Protein load impairs factor H binding promoting complement-dependent dysfunction of proximal tubular cells

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Intrarenal complement activation plays an important role in the progression of chronic kidney disease. A key target of the activated complement cascade is the proximal tubule, a site where abnormally filtered plasma proteins and complement factors combine to promote injury. This study determined whether protein overloading of human proximal tubular cells (HK-2) in culture enhances complement activation by impairing complement regulation. Addition of albumin or transferrin to the cells incubated with diluted human serum as a source of complement caused increased apical C3 deposition. Soluble complement receptor-1 (an inhibitor of all 3 activation pathways) blocked complement deposition while the classical and lectin pathway inhibitor, magnesium chloride–EGTA, was, ineffective. Media containing albumin as well as complement had additive proinflammatory effects as shown by increased fractalkine and transforming growth factor-β mRNA expression. This paralleled active C3 and C5b-9 generations, effects not shared by transferrin. Factor H, one of the main natural inhibitors of the alternative pathway, binds to heparan sulfate proteoglycans. Both the density of heparan sulfate and factor H binding were reduced with protein loading, thereby enhancing the albumin- and serum-dependent complement activation potential. Thus, protein overload reduces the ability of the tubule cell to bind factor H and counteract complement activation, effects instrumental to renal disease progression.

Kidney International (2009) **75,** 1050–1059; doi:10.1038/ki.2009.8; published online 25 February 2009

KEYWORDS: albumin; chemokines; complement; cytokines; factor H; transferrin

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Received 23 April 2008; revised 19 November 2008; accepted 23 December 2008; published online 25 February 2009

Progressive nephropathies with severe defects of the glomerular filtration barrier to proteins are accompanied by tubulointerstitial damage eventually contributing to loss of renal function.[1] The clinical problem has grown enormously[1,2] and understanding the basic mechanisms is mandatory. Experimental observations suggested that ultrafiltered plasma proteins exert renal toxicity, which is partly mediated by activation of inflammatory and fibrogenic pathways by proximal tubular cells.[3–9] Among processes underlying injury, the activation of the complement cascade in the renal tubule has major effects.[10] Complement proteins can be abnormally filtered across the altered glomerular barrier leading to intratubular deposition of C3 and formation of membrane attack complex (MAC).[11–13] Proximal tubular epithelial cells also synthesize most components of the activation cascade.[14,15] Investigation using proteinuric models of glomerular injury showed less tubulointerstitial damage in complement-depleted rats[12,13] and C6-deficient rats,[16] or upon treatment with complement inhibitory molecules.[13,17] Inflammation and a fibrogenic reaction ensue upon proximal tubule overloading by C3 and ultrafiltered proteins in rats with remnant kidneys.[18,19] We have recently found that ultrafiltered complement factors are a key component of proteinuria-associated renal injury. Thus, plasma-derived C3 is recruited with other ultrafiltered proteins in the tubular compartment to mediate interstitial macrophage accumulation and renal structural damage in the absence of C3 synthesis by the kidney, in a mouse model of protein-overload proteinuria.[20] Overall, proteinuria is required for complement to fully exert harmful effects on the kidney,[21] possibly by enhancing the vulnerability of proximal tubular cells to luminal attack by complement. This makes it difficult to dissect mechanisms by which complement could be engaged with other ultrafiltered proteins to induce tubular cell dysfunction. However, approaches using cultured proximal tubular epithelial cells are most appropriate to address this issue.

In human proximal tubular cells exposed to serum, complement activation through the alternative pathway leads to surface deposition of C3 and MAC and upregulation of

cytokines.[22,23] Complement activation might be dependent on relative lack of complement inhibitors. Cell-associated regulators at C3 level, decay accelerating factor and membrane cofactor protein, are hardly detectable on apical membranes of proximal tubular cells. Conversely, human proximal tubular cells produce factor H,[24] a 150-kDa plasma glycoprotein acting as the major soluble inhibitor of the formation of alternative pathway C3 convertase on the cell surface[25,26] and possibly representing a unique defense mechanism against complement activation. No studies have yet documented actual binding of factor H by tubular epithelial cells. Relevant binding sites on the cell surface, as shown in endothelial cells, may include heparan sulfate proteoglycans.[27]

The aim of this study was to investigate whether (1) protein overload may influence complement activation and deposition on the surface of proximal tubular cells exposed to serum complement, a condition that mimics the exposure of tubular cells to ultrafiltered complement in proteinuric disease; (2) this effect was related to reduced binding of factor H on the cell surface accounting for reduced capability of the cell to counteract complement activation; and (3) loss of heparan sulfate sites contributed to reduced factor H binding to the cell.

RESULTS

Protein overload enhances the activation of serum-derived complement by human proximal tubular cells

With the aim of studying the effect of plasma protein challenge on complement deposition, preliminary experiments were carried out to identify the sublytic concentration of human serum (HS), used as source of complement. Cell viability, as assessed by trypan blue dye exclusion at 90 min, was not affected by exposure to 10% HS, whereas a significant ($P<0.01$) reduction was observed upon exposure to 25% HS in comparison with control cells (10% HS: 91 ± 5, 25% HS: 71 ± 4 versus control: 100% viable cells). The concentration of 10% HS was therefore established to be sublytic. HK-2 cells upon exposure to 10% HS activated and fixed C3 and MAC on the apical side of the cell surface as showed by appearance of granular staining by immunofluorescence analysis (Figure 1) on cell surface. In contrast, unstimulated cells showed no fluorescent signal (Figure 1). Addition of sCR-1, a soluble inhibitor of all the three pathways of complement activation, completely blocked C3/MAC deposition on cell surface, indicating specificity of C3 and MAC signals (Figure 1). Similarly, no complement protein deposition was detected using heat-inactivated HS (data not shown).

