Warfarin-Related Nephropathy Modeled by Nephron Reduction and Excessive Anticoagulation

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ABSTRACT

An acute increase in international normalized ratio (INR) to >3.0 in patients with chronic kidney disease (CKD) can associate with an unexplained acute increase in serum creatinine and accelerated progression of CKD. A subset of these patients have renal tubular obstruction by casts of red blood cells, presumably the dominant mechanism of the acute kidney injury described as warfarin-related nephropathy. Here, we developed an animal model of this acute kidney injury that is based on the 5/6-nephrectomy model to aid future investigation of the pathogenesis of this condition. We found that acute excessive anticoagulation with brodifacoum ("superwarfarin") increased serum creatinine levels and hematuria in 5/6-nephrectomized rats but not in controls. In addition, morphologic findings in 5/6-nephrectomized rats included glomerular hemorrhage, occlusive red blood cell casts, and acute tubular injury, similar to the biopsy findings among affected patients. Furthermore, in the rat model, we observed an increase in apoptosis of glomerular endothelial cells. In summary, the 5/6-nephrectomy model combined with excessive anticoagulation may be a useful tool to study the pathogenesis of warfarin-related nephropathy.


The significance of this study derives from the fact that this is the first successful attempt to reproduce in an animal model the morphologic findings seen in patients with a newly recognized syndrome of warfarin-related nephropathy (WRN). WRN can have dire consequences, particularly in chronic kidney disease (CKD) patients. WRN is a not an uncommon complication of warfarin therapy, which is the most commonly used oral anticoagulant in North America.

We recently reported that warfarin therapy can result in acute kidney injury (AKI) by causing glomerular hemorrhage and renal tubular obstruction by red blood cell (RBC) casts.1 Subsequent analysis of 103 patients with CKD revealed that 37% experienced an unexplained increase in serum creatinine (SC) of ≥0.3 mg/dl within 1 week of an international normalized ratio (INR) > 3.0.2 Also, patients with WRN had accelerated progression of CKD, as compared with patients without WRN. Moreover, our recent analysis of more than 15,000 warfarin-treated patients showed that WRN affects approximately 33% of CKD patients and 16% of non-CKD patients who experienced an INR > 3.0.3 We also found that mortality rate in patients with WRN was significantly higher than in patients without WRN.

Hitherto, there is no animal model available to study WRN. The need for an animal model to study WRN is substantial. An animal model could provide a clear understanding of the mech-
anisms of WRN. It may also provide insights into strategies for WRN prevention and treatment. Herein, we report that excessive anticoagulation in rats with 5/6-nephrectomy, a model of ablative nephropathy, results in increased SC levels and reproduces the morphologic findings found in patients with WRN. In contrast, excessive anticoagulation in control animals was not associated with changes in SC levels, and kidney morphology was unremarkable.

RESULTS

Treatment with Brodifacoum Results in Increased SC in 5/6-Nephrectomy, but Not in Control Rats
We investigated whether acute excessive anticoagulation induced by brodifacoum (superwarfarin) results in acute kidney injury in experimental animals. Administration of brodifacoum resulted in a significant prothrombin time (PT) increase in each of the 5/6-nephrectomy and control animals. By day 2 there was as much as a 5-fold increase. By day 3, the increase was >10-fold. No animal survived beyond 4 days. In control animals, brodifacoum did not affect SC levels or kidney morphology. In contrast, excessive anticoagulation in control animals was not associated with changes in SC levels, and kidney morphology was unremarkable.

Treatment with warfarin was chosen because (1) treatment with brodifacoum results in a very rapid increase in PT time and it is very difficult to investigate dose-response relationships because there might not be sufficient time for the effects of the coagulopathy on SC to be fully expressed, and (2) mechanisms of action are similar for warfarin and brodifacoum.

For these studies we used a "surrogate" INR by comparing PT time after and before the treatment. The average PT time in 50 rats (25 control and 25 5/6-nephrectomy rats) was used as the normal PT time. Changes in SC were calculated from baseline in the same animal. Squares represent 5/6-nephrectomy rats; triangles represent control rats.

