Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinurina

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Thomas, Mark E., Kevin P. G. Harris, John Walls, Peter N. Furness, and Nigel J. Brunskill. Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinurina. Am J Physiol Renal Physiol 283: F640–F647, 2002.—The role of the albumin-carried fatty acids in the induction of tubulointerstitial injury was studied in protein-overload proteinuria. Rats were injected with fatty acid-carrying BSA [FA(+)-BSA], fatty acid-depleted BSA [FA(−)-BSA], or saline. Macrophage infiltration was measured by immunohistochemical staining, apoptotic cells were detected by in situ end labeling, and proliferating cells were identified by in situ hybridization for histone mRNA. Macrophage infiltration was significantly greater in the FA(+)-BSA group than in the FA(−)-BSA and saline groups. The infiltrate was largely restricted to the outer cortex. Apoptosis was greater in the FA(+)-BSA group than in the FA(−)-BSA and saline groups. Compared with the saline group, apoptosis was significantly increased in the FA(+)-BSA group but not in the FA(−)-BSA group. Cortical cells proliferated significantly more in the FA(+)-BSA and FA(−)-BSA groups than in the saline group. FA(−)-BSA is therefore a more potent inducer of macrophage infiltration and cell death than FA(+)-BSA. The fatty acids carried on albumin may be the chief instigators of tubulointerstitial injury in protein-overload proteinurina.

lipid; albuminuria; proximal tubule; apoptosis; macrophage; proliferation

ONE CONSISTENT FINDING in animals with experimental renal disease and humans is that even in primary glomerular diseases, declining renal function correlates most strongly with pathological changes seen in the tubulointerstitium of the kidney (25, 30, 32). Mounting evidence indicates that proteinuria is an independent predictor of renal failure progression. This evidence is derived from epidemiological (17) and larger retrospective studies (for review see Ref. 36), as well as post hoc analyses of large randomized controlled trials (1, 22, 28, 31). Taken together, these observations have led to the hypothesis that proteinuria may directly provoke tubulointerstitial injury and, thus, progressive chronic renal disease.

Various mechanisms have been proposed to explain the link between proteinuria, tubulointerstitial injury, and progressive chronic renal failure (25). Some authors postulate that albuminuria may exert a toxic effect on proximal tubular epithelial cells (PTEC), thus damaging the cells and initiating the process of interstitial fibrosis and scarring (3, 36). Certainly, nephrotic patients demonstrate markedly elevated urinary excretion of N-acetyl-β-glucosaminidase, highly suggestive of PTEC injury (21).

A variety of in vitro approaches have been used to assess the effects of albumin on cultured PTEC. These techniques have been used to show that albumin has important effects on growth and proliferation of PTEC (2). Furthermore, albumin induces PTEC to secrete a variety of chemoattractants (29), providing a potential explanation for the marked interstitial inflammatory response seen in nephrosis. These in vitro data are supported by previous studies from our laboratory that have demonstrated the development of this tubulointerstitial inflammation in rats with protein-overload proteinuria associated with not only PTEC proliferation but also PTEC apoptotic death (34). These observations are therefore fully consistent with the hypothesis that albuminuria is able to exert a pathophysiological effect on PTEC. Whether albumin per se is toxic to tubular cells remains unresolved. For instance, Iglesias et al. (16) suggested that albumin acts as a survival factor for proximal tubular cells in culture. Conversely, other authors suggest that albumin causes apoptosis of cultured tubular cells (8). Therefore, the explanation for the PTEC apoptosis observed in albuminuria requires further clarification.

It has been proposed that the nephrotoxicity of albuminuric states is not directly due to the protein molecule itself but that the toxicity resides in lipid carried on albumin. Albumin normally carries >99% of plasma fatty acids in a molar ratio of ≈0.7:1 (fatty acid to albumin), and this ratio is raised in nephrotic syndrome (36). In nephrotic individuals, the load of fatty acid carried by urinary albumin very closely reflects...
that carried by serum albumin (12). An interesting and important exception to this rule is urinary albumin in minimal change disease. In this condition, urinary albumin is relatively devoid of fatty acids compared with its serum counterpart (12), and this may explain the generally benign nature of this condition. In most proteinuric states, filtered albumin thus presents fatty acids to PTEC in large quantities by an unusual and unregulated apical route. These fatty acids are known to provoke major changes in the lipid metabolism of the PTEC (35).