To study whether protein overload could enhance the ability of proximal tubular epithelial cells to fix complement molecules, immunofluorescence analysis of C3 and MAC deposits was performed on HK-2 cells, first challenged with albumin or transferrin and then incubated with 10% HS as described above. Both plasma proteins caused significant increases in C3 staining as detected at the end of the experiment, in comparison with cells exposed to HS alone (Figure 2a). Exposure of HK-2 cells to plasma proteins in the absence of HS resulted in no significant changes in C3 staining as compared with unstimulated cells, thus excluding that the abnormal accumulation of C3 on the cell surface in the presence of HS could be attributed to the induction of C3 synthesis in response to albumin and transferrin. To test whether complement activation may occur through the alternative pathway, cells were incubated with HS containing sCR-1 or with $MgCl_2$–EGTA that blocks the activation of the classic and the lectin pathways. The addition of sCR-1 resulted in complete inhibition of C3 deposition. In contrast, $MgCl_2$–EGTA had no significant effect (Table 1).

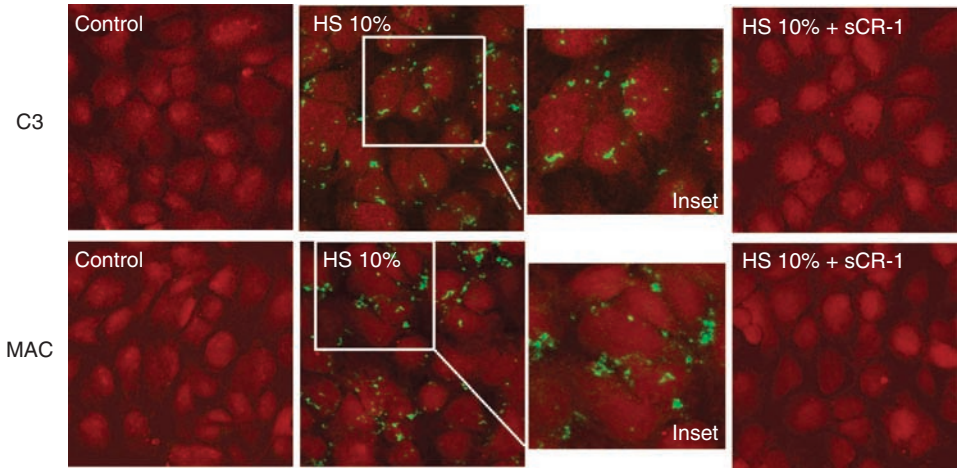

Figure 1 | C3 and MAC (membrane attack complex) deposition on tubular epithelial cell surface in response to human serum (HS). Immunofluorescence staining of C3 and MAC (green) on HK-2 cells exposed for 90 min to medium or 10% HS in the presence or absence of soluble complement receptor-1 (sCR-1, 100 μg/ml). No C3 or MAC fixation was observed on unstimulated cells. HK-2 cells incubated with 10% HS exhibited C3 and MAC staining with granular distribution on cell surface (inset: two-fold enlargement of selected areas). sCR-1 treatment completely inhibited C3 and MAC signals. Living cells were stained in red using 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine probe. Original magnification × 630.

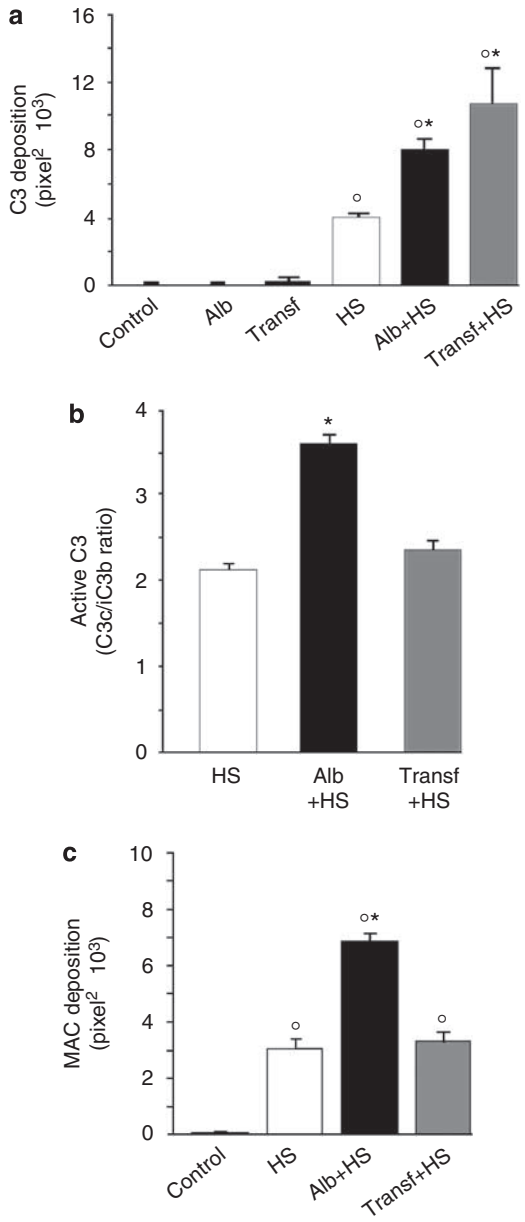

Figure 2 | Effect of plasma proteins on serum-induced C3 and MAC deposition. (**a**) C3 deposits were quantified (pixel$^2 \times 10^3$) by confocal microscopy on HK-2 cells preexposed to medium (control), albumin (alb) or transferrin (transf) (10 mg/ml) for 24 h followed by replacing with medium or 10% HS (90 min). (**b**) Active C3 deposition was assessed in HK-2 cells exposed to the same stimuli as above and expressed as the ratio of total C3 (C3c)/inactive C3b (iC3b) deposit areas. (**c**) MAC deposits were quantified (pixel$^2 \times 10^3$) on HK-2 cell surface by confocal microscopy. Results are expressed as mean ± s.e. ($n = 7$ independent experiments for C3 and MAC deposition, and $n = 4$ for active C3 deposition). °$P < 0.01$ versus control, *$P < 0.01$ versus HS.