Figure 1A shows the change in SC after brodifacoum (superwarfarin) treatment. Control rats (n = 9, ■), 5/6-nephrectomy rats at 3 weeks (n = 8, ▲), and 5/6-nephrectomy rats at 8 weeks (n = 10, ●) after the ablative surgery were treated with a single dose of brodifacoum (BF). SC levels were measured before (day 0) and daily after the treatment with brodifacoum (arrow). Figure 1B shows changes in SC in control and 5/6-nephrectomy rats treated with different doses of warfarin. Animals were treated with different doses of warfarin in drinking water. PT was measured before and after the treatment. A "surrogate" INR was used by comparing PT time after and before the treatment. The average PT time in 50 rats (25 control and 25 5/6-nephrectomy rats) was used as the normal PT time. Changes in SC were calculated from baseline in the same animal. Squares represent 5/6-nephrectomy rats; triangles represent control rats.

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Figure 1. Treatment with brodifacoum (superwarfarin) results in increased SC levels in 5/6-nephrectomy rats but not in controls. Changes in PT are associated with SC level increase in 5/6-nephrectomy rats only. (A) Change in SC after brodifacoum (superwarfarin) treatment. Control rats (n = 9, ■), 5/6-nephrectomy rats at 3 weeks (n = 8, ▲), and 5/6-nephrectomy rats at 8 weeks (n = 10, ●) after the ablative surgery were treated with a single dose of brodifacoum (BF). SC levels were measured before (day 0) and daily after the treatment with brodifacoum (arrow). (B) Animals were treated with different doses of warfarin in drinking water. PT was measured before and after the treatment. A "surrogate" INR was used by comparing PT time after and before the treatment. The average PT time in 50 rats (25 control and 25 5/6-nephrectomy rats) was used as the normal PT time. Changes in SC were calculated from baseline in the same animal. Squares represent 5/6-nephrectomy rats; triangles represent control rats.

5/6-Nephrectomy Itself Results in Chronic Hematuria
Animals with 5/6-nephrectomy developed progressive hematuria (Figure 2A). As measured by dipstick, no hematuria was seen 3 weeks after the ablative surgery; by 6 weeks after the ablative surgery mild hematuria was noted in approximately one-third of the rats. By 8 weeks after the ablative surgery, mild to moderate hematuria was noted in all 5/6-nephrectomy animals. The hematuria likely is related to the progression of focal segmental glomerulosclerosis, which we documented in the 5/6-nephrectomy animals (Table 1). Also, it is well documented that hematuria is a manifestation of focal segmental glomerulosclerosis.
Morphologic findings in the kidneys obtained from animals treated with brodifacoum are shown in Table 1. Glomeruli in control or 5/6-nephrectomy animals were mildly to moderately enlarged by 8 weeks after the ablative surgery. Glomeruli in control rats treated with brodifacoum showed only a few apoptotic endothelial cells (Figure 4D). However, the number of apoptotic cells increased in glomeruli and tubules in 5/6-nephrectomy rats treated with brodifacoum 3 and 8 weeks after the ablative surgery. Thus, the number of apoptotic endothelial cells in glomeruli in 5/6-nephrectomy rats treated with brodifacoum at 3 and 8 weeks after the ablative surgery became minimal (Figure 2B).

**Brodifacoum Coagulopathy Results in Kidney Morphologic Changes Comparable to That Seen in Humans with WRN**

Morphologic findings in the kidneys obtained from animals treated with brodifacoum are shown in Table 1. Glomeruli became slightly enlarged already 3 weeks after the ablative surgery; they were mildly to moderately enlarged by 8 weeks after the ablative surgery. The number of glomeruli with segmental sclerosis also progressively increased after the ablative surgery. There were no RBCs seen in Bowman’s space or tubules in any of the kidneys obtained from animals not treated with brodifacoum, including control and 5/6-nephrectomy rats.

