{125}I-HSA or ^{125}I-AOPPs-HSA in either the presence or the absence of 50-fold excess amounts of the unlabeled ligands. For inhibition experiments, the incubation medium also included anti–human CD36 monoclonal antibody (FA6-152; 80 μg/ml) or control IgG (anti-human IgG monoclonal antibody; 80 μg/ml). CD36-CHO cells and mock-CHO cells were maintained with Ham's F-12 medium (Gibco) supplemented with 10 μg/mL of blasticidin S (Funakoshi, Tokyo,

Japan) and 10% fetal calf serum (FCS) (medium B) in humidified air with 5% CO_2. Cells (1×10^6) were seeded in a 24-well plate and cultured for 1 day in 1.0 mL of medium B, which was then replaced by Dulbecco's modified Eagle's medium containing 3% BSA (medium C). After culturing for 1hr, each well received 0.5 mL of medium C containing various concentrations of ^{125}I-AOPPs-HSA in either the presence or the absence of 50-fold excess amounts of the unlabeled ligand to be tested. After incubation for the indicated times, an aliquot (0.375 mL) of the culture medium was mixed with 0.15 mL of 40% trichloroacetic acid (TCA). To this solution was added 0.1 mL of 0.7 M $AgNO_3$, followed by centrifugation at 1500 x *g* for 10 min. The resulting supernatant (0.25 mL) was used to determine TCA-soluble radioactivity, which was taken as an index of cellular degradation, since proteins are endocytosed by the cells and delivered to lysosomes where they are degraded and excreted into the culture medium in a TCA-soluble form. Then, each well was washed three times with 1.0 mL ice-cold PBS. The cells were lysed with 1.0 mL of 0.1 N NaOH for 1 hr at 37°C to determine the cell-associated radioactivity. Specific cell-association or degradation was determined by subtracting the non-specific values from the totals.

Immunoblotting technique. HK-2 cells were solubilized with 1% Triton X-100/PBS buffer containing 50x protease inhibitor cocktail. These samples (10 µg) were run on 10% SDS-polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose membrane. The membranes were exposed to a goat anti-CD36 polyclonal antibody (L-17, Santa Cruz Biotechnology), mouse anti-SR-A monoclonal antibody (SRA-E5), humanized anti-human LOX-1 antibody (JTX92), rabbit anti-SR-BI polyclonal antibody

(Novus Biologicals), rabbit anti-human RAGE polyclonal antibody (5505), or rabbit anti-human galectin-3 polyclonal antibody and visualized by horseradish peroxidase-conjugated anti-goat, -mouse, -human or -rabbit IgG antibody using the ECL Western blotting detection reagent (Amasham Biosciences). This membrane was reblotted with goat anti-actin polyclonal antibody (I-19, Santa Cruz Biotechnology) as an internal normal. The molecular sizes of CD36, SR-A, LOX-1, SR-B1, RAGE and galectin-3 detected by this immunoblot were 88, 50, 43 82, 55 and 31 kDa, respectively.

Assessment of intracellular ROS. Intracellular ROS generation was assessed in HK-2 cells by means of an oxidant-sensitive dye, CM-H_2DCFDA. Suspensions of the cells (1×10^6 cells) were incubated with 10 μM CM-H_2DCFDA for 15 min at 37°C in the medium. After centrifugation and washing to remove the unincorporated probe, cells were treated with several concentrations of normal-HSA or AOPPs-HSA medium for 30 min at 37°C and placed on ice. Accumulation of DCF in HK-2 cells was measured with a flow cytometer (FACSCalibular; Becton Dickinson Biosciences, Franklin Lakes, NJ) by monitoring the fluorescence at 526 nm. Intracellular ROS formation was expressed as a ratio of the mean fluorescence intensity of control cells incubated in ligand-free medium.

Measurement of active TGF-β1 proteins. Cells were plated at 1×10^6 cells/well in 12-well plates. The next day, cells were rested in serum-free assay medium for 24 hr (cells cultured to 90 - 100 % confluence), and then treated with or without normal-HSA or AOPPs-HSA for 24 hr. TGF-β released into media was measured with an enzyme immunoassay system (Promega, Madison, WI, USA) according to the manufacturer's

instructions. The inhibitory effect of TGF-β1 secretion in HK-2 cells was induced by treatment with anti–human CD36 monoclonal antibody (FA6-152; 80 μg/mL), N-acetyl-cysteine (NAC; 20 mM), diphenylene iodonium (DPI; 10 μM), or control IgG (anti-human IgG monoclonal antibody; 80 μg/mL).