We further explored to which extent albumin or transferrin may promote active C3 deposition. Total C3b and inactive C3b (iC3b) deposits were evaluated by using antibodies (Abs) specific for C3c (present in both C3b and iC3b molecules) or for iC3b fragments, respectively. Active

Table 1 | Effect of $MgCl_2$–EGTA and sCR-1 on C3 deposition in HK-2 cells

	C3 deposit area (pixel^2)		
	–	+$MgCl_2$-EGTA	+sCR-1
10% HS	4458 ± 231	4770 ± 251	26 ± 1°
Albumin +10% HS	8045 ± 483°	8033 ± 633	23 ± 4#
Transferrin +10% HS	9716 ± 1436°	9939 ± 1456	25 ± 2§

Data are expressed as mean ± s.e. of 15 fields for each sample (n=4).
°$P < 0.01$ versus 10% HS, #$P < 0.01$ versus albumin+10% HS, §$P < 0.01$ versus transferrin+10% HS.
HK-2 cells were exposed for 24 h to medium alone, albumin, or transferrin (10 mg/ml) before incubation with 10% human serum (HS) for 90 min in the presence or absence of $MgCl_2$–EGTA (5 mM) or sCR-1 (100 μg/ml).

C3 deposition on cell surface was defined as ratio of C3c/iC3b > 1. When cells were exposed to HS alone, the C3c/iC3b ratio was 2.15 ± 0.05 (Figure 2b). The addition of albumin significantly increased active C3 deposits (3.60 ± 0.09), whereas transferrin had no significant effect as compared with HS alone (2.41 ± 0.06) (Figure 2b).

Then we investigated whether complement activation on tubular cells by albumin and transferrin proceeded to the formation of MAC. MAC fixation also further increased ($P < 0.01$) in cells exposed to albumin followed by the addition of HS, as compared with exposure to HS alone (Figure 2c), an effect that was not observed with transferrin (Figure 2c). The viability of HK-2 cells was not altered by 24-h exposure to albumin or transferrin alone (albumin: 96.8 ± 13.8, transferrin: 93.2 ± 2.9 versus control: 100% viable cells). No additional effect on cell viability was observed when either proteins were given as pretreatment before 10% HS (albumin + HS: 118.3 ± 10.1, transferrin + HS: 83.9 ± 4.4 versus control: 100% viable cells).

Albumin overload enhances fractalkine and TGF-β mRNA upregulation induced by serum complement

To investigate the functional consequences of protein overload-induced activation of complement on HK-2 cells, we studied the expression of fractalkine and transforming growth factor-β (TGF-β), taken as markers of cell activation and known mediators of tubulointerstitial inflammation and fibrosis.[28] Serum complement enhanced the expression of both cytokines as reflected by combined findings of increased fractalkine and TGF-β mRNA by 10% HS, and abrogation in the presence of complement inhibitor sCR-1 (Figure 3). The combination of HS and albumin challenge had additive effects as shown by further significant ($P < 0.05$) increases in mRNA levels of both mediators as compared with each stimulus alone. The exposure of cells to transferrin alone caused fractalkine and TGF-β mRNA upregulation ($P < 0.05$), and no further increases were observed when combined with HS (Figure 3).

Effects of protein load on factor H binding and production

To identify the mechanisms underlying the increased serum C3 and MAC deposition on HK-2 cell surface in response to

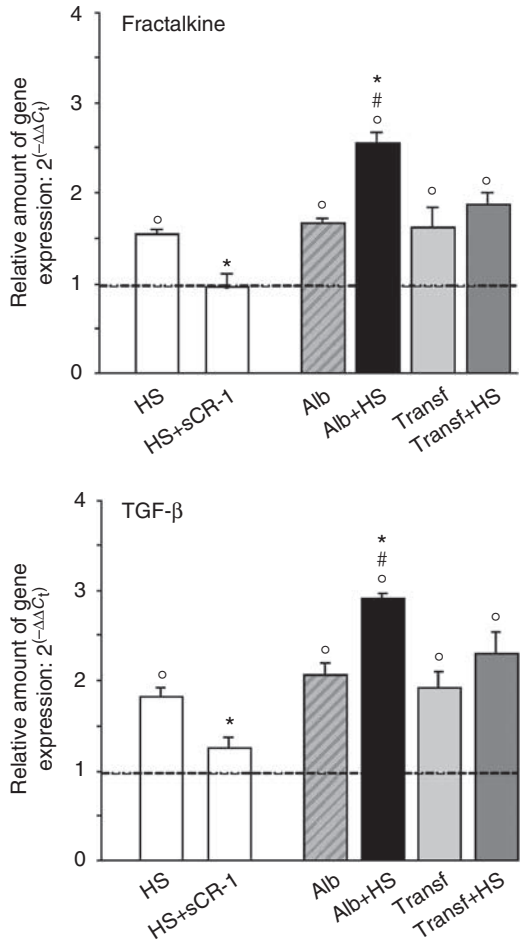

Figure 3 | Effect of serum and protein overload on fractalkine and TGF-β mRNA expression. Expression of fractalkine and TGF-β mRNA evaluated by real-time PCR in HK-2 cells exposed to 10% HS with or without the complement inhibitor sCR-1 or exposed to albumin (alb) or transferrin (transf) (10 mg/ml) followed by medium or 10% HS. Data are relative values as compared with control cells exposed to medium alone (dashed line). Results are expressed as mean ± s.e. of three independent experiments. $^{\circ}P<0.05$ versus control, $^{*}P<0.05$ versus HS, $^{\#}P<0.05$ versus albumin.