Tubular cells in proteinuric states carry large amounts of lipids, seen as urinary oval fat bodies (lipid-laden tubular cells), and fatty casts (tubular cell casts containing lipids). Fatty elements (such as oval fat bodies) are positively correlated with the level of proteinuria (26). Lipids stainable by oil red O are seen in the proximal tubule in biopsies from patients with nephrotic syndrome (9), and intracellular free fatty acids have been implicated in the pathogenesis of hyperoxia-induced renal injury (15). Therefore, glomerular filtrate and the glomerular basement membrane may mediate the injurious effects of proteinuria.

Macrophage infiltration is an early feature of proteinuric human renal disease (11, 23, 27). Foam cells of monocyte/macrophage origin have also been found in these conditions (23, 27). Similarly, macrophage infiltration is seen at the onset of experimental models of proteinuria, such as protein-overload proteinuria (5, 20), and puromycin aminonucleoside nephrosis (7, 6, 18). Experimental measures to prevent the macrophage infiltrate have been widely studied in models of proteinuria. Use of an essential fatty acid-deficient diet is known to abolish the macrophage infiltrate and the acute renal injury in puromycin aminonucleoside nephrosis (13). These findings have led to the hypothesis that macrophages play a key role in tubulointerstitial injury (38). In proteinuric renal disease, the proteinuria may itself provoke the macrophage infiltrate. This may occur as a result of proximal tubular catabolism of filtered fatty acid-carrying albumin, resulting in the release of a lipid chemotactant (20).

Classic atrophic tubules are proximal and undergo tubular injury and regeneration (24). However, the balance of tubular cell proliferation vs. cell death in progressive renal disease has not been well studied. Clearly, because atrophy occurs, there must be a disturbance in the balance of cell proliferation and death. In a model of chronic obstructive uropathy, obstruction is initially followed by tubular cell proliferation, but in the chronic phase, tubular cell apoptosis increases, with net cell loss and tubular atrophy (37). Recent work has shown evidence of tubular cell proliferation in human nephrotic syndrome (14).

To study the role of fatty acids carried on albumin in the development of renal tubulointerstitial injury, we have induced protein-overload proteinuria in rats with fatty acid-bearing BSA [FA(+)-BSA] or delipidated BSA [FA(−)-BSA]. The results indicate that the renal tubulointerstitial lesion is markedly more severe in animals injected with FA(+)-BSA than in those injected with FA(−)-BSA, despite comparable levels of proteinuria in both groups. This supports the proposal that fatty acids bound to albumin contribute substantially to tubulointerstitial injury in proteinuric states.

METHODS

Induction of protein-overload proteinuria. Protein-overload nephropathy was induced in adult female Lewis rats as previously described (5, 34). All animals were obtained from the same supplier and arrived in the same shipment, and all procedures were covered by the appropriate Home Office licenses. Briefly, rats were allowed free access to water and standard rat chow. Rats were given seven consecutive daily intraperitoneal injections of 2 g of FA(+)-BSA (n = 17, with 16 rats completing the protocol) or FA(−)-BSA (n = 17, with 16 rats completing the protocol) or sham sterile normal saline injections (n = 8) using the same volume of saline. Blood samples were taken at baseline (day −7) and on days 2, 4, and 7 (at death), after collection of a 24-h urine specimen. At death, in situ perfusion of the kidneys with saline was carried out by aortic cannulation.

Albumin preparation. Fresh sealed containers of BSA powder were obtained a short time before the study. FA(+)-BSA was obtained from Sigma (catalog no. A4503). Essentially fatty acid-free BSA [FA(−)-BSA] was also obtained from Sigma (catalog no. A6003), and albumin solutions were prepared using sterile saline (150 mmol/l NaCl) as a diluant, as previously described (34).