Treatment with brodifacoum resulted in an increased number of glomeruli with RBCs in Bowman’s space and an increase in the number of tubules containing RBCs and RBC casts. This description is relevant only to the 5/6-nephrectomy animals. No RBCs in Bowman’s space or RBC tubular casts were seen in control animals treated with brodifacoum (Figure 3). Thus, RBCs in tubules and tubular RBC casts were seen in kidneys in 50% of the rats treated with brodifacoum 3 weeks after the ablative surgery. RBCs and RBC tubular casts were present in 100% of the rats treated with brodifacoum 8 weeks after the ablative surgery. Acute tubular necrosis was also more prominent in rats treated with brodifacoum 8 weeks after the ablative surgery (Table 1). Mononuclear interstitial inflammatory cell infiltrates were only seen in the areas of fibrosis. No active inflammatory lesions were noted. There were no glomerular intracapillary hypercellularity, mesangial expansion, or crescents seen in any of the kidney sections examined. No significant tubular calcium deposits were seen either. Vascular changes included slightly increased fibrous intimal thickening in 5/6-nephrectomy animals.

No changes in proteinuria were associated with brodifacoum treatment in either experimental group (data not shown).

**Treatment with Brodifacoum Results in Increased Apoptosis of Endothelial Cells in the Kidney**

To investigate possible targets of WRN beyond those described in Table 1, we analyzed apoptosis in different kidney compartments. We found that in control or 5/6-nephrectomy animals not treated with brodifacoum, the number of apoptotic cells was negligible in all kidney compartments. In contrast, treatment with brodifacoum resulted in an increased number of apoptotic cells in control and 5/6-nephrectomy animals. Glomeruli in control rats treated with brodifacoum showed only a few apoptotic endothelial cells (Figure 4D). However, the number of apoptotic cells increased in glomeruli and tubules in 5/6-nephrectomy rats treated with brodifacoum 3 and 8 weeks after the ablative surgery. Thus, the number of apoptotic endothelial cells in glomeruli in 5/6-nephrectomy rats treated with brodifacoum at 3 and 8 weeks after the ablative surgery was significantly higher than in control rats. The number of apoptotic endothelial cells in peritubular capillaries after brodifacoum treatment was similar in control and 5/6-nephrectomy rats. However, brodifacoum treatment 8 weeks after the ablative surgery resulted in a significant increase in the number of apoptotic epithelial cells in the tubules in 5/6-nephrectomy rats (Figure 4D).

**DISCUSSION**

To the best of our knowledge, this is the first evidence that the WRN reported in humans is reproducible in an animal model.
Table 1. Morphologic findings in kidneys obtained from animals treated with brodifacoum

<table>
<thead>
<tr>
<th>Histologic Parameter</th>
<th>Control (n = 9)</th>
<th>5/6-Nephrectomy 3 Weeks (n = 8)</th>
<th>5/6-Nephrectomy 8 Weeks (n = 10)</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomeruli with global sclerosis (% of glomeruli)</td>
<td>0</td>
<td>0.19 ± 0.19</td>
<td>0.20 ± 0.13</td>
<td>0.4673</td>
</tr>
<tr>
<td>Glomeruli with segmental sclerosis (% of glomeruli)</td>
<td>0</td>
<td>0.25 ± 0.25</td>
<td>2.98 ± 0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0010</td>
</tr>
<tr>
<td>Glomerular enlargement (AU)</td>
<td>0.11 ± 0.11</td>
<td>0.88 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBCs in Bowman’s space (% of glomeruli)</td>
<td>0</td>
<td>0.69 ± 0.34</td>
<td>2.35 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0005</td>
</tr>
<tr>
<td>Acute tubular necrosis (AU)</td>
<td>0.06 ± 0.06</td>
<td>0.38 ± 0.21</td>
<td>1.6 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tubular casts (non-RBC casts, % of tubules)</td>
<td>0.39 ± 0.11</td>
<td>0.50 ± 0.09</td>
<td>0.80 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0281</td>
</tr>
<tr>
<td>Animals with tubular RBCs or tubular RBC casts (% of animals)</td>
<td>0</td>
<td>50</td>
<td>100</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Tubules with RBCs were identified when RBCs were seen in the lumen of tubules without causing luminal occlusion. RBC casts were identified when RBCs in the tubules completely occluded the lumen. Histological findings were scored semiquantitatively for each morphologic change assessed, if possible mostly following the Banff criteria for allograft rejection<sup>14</sup> as follows:

- **Glomerular enlargement**—0 to normal (80 to 110 μm in diameter); 1+ (mild, up to 25% increase in diameter); 2+ (moderate, 26% to 50% increase in diameter); 3+ (severe, >50% increase in diameter).
- Acute tubular necrosis—0 (no acute tubular injury); 1+ (mild patchy acute tubular injury with flattening of the tubular epithelium and/or vacuolization in <25% of tubules); 2+ (acute tubular injury in 25% to 50% tubules with scattered tubules containing apoptotic cell debris and/or few granular casts); 3+ (diffuse, >50% tubules with severe acute tubular injury with sloughed off epithelial cells and scattered granular casts in the lumina).
- Interstitial fibrosis—0 to normal (up to 5% interstitial fibrosis of the cortical area); 1+ (mild, 6% to 25% of cortical area); 2+ (moderate, 26% to 50% of cortical area); 3+ (severe, >50% of cortical area).
- Tubular atrophy—0 to normal (0% to 5% of cortical tubules involved atrophy); 1+ (mild, 6% to 25% of tubules involved); 2+ (moderate, 26% to 50% of tubules involved); 3+ (severe, >50% of cortical tubules involved).

<sup>a</sup>P < 0.005 compared with control; <sup>b</sup>P < 0.05 compared with 5/6-nephrectomy treated with brodifacoum at 3 weeks.

Figure 3. Morphologic findings in the kidneys obtained from experimental animals treated with brodifacoum are similar to those in patients with WRN. (A) Control and (B) 5/6-nephrectomy (ablation nephropathy, 8 weeks postsurgery) rats were treated with brodifacoum. The kidneys were obtained on day 4 post-treatment. The morphologic findings in control rats treated with brodifacoum were mild and nonspecific. In contrast, 5/6-nephrectomy animals had RBCs in Bowman’s space and RBC casts in the corresponding tubules. (C) For comparison, the morphologic findings in a kidney biopsy from a patient with WRN are shown. Numerous RBCs and RBC occlusive casts were noticed in tubules and in Bowman’s space. Magnification, 200×. Hematoxylin and eosin stain.

Here we show that excessive anticoagulation by brodifacoum in 5/6-nephrectomy rats reproduces WRN, as documented by increased SC levels, glomerular hematuria, occlusive RBC casts, and acute tubular injury. These findings closely resemble the findings in kidney biopsies from patients with WRN. In contrast, treatment with brodifacoum did not affect renal function in control animals, which confirmed our previous observation that to develop WRN an underlying kidney condition should be present. It appears that AKI develops shortly after the INR increase because in our study the PT was increased more that 10-fold from baseline by day 3, and the elevation in SC levels in 5/6-nephrectomy animals occurred by day 4. However, the PT time increase occurred very rapidly in animals treated with brodifacoum, and it is possible that deterioration of renal function is developing more gradually. Indeed, in 5/6-nephrectomy animals treated with warfarin, SC changes were correlated with changes in PT time, but the PT time increase occurred within several days after the beginning of treatment. Of note, SC changes were not associated with PT increase in control animals with warfarin or brodifacoum treatment. The possibility that the increase in SC is related to hemodynamic changes after treatment with brodifacoum exists but is unlikely because 5/6-nephrectomy and control animals experienced the same amount of bleeding and SC was significantly increased in 5/6-nephrectomy animals only. Moreover, the increase in SC was also dependent on the progression of CKD because animals treated with brodifacoum at 3 weeks after the ablative surgery had an intermediate increase.
in SC as compared with animals treated 8 weeks after the ablative surgery and with controls. Also, the morphologic findings in 5/6-nephrectomy animals treated 3 weeks after the ablative surgery included less prominent glomerular hemorrhage, occlusive RBC casts, and acute tubular injury as compared with 5/6-nephrectomy animals treated 8 weeks after the ablative surgery (Table 1). This is consistent with our observations in humans that patients with CKD are at much greater risk of WRN than those without CKD.3 Also, the increase in SC in our animal model of WRN is seen within a few days of the onset of brodifacoum-induced coagulopathy, consistent with our WRN studies in humans.