Statistics. Statistical analyses were performed using the Student t-test. A probability value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Interaction of AOPPs-HSA with HK-2 cells.

To examine whether AOPPs-HSA interact with HK-2 cells, endocytosis experiments were conducted using ^{125}I-labeled HSA or AOPPs-HSA in HK-2 cells. Upon incubation at 37°C, cell-association of ^{125}I-AOPPs-HSA with HK-2 cells significantly increased in a dose-dependent manner, by more than ^{125}I-HSA (Fig. 1A and 1B). In addition, endocytic degradation of ^{125}I-AOPPs-HSA by HK-2 cells occurred at a higher level than HSA (Fig. 1C and 1D). These results suggest that AOPPs-HSA undergoes endocytosis at a higher level than does HSA in HK-2 cells. Furthermore, we performed the ligand binding study of AOPPs-HSA in HK-2 cells. The total binding of ^{125}I-AOPPs-HSA to these cells at 4°C was inhibited by >60% by the presence of an excess amount of unlabeled AOPPs-HSA. The specific binding, which was obtained by subtracting nonspecific binding from the total binding, showed a saturation curve for which Scatchard analysis revealed a binding site with an apparent Kd of 1.93 μg/ml and a maximal binding of 111.2 ng/mg of cell protein (data not shown). These results indicated that HK-2 cells possess a high-affinity binding site for AOPPs-HSA. The recognition site for AOPPs-HSA in HK-2 cells was also examined. The cell association of ^{125}I-AOPPs-HSA with HK-2 cells was effectively replaced by a 50-fold excess of unlabeled AOPPs-HSA (>80%, $p < 0.01$ versus control), whereas a 50-fold excess of unlabeled HSA had only a slight effect (<5%, not significant versus control) (Fig.2A). The endocytic degradation of ^{125}I-AOPPs-HSA was also inhibited almost completely by the presence of a 50-fold excess of unlabeled AOPPs-

HSA (>80%, $p < 0.01$), whereas a 50-fold excess of unlabeled HSA had only a slight effect (<10%) (data not shown). These results indicated that AOPPs-HSA was endocytosed differently from HSA. Since it has been suggested that the receptors involved in the uptake of HSA are mainly the megalin/cubilin receptor complex (3, 8), those are unlikely to contribute to the endocytosis of AOPPs-HSA because megalin is negatively charged, thus showing a preferential affinity for positively charged proteins (31), whereas AOPPs-HSA shows a negative charge. To further elucidate the role of megalin/ cubilin complex in the endocytosis of AOPPs-HSA in HK-2 cells, we evaluated the effect of silencing the megalin gene on the endocytosis of AOPPs-HSA. As a result, although the transfection of megalin siRNA (80 pmol) resulted in more than 99% suppression of megalin protein expression in HK-2 cells, the association of ^{125}I-AOPPs-HSA did not decrease in these cells (Fig. 2B and 2C). In addition, the endocytic degradation of ^{125}I-AOPPs-HSA was not also affected by megalin siRNA transfection (data not shown). Li et al. demonstrated that transfection by megalin siRNA (50 pmol) resulted in up to 90% suppression of not only megalin but also cubilin protein and mRNA expression in HK-2 cells (27), suggesting that cubilin protein and mRNA expression are most probably knocked down in cells transfected with megalin siRNA. Therefore, these data demonstrated the evidence that the megalin-cubulin complex is not associated with the endocytosis of AOPPs-HSA in HK-2 cells.

CD36 was involved in the endocytosis of AOPPs-HSA in HK-2 cells.