Serum BSA was measured by radial immunodiffusion (The Binding Site, Birmingham, UK).

Macrophage immunohistochemistry and quantification. Rat kidneys were bivalved, fixed in Carnoy’s fixative for 3 h, and then switched to absolute alcohol before they were embedded in paraffin wax. Paraffin sections (3 μm) were dewaxed and rehydrated, and endogenous peroxidase activity was blocked first with acidified methanol-hydrogen peroxide and then with normal horse serum (Vector Laboratories, Peterborough, UK). Endogenous biotin was blocked by successive addition of avidin D and biotin (avidin-biotin blocking kit, Vector). A murine monoclonal antibody to rat monocytes/macrophages was used as the primary antibody (clone ED1, Serotec, Kidlington, Oxon, UK). Omission of the primary antibody was used as the negative control. The remaining procedure was carried out using an avidin-biotin complex immunoperoxidase method (Vector ABC Elite kit), with appropriate intermediate wash steps. 3,3’-Diaminobenzidine was used as a horseradish peroxidase substrate in the presence of hydrogen peroxide.

Image analysis with NIH Image. NIH Image was used to carry out blinded analysis of video images of coded sections using a Carl Zeiss III RS microscope with a videocamera linked to a Power PC. In our hands, this technique is a reliable means of quantifying the macrophage infiltrate in human renal biopsies (11). Sections were viewed at ×125 under constant lighting. With the use of a graticule, each ×125 field was shown to measure ~0.18 mm². The shareware program NIH Image (version 1.57) captures the color video image of a section (via a plug-in digitizer) and converts it to a gray-scale image. For macrophage analysis, the image was processed to remove all pixels below a fixed threshold (set at 100). Paler staining, representing background, below the

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threshold level is eliminated from the image. NIH Image
then measures the remaining black pixels as a percentage of
the area of total image. Each rat kidney section was counted
in a stereotyped fashion, as six to eight radial cuts, spaced
out around the cortical perimeter of the section. Each cut
consisted of six adjacent but nonoverlapping fields, which
were imaged from subcapsular cortex (field 1), perpendicu-
larly toward the hilum. The innermost region thus measured
(field 6) represents the inner cortex-corticomедullary junc-
tion. The capsule and large blood vessels were excluded from
the quantification.

Quantification of apoptosis and cell proliferation. In situ
end labeling for the detection of apoptosis was carried out as
described previously (34), with apoptotic nuclei stained black
(34). Proliferating cells were detected by in situ hybridization
for histone mRNAs, as described elsewhere (19, 34). Brown/
black staining of the cytoplasm indicated that the cell was in
the S phase. The particle-counting function of NIH Image
was used to count in situ end label-positive cells (34) or
the S phase. The particle-counting function of NIH Image
for histone mRNAs, as described elsewhere (19, 34). Brown/
(34). Proliferating cells were detected by in situ hybridization
described previously (34), with apoptotic nuclei stained black
end labeling for the detection of apoptosis was carried out as

<table>
<thead>
<tr>
<th>Table 1. Kidney and body weights</th>
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</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
</tr>
<tr>
<td>Total wet kidney wt, % bodywt</td>
</tr>
<tr>
<td>FA(+)-BSA</td>
</tr>
<tr>
<td>FA(−)-BSA</td>
</tr>
<tr>
<td>Saline</td>
</tr>
<tr>
<td><strong>Total wet kidney wt, % bodywt</strong></td>
</tr>
<tr>
<td>FA(+)-BSA</td>
</tr>
<tr>
<td>FA(−)-BSA</td>
</tr>
<tr>
<td>Saline</td>
</tr>
</tbody>
</table>

Values are means ± SD. FA(+)BSA, fatty acid-carrying BSA;
FA(−)-BSA, fatty acid-depleted BSA; NA, not applicable. Identical
symbols († and ‡) for 2 groups on the same day indicate statistical
significance: †P < 0.001 after Bonferroni correction (Mann-
Whitney tests). There was no significant difference between groups without identical symbols.