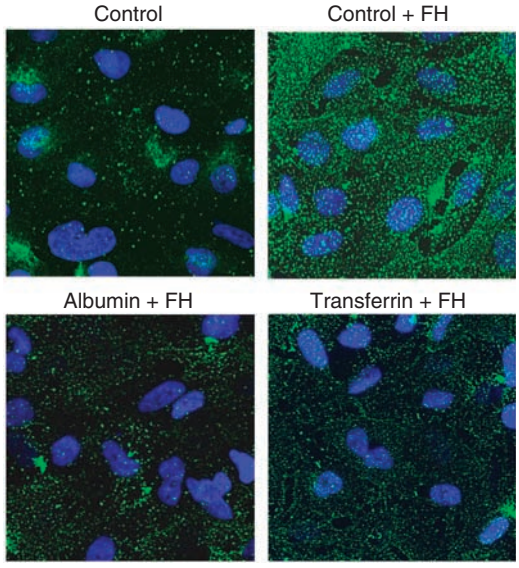

Figure 4 | Effect of plasma protein load on factor H binding to HK-2 cells. HK-2 cells showed a weak granular staining (green) for factor H (FH) on the cell surface as assessed by immunofluorescence analysis. Strong staining for FH was observed after incubation of the cells for 3 h with purified human FH (10 μg/100 μl). Preexposure for 24 h to albumin (10 mg/ml) or transferrin (10 mg/ml) significantly decreased the capability of HK-2 cells to bind FH. Nuclei were counterstained in blue with DAPI. Original magnification × 630.

Table 2 | Plasma proteins decrease exogenous factor H binding on HK-2 cells

	Fluorescent cells (%)	
	–	+Factor H
Control	23.35 ± 1.44	37.87 ± 0.93°
Albumin	21.89 ± 0.78	25.86 ± 1.81**
Transferrin	27.19 ± 1.19	29.28 ± 3.90*

FACS analysis of factor H binding on cell surface. Results (mean ± s.e.) are expressed as % of fluorescent cells (n=8).
$^{\circ}P<0.01$ versus control; $^{*}P<0.05$, $^{**}P<0.01$ versus control+factor H.
HK-2 cells were exposed for 24 h to medium (control), albumin, or transferrin. Then, human factor H (10 μg) was added for 3 h.

protein overload, we focused on factor H, one of the main natural inhibitors of the alternative pathway of complement, which could act on proximal tubular epithelial cells. For this purpose, we studied first whether resting HK-2 cells bind factor H. Unstimulated HK-2 cells expressed low levels of this complement regulatory protein (Figure 4). Exogenous purified factor H added to HK-2 cells markedly bound to the cell surface (Figure 4), as shown by the increased percentage of fluorescent cells detected by flow cytometry (Table 2). The binding of factor H on the cell surface was decreased by pre-exposure of HK-2 cells to albumin or transferrin (Figure 4 and Table 2).

To understand whether plasma proteins could affect endogenous factor H synthesis, western blot analysis was performed for detection of factor H production by HK-2 cells exposed to medium alone, albumin, or transferrin for 24 h. Unstimulated cells constitutively produced factor H, as showed by detection in the cell supernatants of a band of approximately 150 kDa, corresponding to factor H (see Figure S1). This band was not detectable in supernatants derived from HK-2 cells incubated with albumin or transferrin (Figure S1), thereby indicating an inhibitory effect of protein overload on factor H production by proximal tubular cells.

Role of factor H in the control of active C3 and MAC deposition

To define in our experimental setting the relation between factor H binding and complement deposition, we first assessed the level of active C3 deposits on tubular cells after exposure to HS, a source of factor H in addition to complement. Active C3 deposition was evaluated by flow cytometry as the ratio C3c/iC3b. When HK-2 cells were

