Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinuria

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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 proteinuria. 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 proteinuria.

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 hypoxia-induced renal injury (15). Therefore, glomerular filtered lipids, especially fatty acids bound to albumin, 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 nephropathy (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 chemoattractant (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).

The albumin concentration of the solutions was assayed, the solutions were diluted to 33%, and aliquots were stored at 4°C until the time of injection. The pH of the FA(+)-BSA solution was 6.5, and that of the FA(-)-BSA solution was 6.9. The BSA preparations tested negative for endotoxin (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
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), perpendicularly toward the hilum. The innermost region thus measured (field 6) represents the inner cortex-corticomedullary junction. 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 histone mRNA-positive cells on coded sections. Typically, 10 cuts per section were counted, each with four adjacent, nonoverlapping fields. For cell proliferation analysis, staining the size of positive cell cytoplasm (150–300 pixels), above a fixed high threshold of 210, was counted as a single particle. A median of 40 low-power fields (~0.17 mm²) were counted per rat.

**Statistical analysis.** Mann-Whitney tests (with Bonferroni's correction) and Kruskal-Wallis ANOVA with Dunn's posttest for multiple comparisons were used for nonparametric distributions. Box plots show the median line, interquartile range (marked by the box edges), and range of data (shown by the "whiskers").

### 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). However, 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).

**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>
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<tbody>
<tr>
<td><strong>Total protein g/l</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>FA(+)BSA</td>
<td>63 ± 2*</td>
<td>80 ± 6*</td>
<td>77 ± 4*</td>
<td>77 ± 3*</td>
</tr>
<tr>
<td>FA(−)BSA</td>
<td>63 ± 3*</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 immunodiffusion. ND, not done. Identical symbols for 2 groups on the same day indicate statistical significance: \*P < 0.001; †P < 0.05 > P > 0.01; \‡P < 0.01; \§P < 0.001 after Bonferroni correction (Mann-Whitney tests). There was no significant difference between groups with identical symbols.

**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, respectively (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 BSA 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 exposed 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_GFR), but this was not significantly different between baseline and day 7. In the saline-injected animals, C_GFR 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. Urinary 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
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 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-

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**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>
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<tbody>
<tr>
<td>Creatinine clearance, ml/min</td>
<td></td>
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</tr>
<tr>
<td>FA(+)-BSA</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>Saline</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>Urinary protein, mg/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA(+)-BSA</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>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&quot;§</td>
<td>5.8 ± 1.9&quot;§</td>
<td>3.8 ± 2.0&quot;§</td>
</tr>
<tr>
<td>Urinary rat serum albumin, mg/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA(+)-BSA</td>
<td>ND</td>
<td>128 ± 94</td>
<td>122 ± 83</td>
<td>142 ± 49</td>
</tr>
<tr>
<td>FA(−)-BSA</td>
<td>ND</td>
<td>144 ± 57</td>
<td>102 ± 70</td>
<td>149 ± 82</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. Identical symbols for 2 groups on the same day indicate statistical significance: *P < 0.01; †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.

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Fig. 1. Macrophage immunohistochemistry in protein-overload nephrosis. Cortical macrophages were detected using anti-rat macrophage antisera and visualized by immunoperoxidase staining. Representative sections are shown. A: saline-injected control animal. B: animal injected with fatty acid-depleted BSA [FA(−)-BSA]. C: animal injected with fatty acid-carrying BSA [FA(+)-BSA].
FATTY ACIDS AND TUBULOINTERSTITIAL INJURY

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 infiltration with proteinuria in FA(+)-BSA (○, solid line) and FA(−)-BSA (●, dashed line) groups. For FA(+)BSA group, r = 0.28, P = not significant. For FA(−)-BSA group, r = 0.32, P = 0.02.

Cortical 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 correlation 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 r² = 8% (P = 0.29). For animals injected with FA(−)-BSA, the same correlation was somewhat higher, with r² = 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 significantly.

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Fig. 4. Cortical cell proliferation in protein-overload nephrosis. Proliferating 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 > 0.05 vs. saline controls; **P > 0.01 vs. saline controls; *P = not significant for FA(+)-BSA vs. FA(-)-BSA (Kruskal-Wallis ANOVA).

DISCUSSION

Tubulointerstitial injury is a key stage in the progression of chronic renal failure (25), and more recently, proteinuria has been recognized as a mediator of progression, independent of glomerular injury. However, 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 excretion (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 heterologous albumin to rapidly induce very heavy proteinuria, without major glomerular injury, classical immune 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 chemoattractant at the time of peritubular macrophage infiltration. In vitro studies of cultured proximal tubules suggested that the chemoattractant was produced 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 proteinuric renal disease. To date, large-scale studies examining 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 lowering that compare favorably with the protective effects of angiotensin-converting enzyme inhibition seen in proteinuria (10).

Our studies provide further data supporting the concept 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 macrophage infiltration, apoptosis, and cell proliferation in protein-overload proteinuria. The advent of video-linked image analysis has allowed us to more objectively 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 degree 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 Ccr in the BSA-injected groups during the study, saline-injected controls showed a rise in Ccr. 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 confirmation.

Protein overload led to a macrophage infiltrate in both protein-injected groups, which was of significantly 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 suggested that macrophage infiltration with FA(+)-BSA was due to a lipid chemoattractant. Our results indicate that the absence of fatty acids on FA(-)-BSA markedly reduces macrophage infiltration into the kidney, 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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REFERENCES