Recently, there have been reports of several types of receptors that bind to modified albumin (20). However, it is not yet clear which scavenger receptor is involved in the

endocytosis of AOPPs-HSA by HK-2 cells. The expression of scavenger receptors class A-I/II (SR-A), scavenger receptors class B (CD36), lectin-like oxidized LDL receptors (LOX-1), scavenger receptor class B type 1 (SR-B1), receptor for advanced glycation end products (RAGE) and galectin-3 was examined using a Western blotting analysis in HK-2 cells. As shown in Figure 3, CD36, SR-B1 and galectin-3 were expressed in HK-2 cells, whereas SR-A, LOX-1 and RAGE were poorly expressed in HK-cells. Specifically, CD36 is one of important receptors which is involved in the endocytosis of oxidized protein in adipocytes (25), thus suggesting that CD36 may be involved in the uptake of AOPPs-HSA in HK-2 cells. To elucidate this association, the cellular endocytosis of ^{125}I-AOPPs-HSA was also examined using CD36-CHO cells (overexpressed CD36 in CHO cells) and mock-CHO cells (in which only the vector was transfected to CHO cells) (Fig. 4). The specific cell association and degradation of ^{125}I-AOPPs-HSA with CD36-CHO cells exhibited a dose-dependent effect (Fig. 4A and 4C). However, the specific cell association and degradation of ^{125}I-AOPPs-HSA were not observed in mock-CHO cells (Fig.4B and 4D). These results demonstrated that AOPPs-HSA served as a ligand for CD36.

To examine the involvement of CD36 in endocytosis of AOPPs-HSA in HK-2 cells, neutralizing anti-CD36 monoclonal antibody (FA6-152) was used for the endocytic association and degradation of ^{125}I-AOPPs-HSA in HK-2 cells (Fig. 5). Non-specific association, which was measured with a 50-fold excess of unlabeled AOPPs-HSA (Fig. 2), was subtracted from each experiment. As shown in figure 5, the receptor-mediated specific cell association and degradation of ^{125}I-AOPPs-HSA in HK-2 cells was

significantly inhibited by addition of anti-CD36 antibody (inhibitory effect of association: 35%, $p < 0.05$; inhibitory effect of degradation: 45%, $p < 0.01$), while non-immune IgG and excess unlabeled HSA had no effect on this process (<5%, considered not significant versus control). These results indicate that some endocytic pathways of AOPPs-HSA in HK-2 cells are mediated by CD36.

AOPPs-HSA enhances the expression of CD36 in HK-2 cells.

It has been shown that oxidized low-density lipoprotein (ox-LDL) and AGE-proteins, such as CD36 ligands, induce CD36 protein synthesis in macrophages (17, 23). Therefore, the effects of AOPPs-HSA on CD36 protein expression in HK-2 cells were examined. Exposure of cells to 100 μg/mL AOPPs-HSA at 37°C for 24 hr, but not exposure to HSA, significantly increased levels of CD36 protein expression in cell lysates (Fig. 6). This result suggested that proteinuria, including a large amount of AOPPs-HSA, causes a series of adverse effects in proximal tubular cells, resulting from upregulation of CD36 expression and increased uptake of AOPPs-HSA.

AOPPs-HSA increased the generation of ROS in HK-2 cells.

To assess whether AOPPs-HSA could also induce oxidative stress like HSA, ROS in HK-2 cells was measured by the CM-H_2DCFDA method and fluorescence-activated cell-sorter (FACS) analysis. After addition of HSA or AOPPs-HSA (1.0, 2.5 and 5.0 mg/mL) to HK-2 cells, ROS was generated in a dose-dependent manner by AOPPs-HSA. To a lesser extent, HSA also generated ROS in HK-2 cells (Fig. 7). To examine the mechanism of ROS generation by AOPPs-HSA, the authors examined the effects of the

following: NAC (20 mM), a ROS scavenger; DPI (10 μM), an inhibitor of membrane NADPH oxidase; and, staurosporine (100 nM), a PKC inhibitor for ROS generation. As shown in Fig. 7, all three inhibitors were able to block ROS generation induced by AOPPs-HSA, suggesting that AOPPs-HSA produced intercellular ROS by the activation of PKC and membrane NADPH oxidase.

AOPPs-HSA activated secretion of TGF-β1 by the CD36 pathway in HK-2 cells.