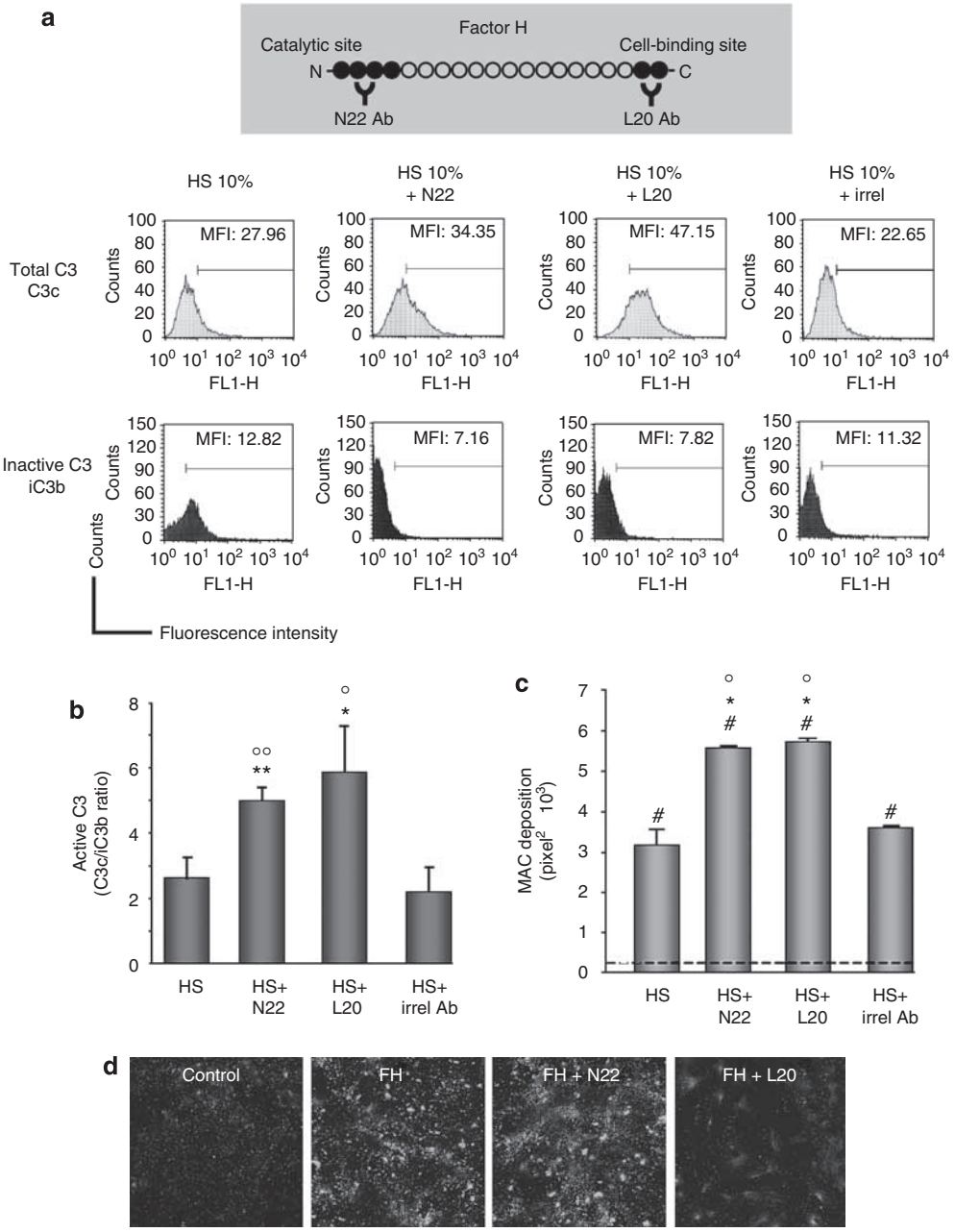

Figure 5 | Role of factor H in the control of active C3 and MAC deposition on HK-2 cells. (**a**) Flow cytometric analysis of total C3 (C3c) and inactive C3 (iC3b) deposition on HK-2 cells exposed to 10% HS for 90 min in the presence or absence of anti-factor H (FH) N22 Ab, which blocks the catalytic site, or L20 Ab that inhibits the cell membrane-binding site (top). Histograms are representative of mean fluorescence intensity (MFI) for C3c and iC3b (bottom). (**b**) Active C3 deposition is defined as ratio of C3c and iC3b MFI >1. Results are expressed as mean $\pm$ s.e. ($n=4$ independent experiments). $^{*}P<0.05$, $^{**}P<0.01$ versus HS; $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$ versus HS + irrel. (**c**) MAC deposition was analyzed by confocal microscopy and quantified as pixel$^2 \times 10^3$ (dashed line represents control value). Results are expressed as mean $\pm$ s.e. ($n=3$ independent experiments). $^{\#}P<0.01$ versus control, $^{*}P<0.01$ versus HS; $^{\circ}P<0.01$ versus HS + irrel. (**d**) Effects of anti-FH Abs on purified FH binding to HK-2 cell surface. Blocking the FH catalytic site had no effect, in contrast to the marked inhibition obtained by blocking FH cell binding site by L20 Ab. Original magnification $\times 630$. Irrel Ab, irrelevant antibodies (control).

exposed to HS, the mean fluorescence intensity (MFI) of C3c was higher than iC3b MFI (Figure 5a) and the ratio C3c/iC3b was 2.3 ± 0.4 (Figure 5b). Active C3 level in this condition results from a balance between complement activation spontaneously occurring on cell surface and its inactivation by factor H. Serum factor H was capable of binding to HK-2 cells, as showed by immunofluorescence staining of factor H on tubular cell surface after 90-min HS exposure (data not shown). The blockade of the factor H catalytic site by pretreating HS with N22 Ab[29] significantly impaired factor H-dependent C3b inactivation leading to an increase of total C3 (Figure 5a) and active C3 deposits as indicated by the

ratio C3c/iC3b = 5.0 ± 0.3 (Figure 5b). Similarly, L20 Ab, which blocks the factor H cell membrane-binding site,[29] increased both total C3 (Figure 5a) and the C3c/iC3b ratio (5.9 ± 1.4) (Figure 5b), indicating more deposition of active C3 on the tubular cell surface. In contrast, addition of irrelevant control anti-CD13 Ab had no effects as compared with HK-2 cells exposed to HS alone (Figure 5a and b).

The influence of factor H activity on the presence of MAC deposits on HK-2 cells was also evaluated. The inhibition of either the catalytic site or the cell-binding site of factor H by using N22 and L20 Abs, respectively, significantly increased the amount of MAC deposits on cell surface as compared with cells incubated with HS either alone or plus control irrelevant Ab (Figure 5c).

Immunofluorescence experiments showed no inhibitory effects of N22 Ab on exogenous factor H binding to the cell surface, which instead was almost completely prevented by L20 Ab (Figure 5d). Both Abs showed no binding to the cell surface in the absence of factor H (data not shown).

Protein load decreases heparan sulfate density responsible for factor H binding to HK-2 cells

Polyanionic molecules and specifically cell-associated proteoglycans are major binding sites for factor H, as shown by studies in cell-free systems[30] and endothelial cells.[26,27] To assess whether heparan sulfate residues could mediate factor H binding to tubular cell surface, HK-2 cells were exposed to an Ab against N-sulfated glucosamine residues of heparan sulfate before the addition of factor H. In comparison with cells exposed to factor H alone (Figure 6), a marked reduction of factor H binding was observed in cells treated with anti-heparan sulfate Ab (Figure 6). No decrease in factor H binding was found in the presence of an irrelevant control Ab (anti-CD26) that binds to a tubular epithelial surface target (Figure 6).

To further characterize the effect of protein load on factor H binding capability, we investigated by flow cytometry whether plasma protein challenge could modify the surface expression of heparan sulfate on HK-2 cells. Incubation with albumin or transferrin for 24 h caused a significant reduction of heparan sulfate on HK-2 cell surface as reflected by lower mean fluorescence intensity in comparison with unstimulated cells (Figure 7). To identify the possible mechanism(s) by which plasma proteins lead to a reduction of heparan sulfate residues on the HK-2 cell surface, we focused on heparanase 1 (HPSE-1), an enzyme involved in the heparan sulfate cleavage.[31] Western blot analysis of HPSE-1 protein expression was performed in protein cell lysates obtained from HK-2 cells incubated for 15 h with medium (control), albumin, or transferrin. HPSE-1 was constitutively expressed in untreated cells (see Figure S2). In response to plasma protein exposure, an increase in the level of the 65-kDa form of HPSE-1 was observed and confirmed by densitometric analysis (Figure S2).