Table 2. Serum protein levels

<table>
<thead>
<tr>
<th></th>
<th>Day −7</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total protein g/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA(+)BSA</td>
<td>63 ± 2‡</td>
<td>60 ± 6*</td>
<td>77 ± 4‡</td>
<td>77 ± 3*</td>
</tr>
<tr>
<td>FA(−)-BSA</td>
<td>63 ± 1‡</td>
<td>77 ± 7‡</td>
<td>73 ± 4‡</td>
<td>74 ± 4‡</td>
</tr>
<tr>
<td>Saline</td>
<td>59 ± 2‡</td>
<td>56 ± 3‡</td>
<td>62 ± 3‡</td>
<td>58 ± 3‡</td>
</tr>
<tr>
<td><strong>BSA, g/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA(+)BSA</td>
<td>ND</td>
<td>61 ± 16</td>
<td>66 ± 13‡</td>
<td>52 ± 18</td>
</tr>
<tr>
<td>FA(−)-BSA</td>
<td>ND</td>
<td>58 ± 10</td>
<td>51 ± 13‡</td>
<td>56 ± 22</td>
</tr>
<tr>
<td>Saline</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SD. BSA was determined by radial immuno-
diffusion. ND, not done. Identical symbols for 2 groups on the same
day indicate statistical significance: *P < 0.001; †P < 0.01;‡ P < 0.001;§ P < 0.01 after Bonferroni correction (Mann-
Whitney tests). There was no significant difference between groups without identical symbols.

RESULTS

Food intake and kidney and body weights. There was no
difference between groups in food intake during the run-in period (days −14 to −1, data not shown). How-
ever, during the experimental period (days 0–7), food
intake of the FA(+)BSA and FA(−)-BSA groups (44 ±
11 and 48 ± 15 g/day, respectively) was significantly
lower than that of the saline group (67 ± 13 g/day, P <
0.005 and P < 0.02, respectively, compared with BSA
groups).

There was no significant difference between groups in
body weights (Table 1). However, total wet kidney
weight was significantly higher in the BSA-injected
groups when expressed as a percentage of body weight (Table 1). Wet kidney weight was higher in the
FA(+)BSA than in the FA(−)-BSA group, but this did
not reach statistical significance (P = 0.10).

Serum biochemistry. The serum biochemical results
are depicted in Table 2. There were minor differences
between groups at baseline. The saline-injected group
had lower total protein levels, with higher urinary
protein excretion. These differences were statistically,
but not biologically, significant.

BSA injections produced a marked rise in serum
total protein and serum albumin levels in the
FA(+)BSA and FA(−)-BSA groups. The change in total
protein levels was +27 ± 9, +22 ± 11, and −4 ± 4% for
the FA(+)BSA, FA(−)-BSA, and saline groups, respec-
tively (day 2 vs. day −7). Total protein levels during
the experimental period were significantly higher in
both albumin-injected groups than in the saline group.
Both types of albumin injections produced comparable
rises in total protein and serum albumin levels, with the
sole exception of significantly higher serum BSA levels in
the FA(+)BSA group at day 4. Nevertheless, overall
the kidneys of the albumin-injected groups were ex-
posed to similar levels of heterologous albumin.

Urine biochemistry. Results of urine biochemistry are
shown in Table 3. In the BSA-injected groups, there
was a rise in creatinine clearance (C_{Cr}), but this was
not significantly different between baseline and day 7.
In the saline-injected animals, C_{Cr} rose substantially
during the experimental period and was significantly
increased at day 7 compared with baseline (P < 0.005).
This increase may have been due to the volume-loading
effect of saline injections (~7 ml/day).

Both BSA-injected groups had heavy proteinuria,
equivalent to almost half of the daily injected dose. Urine protein excretion tended to be slightly higher in
the FA(+)BSA than in the FA(−)-BSA group, but this
did not reach statistical significance at any point. Ur-
inary rat serum albumin excretion represented 13–21% of the total urine protein excretion at day 1. There was
no significant difference in urinary rat serum albumin
excretion in the two BSA-injected groups during the

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experimental phase. These results are similar to previous work on this model (5).