In our studies of human WRN, we did not find a significant relationship between the degree of INR elevation above normal and the risk of WRN.3 However, in our animal model of WRN we found that in the 5/6-nephrectomy rats treated with brodifacoum 8 weeks after the ablative surgery, there was a significant relationship between the degree of INR elevation and the degree of SC elevation. We suggest that this difference between human WRN and animal WRN can be explained by the much wider range in INR achieved in the animal model.

With regard to the mechanism of AKI in the animal model of WRN, we suggest that the coagulopathy increases glomerular hematuria in the 5/6-nephrectomy rats, which results in the formation of obstructing tubular RBC casts. Although this is probably the dominant mechanism of the AKI, we suggest that there may also be other important mechanisms. For example, warfarin has been shown to affect glomerular mesangial cells by interfering with the activation of the product of growth arrest-specific gene 6. This could affect glomerular hemodynamics or aggravate the underlying glomerular disease.7,8 However, we did not find significant differences in growth arrest-specific gene 6 expression in the kidneys obtained from animals treated with brodifacoum and control (data not shown). However, we did find that excessive anticoagulation results in an increased number of apoptotic cells in the kidney of endothelial and nonendothelial origin, especially in the glomeruli. Thus, apoptosis of glomerular cells may also contribute to the pathogenesis of WRN. We also suggest that warfarin-related endothelial damage may play a role in the acute increase in mortality in human WRN.3

In conclusion, we are entering the terra incognita of a previously unrecognized kidney condition. At this moment, very little is known about the pathogenesis of WRN and therapeutic approaches. Nevertheless, we had demonstrated that this is not an uncommon disease, involving at least 30% of CKD patients on warfarin therapy who experienced excessive anticoagulation with INR > 3.0. These patients also have an increased mortality rate. The need of an animal model to better understand the pathogenic mechanisms of WRN is obvious. We suggest that the work presented here is an important step forward in that regard.

CONCISE METHODS

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.9 Male Sprague-Dawley rats weighing 140 to 160 g were allowed food and water ad libitum. The 5/6-nephrectomy was performed under a ketamine/xylazine (6.0 mg/0.77 mg/100 g) anesthesia by a nephrectomy of the right kidney and resection of two-thirds of the left kidney, as described previously.10–12 Weekly monitoring of SC, proteinuria, and hematuria (by DiaScreen [Chronimed, Inc., Minnetonka, MN] dipstick) was performed. Brodifacoum was given in pellets at 3 (n = 8) or 8 (n = 10) weeks
after the ablative surgery, according to the manufacturer protocol. Briefly, animals had free access to brodifacoum-containing pellets at day 0 for 24 hours, which was given instead of regular rodent food. Animals of the same age and gender were used as controls at day 0 for 24 hours, which was given instead of regular rodent food. Animals of the same age and gender were used as control at day 0 for 24 hours, which was given instead of regular rodent food.

Figure 5. Experimental protocol of the study. Two- to-3-month-old Sprague–Dawley rats were subjected to 5/6-nephrectomy (ablative nephropathy). SC, proteinuria, and hematuria were measured weekly after the ablative surgery. Animals were treated with brodifacoum (superwarfarin) 3 and 8 weeks after the ablative surgery. SC, proteinuria, and hematuria were measured daily after the treatment. All animals died by day 4 after the treatment with brodifacoum was initiated.