Finally, we assessed whether heparan sulfate, by binding factor H, may be implicated in its inhibitory activity on complement activation on HK-2 cells. Treatment of cells with a specific Ab blocking the heparan sulfate residues before HS addition significantly increased active C3 (Figure 8a) and MAC deposition (Figure 8b) as compared with cells exposed to HS alone or to HS plus the irrelevant control Ab (Figure 8a and b).

DISCUSSION

We established a model to evaluate the effects of sublethal challenge by complement on proximal tubular epithelial cells under conditions that mimic the renal tubule in proteinuric nephropathy. This allowed us to investigate whether excess plasma proteins may lead to proximal tubular cell dysfunction induced by the abnormal presence of complement as found in the ultrafiltrate, and by which mechanisms. Findings that albumin overload enhances the deposition of serum-derived C3 on the cell surface through the alternative pathway leading to enhanced assembly of MAC suggest a role of excess proteins within the ultrafiltrate in facilitating the activation of the complement cascade. In particular, these data explain the high vulnerability of proximal tubular cells to abnormal exposure to complement in chronic proteinuric diseases. Along this line, C6 deficiency failed to significantly impact on tubulointerstitial damage in nonproteinuric models of renal disease, suggesting that in the absence of proteinuria, C5-b9 was not sufficient to enhance injury.[21] If

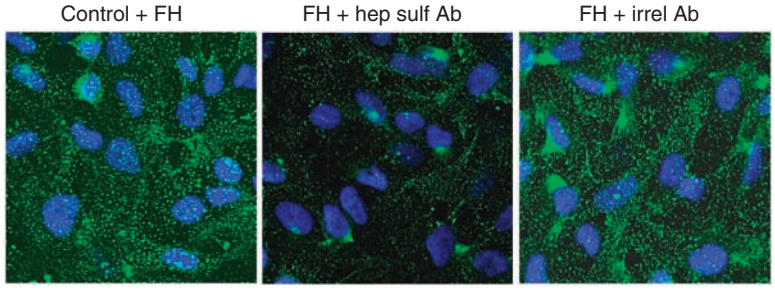

Figure 6 | Factor H binds to heparan sulfate residues on HK-2 cell surface. Immunofluorescence analysis of factor H (FH) binding to HK-2 cell surface. With respect to cells exposed to FH alone, a marked reduction of FH binding was observed in cells treated with an Ab specifically recognizing heparan sulfate residues (hep sulf Ab). No decrease in FH binding was found in the presence of a control Ab (irrel Ab). Nuclei were counterstained in blue with DAPI. Original magnification × 630.

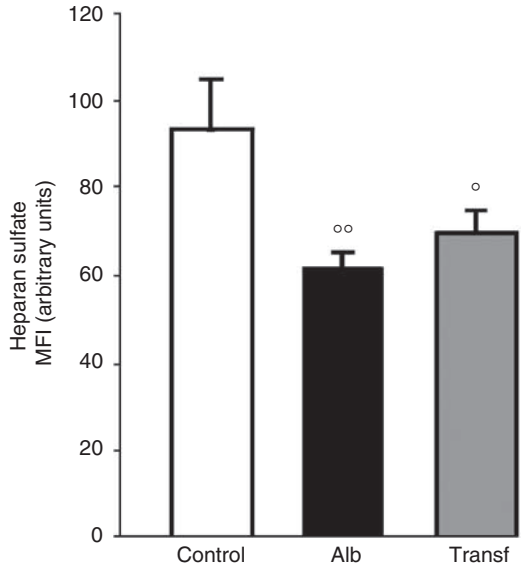

Figure 7 | Protein load decreases heparan sulfate density on HK-2 cell surface. Flow cytometry of heparan sulfate expression measured by mean fluorescence intensity (MFI) in HK-2 cells exposed to medium (control), albumin (alb), or transferrin (transf) (10 mg/ml) for 24 h. Results are expressed as mean ± s.e. ($n = 8$ independent experiments). Results are mean ± s.e. ($n = 3$ experiments). $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ versus control.

translated *in vivo*, increased complement deposition and activation by protein load on tubular cell surface may play a pivotal role in exacerbating tubulointerstitial injury.

We previously documented that antiproteinuric treatment by ameliorating the damage of the glomerular barrier to proteins limits tubular and interstitial injury in chronic proteinuric nephropathy.[18–20] The reduced tubular overload with filtered proteins and C3 was associated with reduced proinflammatory activation of tubular cells, which could account for renoprotective effects.[18,19] In fact, the abnormal exposure of proximal tubular cells to ultrafiltered C3 is a major factor promoting tubulointerstitial injury in mice with protein overload proteinuria even in the absence of local C3 synthesis.[20] In light of these findings, protein overload and activation of complement on luminal surface are strictly interrelated processes, in line with the concept that chronic proteinuria provides both a stimulus for C5b-9 assembly and an environment favoring its sublytic effect. The latter includes increased cytokine expression[23] and upregulation of collagen type IV by proximal tubular cells, contributing to the fibrotic process *in vivo*.[32] Here, both albumin and serum complement upregulated fractalkine and TGF-β mRNA expression, and in an additive manner when given in combination. These findings indicate that albumin load-enhanced complement activation acts to sustain proinflammatory and fibrogenic cellular phenotypic changes. The evidence for protein and complement-induced TGF-β upregulation reinforces the role of TGF-β signaling in myofibroblast accumulation in the interstitium surrounding proximal tubules engaged by ultrafiltered proteins.[19] In agreement with our data, the addition of serum or C3a and