Microscopy. As expected, light microscopy suggested that glomerular structure was well preserved. The tubules of animals injected with FA(+)-BSA or FA(−)-BSA showed patchy intense vacuolation. Some tubules showed proteinaceous casts, with loss of tubular cell height, suggesting possible tubular necrosis (not shown). Transmission electron microscopy confirmed very marked tubular vacuolation (not shown).

Macrophage infiltration. A marked macrophage infiltrate was seen in animals injected with FA(+)-BSA. The macrophage infiltrate is illustrated in Fig. 1, which shows the immunoperoxidase staining for the ED1 rat monocyte/macrophage antigen. The images depict similar low-power views of outer cortex of rats injected with saline (Fig. 1A), FA(−)-BSA (Fig. 1B), or FA(+)-BSA (Fig. 1C). The macrophage infiltrate in the FA(+)-BSA-injected animal is clearly seen. It is peritubular, with relative sparing of glomeruli. The much reduced degree of macrophage infiltration in rats injected with FA(−)-BSA is also clear. Very few cortical macrophages were seen in animals injected with saline.

In the animals injected with FA(+)-BSA, immunoperoxidase staining for the ED1 rat monocyte/macrophage marker represented ~0.5% of the total area. Box plots of the percent area of the macrophage infiltrate in all three groups are shown in Fig. 2A; each group was significantly different from the others. Injection with FA(−)-BSA was accompanied by a lesser degree of macrophage infiltrate, about one-third of that observed in FA(+)-BSA animals. The macrophage infiltrate in FA(−)-BSA animals was still significantly greater than that in saline controls, where macrophages accounted for only ~0.05% of area across the six cortical fields.

Demonstration of the distribution of the infiltrate. The macrophage infiltrate was studied at various levels in the rat kidney cortex (Fig. 2B). In the two protein-overloaded groups, the infiltrate declined steadily from the outermost cortical field. The animals injected with FA(+)-BSA showed a significantly greater infiltrate than those injected with FA(−)-BSA across all six fields studied (P < 0.05; Fig. 2B). The animals injected with FA(−)-BSA, in turn, had a significantly greater infiltrate than saline-injected animals in the outer four of the six fields studied (Fig. 2B). Although removal of the fatty acids from the BSA reduced mac-

Table 3. Urine chemistry

<table>
<thead>
<tr>
<th></th>
<th>Day −8</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.8</td>
<td>1.6 ± 1.1†</td>
</tr>
<tr>
<td>FA(+)-BSA</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.7†</td>
<td>1.7 ± 0.9</td>
<td>1.9 ± 1.2‡</td>
</tr>
<tr>
<td>FA(−)-BSA</td>
<td>1.5 ± 0.5</td>
<td>2.3 ± 1.0†</td>
<td>2.2 ± 0.5</td>
<td>3.7 ± 1.2‡</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary protein, mg/24 h</td>
<td>3.4 ± 0.7</td>
<td>956 ± 476‡</td>
<td>1,140 ± 229‡</td>
<td>849 ± 258‡</td>
</tr>
<tr>
<td>FA(+)-BSA</td>
<td></td>
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<tr>
<td>FA(−)-BSA</td>
<td>3.2 ± 0.7†</td>
<td>690 ± 303*</td>
<td>708 ± 498*</td>
<td>824 ± 273*</td>
</tr>
<tr>
<td>Saline</td>
<td>5.5 ± 3.1†</td>
<td>7.4 ± 3.2*</td>
<td>5.8 ± 1.9*</td>
<td>3.8 ± 2.0*</td>
</tr>
<tr>
<td>Urinary rat serum albumin, mg/24 h</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>FA(+)-BSA</td>
<td></td>
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<tr>
<td>FA(−)-BSA</td>
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<tr>
<td>Saline</td>
<td></td>
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</table>

*Values are means ± SD. Identical symbols for 2 groups on the same day indicate statistical significance: *P < 0.001; †P < 0.05 > P > 0.01; ‡P < 0.001; §0.01 > P > 0.001 after Bonferroni correction (Mann-Whitney tests). There was no significant difference between groups without identical symbols.
mrophage infiltration, the predominantly outer cortical nature of the infiltrate was unchanged.