SC, proteinuria, and hematuria were measured daily after the ablative surgery. According to the manufacturer protocol. Briefly, animals had free access to brodifacoum-containing pellets at day 0 for 24 hours, which was given instead of regular rodent food. Animals of the same age and gender were used as control (n = 9) and received the same treatment with brodifacoum (Figure 5). For dose-response studies, warfarin was given to control and 5/6-nephrectomy rats 8 weeks after the ablative surgery in drinking water. The amount of warfarin consumed by the animals was measured daily. Several doses of warfarin (0.25, 0.5, and 1.0 mg/kg per day) were used. After the brodifacoum or warfarin administration, monitoring of the PT, SC, proteinuria, and hematuria was performed.

SC was measured using a creatinine reagent assay (Raichem, San Marcos, CA) according to the manufacturer’s protocol. The detection method is based on the Jaffe reaction. Briefly, serum was mixed with working reagent at 37°C at a ratio of 1:10 in a 96-well plate and the absorbance was read at 510 nm at 40 and 100 seconds using a Bio-Tek PowerWave 340 plate reader (BioTek, Winooski, VT).

PT was measured using an Electra 750 coagulation analyzer (Medical Laboratory Automation, Pleasantville, NY) according to the manufacturer’s protocol. Briefly, blood was collected from the tail vein in an Eppendorf tube with 3.8% sodium citrate as the anticoagulant at a ratio of 9 parts blood to 1 part anticoagulant. The blood specimen is then centrifuged at 1000 RCF for 15 minutes. Thromboplastin is then reconstituted as the manufacturer recommends and warmed on the MLA Electra 750 before use for 15 minutes. Then 0.1 ml of plasma is transferred to the bottom of a cuvette and placed in the incubation station for 3 minutes. The sample is then transferred to the test station. Warm thromboplastin (0.2 ml) is aspirated and placed over the test station. The pipette plunger is pushed down as the test is started. When the timer stops, clotting time is recorded.

Hematuria and proteinuria were measured using DiaScreen (Chronimed, Inc., Minnetonka, MN) reagent strips in the urine. Hematuria was graded using a semiquantitative scale of 0 to 3+. Score 0 was designated for negative hematuria, score 1+ for mild hematuria, score 2+ for moderate hematuria, and score 3+ for large hematuria.

Kidneys were fixed in 10% buffered formalin, embedded in paraffin, and cut as 3-μm sections. Hematoxylin and eosin stained slides were analyzed by two independent pathologists who were not aware of the source of the kidney sections. In each animal an entire area of longitudinal sections of one kidney was evaluated. In 5/6-nephrectomy animals, the scarred areas related to the surgical procedure were excluded. In general, each section contained >50 glomeruli, >500 tubules, and >10 small arteries.

Interobserver agreement was calculated based on kappa statistics. Rates were categorical from 0 to 3 with 0.5 steps. Agreement: 90.38%

Expected agreement: 65.69%
Kappa: 0.7197
SEM: 0.0397
Z: 18.15
Prob > Z: 0.0000

The results show an almost perfect agreement between the two observers (90.38%) with a highly significant P value (P < 0.0001).

Statistical Analysis

Results are presented as mean ± SEM if not otherwise specified. Differences between groups were analyzed by the two-paired t test or ANOVA test where it was applicable. Tukey post test was performed to analyze the differences between groups in conjunction with ANOVA. Association between SC changes and PT time increase was analyzed using Pearson correlation analysis with a twotailed P value. Kappa statistics were used to study the interobserver agreement.

ACKNOWLEDGMENTS

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DISCLOSURES

None.

REFERENCES

Evidence that warfarin related nephropathy occurs commonly in both those with and without chronic kidney disease and is associated with increased mortality rate. 2011 Kidney Int, 2011, in press