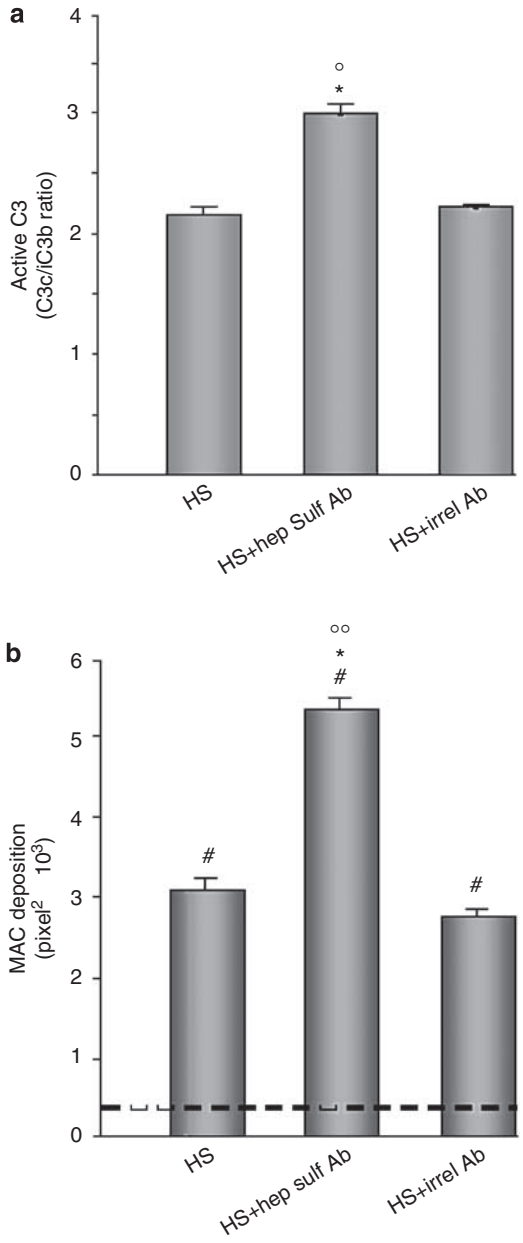

Figure 8 | Role of heparan sulfate in serum-derived active C3 and MAC deposition. (**a**) Active C3 and (**b**) MAC deposition on HK-2 cells treated with an anti-heparan sulfate Ab (hep sulf Ab) or irrelevant Ab (irrel Ab) before the addition of 10% HS for 90 min. Results are expressed as mean ± s.e. ($n = 5$ independent experiments for active C3; $n = 3$ for MAC deposition). Dashed line represents control value. $^{\#}P < 0.01$ versus control, $^{*}P < 0.01$ versus HS; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ versus HS + irrel.

C5a to human proximal tubular cells resulted in a fibrogenic phenotype with increased α-smooth muscle actin and collagen I expression, consistent with complement-dependent epithelial–mesenchymal transition.[33]

The exposure to complement did not further enhance transferrin-induced fractalkine and TGF-β gene upregulation. This difference with respect to albumin was related to the strong ability of albumin of enhancing active C3 deposition, as reflected by simultaneous increase in total C3

and reduction in iC3b, with increased total C3/inactive iC3b ratio. Active C3 deposition is pivotal for C5b-9 generation and complement-dependent injury. Molecular mechanisms underlying this differential response were not further characterized here and might include differences in albumin and transferrin receptor affinity and/or downstream intracellular events. It is noteworthy that albumin overload[9] and activated complement proteins stimulate cytokine expression through the nuclear factor-κB-dependent pathway. This response is most likely to be enhanced in the presence of both C3a[34] and C5b-9,[35] which can be formed in excess upon exposure to albumin but not transferrin load.

The second purpose of this study was to assess whether protein overload may influence the capability of proximal tubular cells to counteract complement activation by altering their complement regulatory potential. We studied the role of factor H, the main soluble inhibitor of alternative pathway shown to be produced by human proximal tubular cells.[24] Factor H binds to the cell surface serving as a cofactor for factor I in the degradation of newly formed C3b to inactive iC3b, thereby limiting the formation of C3 convertase.[36] Here, the evidence for factor H binding to unstimulated proximal tubular cells identifies this cell type as a novel candidate target for its protective action. Moreover, cell-bound serum-derived factor H limited active C3 and MAC deposits on cell surface, which indeed were enhanced in the presence of Abs blocking either the C-terminal cell membrane-binding site or the catalytic site of factor H. Importantly, surface-bound factor H may act as the main shield against complement activation in proximal tubular cells, which have no or very low expression of membrane-anchored regulators, such as CR1, decay accelerating factor, membrane cofactor protein, and CD59.[37]

Factor H is known to bind to few cell types, including endothelial cells, through heparan sulfate/glycosaminoglycan sites.[25,27,38,39] Our data showing factor H binding to heparan sulfate residues on the proximal tubular cell surface are in agreement with the possibility that heparan sulfate chains may behave as a polyanionic structure for factor H binding, as suggested also by studies using cell-free systems.[40,41] Moreover, increased active C3 deposition upon blockade of heparan sulfate residues reflects the ability of heparan sulfate–factor H interaction to reduce complement deposition on HK-2 cells. This function is impaired by protein overload at least partly through a heparanase-1-mediated mechanism so as to enhance the surface deposition of active C3. These results indicate that protein overload alters proximal tubular cell phenotype by reducing the ability of the cell to counteract complement activation. The production of endogenous factor H was also reduced in protein-overloaded cells in the absence of serum. However, the importance of this effect in HK-2 cells here is not clear, as it was not associated with an increase in endogenous C3 binding to the cell surface.

These findings have significant implications in renal disease. First, the evidence that excess plasma proteins prevent factor H binding to proximal tubular cells highlights a potential mechanism underlying uncontrolled complement activation at the tubular level. This would predict more protection by targeting the tubule with complement inhibitory molecules, such as soluble complement receptor-1 and targeted-Crry and CD59, if given in combination with antiproteinuric therapy.[13,17] Second, investigation of factor H in renal disease has been mostly confined to the pathogenetic role of intravascular/intracapillary factor H loss or dysfunction.[42,43] Notably, tubular cell injury has been suggested to contribute to renal dysfunction in hemolytic uremic syndrome,[44] a disease often associated with factor H defective activity. Impaired tubular cell protection against complement activation may contribute to this disease process and possibly to tubular injury in other settings in which ultrafiltered and/or tubular cell-derived factor H could play a regulatory role.