Using a slightly different model with a longer time course, Eddy et al. (5) found that the macrophage infiltrate was correlated with peak proteinuria. In the present study (Fig. 2C), there was no significant corre-

lation between the infiltrate and proteinuria levels for animals injected with FA(+)BSA. For these animals, the correlation of percent area stained with day 6 proteinuria showed $r^2 = 8\%$ ($P = 0.29$). For animals injected with FA(−)BSA, the same correlation was somewhat higher, with $r^2 = 32\%$ ($P = 0.02$).

**Apoptosis in protein-overload proteinuria.** We previously published data showing the occurrence of tubular cell apoptosis in protein-overload proteinuria (34). This earlier work found notable apoptosis in animals injected with FA(+)BSA compared with a low rate of apoptosis in saline-injected controls.

Figure 3 shows box plots of the numbers of in situ end label-positive apoptotic cortical cells per low-power field in animals injected with FA(+)BSA, FA(−)BSA, or saline. Rats injected with FA(+)BSA (median 15.5 per low-power field) had significantly more cortical apoptotic nuclei than animals sham injected with saline (median 0.5 per field, $P < 0.01$). Animals injected with FA(−)BSA had more apoptotic nuclei (median 2.0 per field) than those injected with saline, but this difference did not reach significance ($P > 0.05$). The FA(−)BSA group had significantly less apoptotic nuclei than the FA(+)BSA group ($P < 0.05$).

**Cell proliferation in protein-overload proteinuria.** Cortical cell proliferation was studied using NIH Image to count cells, in blinded fashion, the cytoplasm of which was labeled by histone mRNA in situ hybridization. The cell proliferation in the three groups is shown in Fig. 4. In the saline group, background levels of cortical cell proliferation were barely detectable. Cortical cell proliferation was significantly increased in the FA(+)BSA and FA(−)BSA groups (median 6.0 and 3.0 histone-positive cells per field, respectively) compared with the saline group ($P < 0.01$ and $P < 0.05$, respectively). The numbers of histone-positive cells in the two BSA-injected groups did not differ signifi-

![Fig. 2.](image-url) Fig. 2. Cortical infiltration of macrophages in protein-overload nephrosis. Area stained by rat monocyte macrophage marker is shown as percentage of total cortical area measured using NIH Image. A: box plots of macrophage infiltration in groups of rats injected with FA(+)BSA, FA(−)BSA, or saline. *$P > 0.05$ vs. FA(−)BSA; **$P > 0.05$ vs. saline controls; ***$P < 0.001$ vs. saline controls (Kruskal-Wallis ANOVA). B: distribution of macrophage infiltration across cortex. Values (means ± SE) are shown as percent area stained for each group in 6 fields moving from outer to inner cortex. • FA(+)BSA group; ▲ FA(−)BSA group; ○ saline group. +$P > 0.05$ vs. saline controls (Kruskal-Wallis ANOVA). C: correlation of macrophage infiltrate with proteinuria in FA(+)BSA (○, solid line) and FA(−)BSA (■, dashed line) groups. For FA(+)BSA group, $r = 0.28$, $P = 0.01$; for FA(−)BSA group, $r = 0.32$, $P = 0.02$.

![Fig. 3.](image-url) Fig. 3. Cortical apoptosis in protein-overload nephrosis. Apoptotic cells detected by in situ end labeling were counted using NIH Image. Box plots of apoptotic cells in groups of rats injected with FA(+)BSA, FA(−)BSA, or saline are shown. *$P > 0.01$ vs. saline controls; **$P > 0.05$ vs. FA(+)BSA group; $P = 0.01$ vs. FA(−)BSA group; $P = 0.02$ vs. saline control (Kruskal-Wallis ANOVA).
FIGURE 4. Cortical cell proliferation in protein-overload nephrosis. Proli-
ferating cells were detected by in situ hybridization for histone
mRNA, and positive cells per field were counted using NIH Image.
Box plots of proliferating cells in groups of rats injected with
FA(+)-BSA, FA(-)-BSA, or saline are shown. *P > 0.01 vs. saline
controls; **P > 0.05 vs. saline controls; P = not significant for
FA(+)-BSA vs. FA(-)-BSA (Kruskal-Wallis ANOVA).