To confirm whether AOPPs-HSA actually causes injury of HK-2 cells via the CD36 pathway, the secretion of TGF-β1 proteins, which causes fibrosis on proximal tubular cells, was examined using an enzyme immunoassay. After the incubation of HK-2 cells with 100 μg/mL of HSA or AOPPs-HSA for 24 hr, TGF-β1 proteins in the medium were measured. Higher levels of TGF-β1 proteins were secreted in the medium by cells exposed to AOPPs-HSA. To a lesser extent, HSA also produced TGF-β1 (Fig. 8). The involvement of CD36 in the secretion of TGF-β1 by AOPPs-HSA was also examined, using an anti-CD36 monoclonal antibody (FA6-152). As expected, FA6-152 significantly inhibited secretion of TGF-β1 induced by AOPPs-HSA in HK-2 cells. In addition, we examined whether NAC and DPI could suppress the secretion of TGF-β1 induced by AOPPs-HSA. These inhibitors significantly inhibited the secretion of TGF-β1 induced by AOPPs-HSA, thus suggesting that ROS generated by AOPPs-HSA act as signaling molecules in HK-2 cells and lead to the activation of TGF-β1.

DISCUSSION

CKD is a worldwide health problem and the number of patients with CKD is increasing rapidly (7). Progressive renal disease is caused by the development of glomerulosclerosis and interstitial fibrosis. Therefore, it is important to explore the factor(s) that promote the process in order to develop new strategies for suppressing CKD. Recent studies have found that plasma concentration of AOPPs significantly increased with the progression of renal dysfunction in patients (48) and that AOPPs may contribute to the progression of CKD (26). However, the mechanisms by which AOPP accelerates renal fibrosis remain poorly understood. Therefore, we examined the mechanisms of AOPPs and the detrimental effects induced by AOPPs in HK-2 cells. Taking this approach, the novel findings of the present study were as follows. 1) AOPPs-HSA undergoes endocytic uptake and subsequent lysosomal degradation by proximal tubular cells, HK-2 cells. 2) These processes were effectively inhibited by anti-CD36 antibody. 3) AOPPs-HSA upregulated the generation of intracellular ROS via a mechanism that involves PKC and membrane NADPH oxidase signaling pathways, and the secretion of TGF-β1 in HK-2 cells. 4) AOPPs-HSA induced secretion of TGF-β1 is mediated by CD36. Thus, this study provides new evidence for a detailed mechanism by which AOPPs cause proximal tubular cell injury via CD36.

CD36 is an 88-kDa transmembrane glycoprotein of the class B scavenger receptor family, and is involved in multiple biological processes (15). It is broadly expressed by

renal tubular cells, platelets, monocytes, adipocytes, endothelial cells, erythroblasts, epithelial cells and several tumor cell lines (15, 50). In addition, CD36 interacts with multiple extracellular ligands, including thrombospondin-1, long-chain free fatty acids, modified (oxidized) low-density lipoprotein (ox-LDL), collagens I and IV and advanced glycation end products (AGE) (15). Previous reports showed that the expression of CD36 is upregulated by its own ligand. For instance, Iwashima et al. showed that AGE induced the expression of CD36 in human monocyte-derived macrophages and in THP-1 cells (23). Susztak et al. reported that CD36 expression was induced by D-glucose in proximal tubular cells (42). In this study, AOPPs-HSA, a ligand for CD36, significantly induced protein expression of CD36 in a dose-dependent manner. Interestingly, HSA (100μg/mL), not a ligand for CD36, slightly increased the expression of CD36 in HK-2 cells in this study. Recently, Yang et al. showed that high concentrations of normal albumin (1.0 and 10 mg/mL) induced the expression of CD36 in LLC-PK1 cells, and enhanced the secretion of bioactive TGF-β1 and fibronectin with the upregulation of CD36 (50). Thus, this report speculates that high concentrations of HSA (> 1.0 mg/mL) may increase the expression of CD36 in HK-2 cells. These findings suggested the possibility that CD36 may be an important receptor in proximal tubular injury and AOPPs and normal albumin may be deleterious mediators in this disease.

Recent evidence has clearly shown rapid and significant increases in intracellular ROS after growth factor and cytokine stimulation. These types of ROS appear to be essential for a host of downstream signaling events, including cell proliferation, apoptosis, and fibrosis, and thus contribute to the development of disease (16). Nakajima

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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