MATERIALS AND METHODS

Cell culture and incubation

HK-2 cells, a human proximal tubular epithelial cell line[45] (American Type Culture Collection, Rockville, MD, USA), were grown as described previously.[4] For a detailed description, see Supplementary Methods. Confluent cells were maintained overnight in Dulbecco's modified Eagle's medium/F-12 without serum before the experiments. For immunofluorescence studies, experiments were conducted on cells grown on glass coverslips (Bio-optica Milano Spa, Milano, Italy). HS from a pool of plasma from five healthy volunteers was used as a source of complement.

The concentration-dependent effect of HS on cell viability was tested by incubation (90 min) with control medium (Dulbecco's modified Eagle's medium/F-12 serum free), 10 or 25% HS. Viability was assessed by trypan blue dye exclusion (Sigma, Chemical Co., St Louis, MO, USA). C3 and MAC deposits were assessed by immunofluorescence in HK-2 cells incubated for 90 min with control medium, 10% HS in the presence or absence of sCR-1 (100 μg/ml, Avant Immunotherapeutics Inc., Needham, MA, USA). To visualize living cells, the cell tracker 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (10 μM, Molecular Probes Inc., Eugene, OR, USA) was added to the medium for 3 h before 10% HS incubation. To investigate whether preexposure to plasma proteins could potentiate HS cytotoxicity and complement deposition, cells were incubated for 24 h with control medium, human albumin (low endotoxin), or transferrin (10 mg/ml, Sigma, Chemical Co., St Louis, MO, USA) followed by 10% HS stimulation. C3 and MAC deposits were quantified by confocal microscopy. The albumin and transferrin concentrations and time of exposure were chosen on the basis of previous studies by us and other groups reporting *in vitro* conditions that are most likely to mimic the chronic proteinuric setting.[4,5,9,46,47] In particular, we selected a high dose of albumin to duplicate a condition reflecting severe and prolonged injury of the glomerular barrier in which proteins with a higher molecular weight, such as C3 (MW 185 kDa) and factor H (MW 150 kDa), would become filtered and reach the tubule. Experiments using a lower concentration of albumin (100 μg/ml) before 10% HS addition showed no significant increase in C3 deposition as compared with the exposure to HS alone (control: 34 ± 7; HS: 3668 ± 639; HSA 100 μg/ml + HS: 4031 ± 710 pixel^2).

To identify the activation pathway(s), HS was supplemented with $MgCl_2$–EGTA (5 mM, Sigma) or sCR-1 (100 μg/ml). Fractalkine and TGF-β mRNA expression were assessed in cells exposed to medium

or 10% HS, with or without albumin or transferrin preexposure. The effect of 10% HS pretreatment with sCR-1 (100 μg/ml) was also evaluated.

C3 and MAC immunofluorescence staining and flow cytometry

Cells were fixed in 4% paraformaldehyde and 2% sucrose (20 min, 37°C). For C3, HK-2 cells were incubated with fluorescein isothiocyanate-conjugated rabbit anti-human C3c Ab (11.3 μg/ml, Dako A/S, Glostrup, Denmark). MAC was detected by using rabbit IgG anti-human C5b-9 Ab (27.6 μg/ml, Calbiochem, La Jolla, CA, USA) for 1 h followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (13.6 μg/ml, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Fifteen fields per sample were randomly acquired in a blinded manner using an inverted confocal laser microscopy (LSM 510 meta; Zeiss, Jena, Germany). Digitized images were binarized using a threshold for areas of staining corresponding to C3 and MAC deposition. Fluorescent areas were calculated automatically in pixel^2 using analysis software Image J1.32.

Active C3 deposition on HK-2 cell surface was assessed by confocal microscopy (see Supplementary Methods) and by flow cytometry (FACSort, Becton-Dickinson, San Jose, CA, USA). Briefly, HK-2 cells were exposed for 90 min to 10% HS in the presence or absence of the anti-factor H blocking Abs N22 and L20 (mouse IgG1, 10 μg/ml)[29] against the catalytic site and cell membrane-binding site of factor H, respectively. Cells were detached and stained with either fluorescein isothiocyanate-conjugated anti-human C3c Ab or with mouse monoclonal anti-iC3b (10 μg/ml, Quidel, San Diego, CA, USA) followed by fluorescein isothiocyanate-conjugated secondary Ab. As an irrelevant control Ab, mouse anti-CD13, which binds to the tubular epithelial cell surface, was used (IgG1, 10 μg/ml, AbD Serotec, Oxford, UK).

Statistical analysis

Results are expressed as mean ± s.e. Statistical analysis was performed using analysis of variance followed by Tukey's test for multiple comparisons or nonparametric Kruskal–Wallis test. Statistical significance level was defined as $P<0.05$.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work received partial support by a Genzyme Renal Innovation Program grant. We have no conflicting financial interests. Dr Simona Buelli is a recipient of a fellowship 'Riva del Garda' through the courtesy of 'Fondazione Aiuti per la Ricerca sulle Malattie Rare.' We thank Fabio Sangalli for his precious contribution during confocal microscopy evaluations, Paola Cassis for the western blot analysis, and Anna Pezzotta for technical assistance. Manuela Passera helped in preparing the manuscript. Part of this work has been presented at the Annual Meeting of the American Society of Nephrology (November 16–19, 2006, San Diego, CA, USA).

SUPPLEMENTARY MATERIAL

Figure S1. Effect of plasma protein load on factor H production by HK-2 cells.

Figure S2. Protein load promotes heparanase 1 (HPSE-1) overexpression in HK-2 cells.

Supplementary Methods.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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