DISCUSSION

Tubulointerstitial injury is a key stage in the pro-
gression of chronic renal failure (25), and more re-
cently, proteinuria has been recognized as a mediator
of progression, independent of glomerular injury. How-
ever, only in the last few years has evidence emerged to
link these two steps in progressive renal disease. Eddy
et al. (5) found that the interstitial infiltrate in acute
puromycin aminonucleoside nephrosis waxed and
waned in parallel with the transitory proteinuria. In
protein-overload proteinuria, the mononuclear infiltrate
was found to correlate with urinary protein excre-
tion (27). These studies raised the possibility of a
link between proteinuria and the interstitial infiltrate,
which is dominated by macrophages.

The protein-overload proteinuria model uses heter-
oalogous albumin to rapidly induce very heavy protein-
uria, without major glomerular injury, classical immu-
ne response, or acute renal impairment. Kees-Folts
et al. (20) found that protein-overload proteinuria led
to the in vivo elaboration of a lipid macrophage che-
omacrotactant at the time of peritubular macrophage
infiltration. In vitro studies of cultured proximal tubu-
ules suggested that the chemoattractant was pro-
duced as a result of tubular catabolism of FA(+)-BSA,
but not exposure to FA(-)-BSA.

In nephrosis, the fatty acid load carried by albumin is
increased considerably (33), and apical membrane of
proximal tubular cells is exposed to this heavily fatty
acid-laden albumin (12). Given the potential for this
lipid to stimulate a proinflammatory environment in
the kidney, it would appear intuitively reasonable to
suggest that lipid lowering may be beneficial in pro-
teinuric renal disease. To date, large-scale studies ex-
amining the effects of lipid lowering on the progression
of renal failure in humans have not been performed.
However, a recent meta-analysis of 13 smaller studies
reveals significant renoprotective effects of lipid lower-
ing that compare favorably with the protective effects
of angiotensin-converting enzyme inhibition seen in
proteinuria (10).

Our studies provide further data supporting the con-
cept of a detrimental effect of lipid on renal disease by
examining the contrasting effects in vivo of FA(+)-BSA,
FA(-)-BSA, or saline administration on cortical macro-
phage infiltration, apoptosis, and cell proliferation in
protein-overload proteinuria. The advent of video-
linked image analysis has allowed us to more objec-
tively quantify these processes in a large number of
fields over a wide area. The histological staining was
analyzed in a stereotyped fashion, so the average de-
gree of staining at any given level could be calculated.
This allowed us to show the geographical distribution
of these processes within the kidney.

The urine protein excretion was similarly raised to
grossly elevated levels in both BSA groups studied.
This suggests that the tubules in both groups were
exposed to equivalent filtered albumin levels. Overall,
this suggests that differences between the groups were
due to the quality, rather than the quantity, of the
filtered albumin. We cannot completely exclude the
possibility that injected FA(-)-BSA may pick up free
fatty acids from the rat circulation and carry them into
the kidney tubule. However, it is unlikely that large
quantities of free fatty acid are available to injected
albumin. Furthermore, because FA(+)-BSA is not fully
saturated with fatty acids, FA(+)-BSA and FA(-)-BSA
would pick up scarce free fatty acids to an equivalent
extent. Thus tubules will be exposed to much higher
quantities of fatty acid in animals injected with
FA(+)-BSA than in those injected with FA(-)-BSA.

Although there was no change in C Cr in the BSA-
injected groups during the study, saline-injected con-
trols showed a rise in C Cr. This may have been due to
volume loading. It suggests that the BSA injected into
the other two groups may have had a counterbalancing
injurious effect. Previous studies have not used saline-
injected controls, so this finding will require confirma-
tion.

Protein overload led to a macrophage infiltrate in
both protein-injected groups, which was of significa-
cantly greater magnitude in the FA(+)-BSA than in the
FA(-)-BSA group. The latter group, in turn, had a
significantly higher infiltrate than the saline-injected
group. This is the first in vivo study to confirm the in
vitro findings of Kees-Folts et al. (20). Their data sug-
gested that macrophage infiltration with FA(+)-BSA
was due to a lipid chemoattractant. Our results indi-
cate that the absence of fatty acids on FA(-)-BSA
markedly reduces macrophage infiltration into the kid-
ney, possibly because of reduced production of such
chemoattractant(s). The residual infiltrate seen in the
FA(-)-BSA rats seems likely to be due to a second
chemoattractant mechanism. We cannot exclude the
trace of fatty acids remaining on FA(-)-BSA as a cause
of the residual infiltrate, although this seems unlikely.
More likely is the possibility that albumin itself causes
release of some chemoattractants by proximal tubular
cell, as demonstrated in vitro by several authors
(39, 40).
The image analysis gives an accurate one-dimensional view of the macrophage infiltrate at different levels of the cortex and medulla, whereas previous studies have only examined it in the cortex as a whole. The analysis clearly showed that the infiltrate is largely confined to the outer cortex, which tails off toward the inner cortex and medulla. The reason for this geographical distribution of the infiltrate is unknown. Interestingly, the infiltrate appears to localize to areas where resident macrophages are already found in the normal kidney. Saline-injected rats had a sparse resident macrophage population that was most notable in the outer cortex. Although this analysis is considerably more sophisticated than previous studies, it remains an approximation to a complex three-dimensional process.

The data are analogous in humans. Using quantification of macrophage immunohistochemistry with NIH Image, we previously showed the presence of a macrophage infiltrate in proteinuric human glomerular disease (11). There was a heavier infiltrate in membranous glomerulonephritis that was similar in magnitude to the infiltrate in the FA(+)BSA group. In minimal change disease, where urinary albumin is relatively devoid of fatty acid (12), the infiltrate was comparable to that seen with FA(-)BSA and significantly less than that seen in membranous glomerulonephritis (11). A similar gradient of macrophage numbers has also been noted in human renal biopsies of cases with heavy proteinuria (Furness PN, unpublished observations). Normal human renal biopsies showed a sparse macrophage infiltrate of low intensity similar to the saline-injected rats.

The FA(-)BSA group showed a significant correlation between infiltrate and proteinuria. This observation supports the contention that urinary protein per se may induce inflammation. The lack of significant correlation between proteinuria and macrophage infiltration in the FA(+)BSA group suggests that fatty acids bound to albumin complicate and confound this relationship by exposing the kidney to additional pathophysiological stimuli.

We recently showed increased tubular cell turnover in this model, manifest as significantly increased cortical apoptosis and cell proliferation in animals injected with FA(+)BSA compared with saline controls (34). We now demonstrate that induction of proteinuria with FA(-)BSA results in significantly lower levels of apoptosis than observed in animals with FA(+)BSA proteinuria. This observation indicates that fatty acids induce tubular apoptosis directly or indirectly via another mechanism possibly related to their proinflammatory properties. Apoptosis in FA(-)BSA animals was not significantly greater than in saline controls, and therefore the data do not support a directly proapoptotic effect of albumin in kidney tubules, as suggested by previous in vitro studies (8). Conversely, proliferation in both proteinuric groups was similar and significantly greater than in controls. Thus albumin per se, and not bound fatty acids, appears to mediate the proliferative response of tubular cells in proteinuria, in accordance with our previous in vitro observations (4). It seems probable that the pathological changes observed in this short-term study represent the precursors of later scarring, destined to develop in a similar distribution. However, in the model used in these experiments, scarring and fibrosis do not occur, and longer-term studies are required to address this possibility more directly.

Our studies show a consistent pattern across the three groups of animals. The effects on macrophage infiltration and apoptosis were most marked in the FA(+)/BSA group, with a progressive diminution in the FA(-)BSA and saline groups. The results indicate that fatty acids carried on filtered albumin play a leading role in the pathophysiology of proteinuria and provide fresh impetus for the study of lipid-lowering strategies in renal disease.

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