Activated Omentum Slows Progression of CKD

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ABSTRACT

Stem cells show promise in the treatment of AKI but do not survive long term after injection. However, organ repair has been achieved by extending and attaching the omentum, a fatty tissue lying above the stomach containing stem cells, to various organs. To examine whether fusing the omentum to a subtotally nephrectomized kidney could slow the progression of CKD, we used two groups of rats: an experimental group undergoing 5/6 nephrectomy only and a control group undergoing 5/6 nephrectomy and complete omentectomy. Polydextran gel particles were administered intraperitoneally before suture only in the experimental group to facilitate the fusion of the omentum to the injured kidney. After 12 weeks, experimental rats exhibited omentum fused to the remnant kidney and had lower plasma creatinine and urea nitrogen levels; less glomerulosclerosis, tubulointerstitial injury, and extracellular matrix; and reduced thickening of basement membranes compared with controls. A fusion zone formed between the injured kidney and the omentum contained abundant stem cells expressing stem cell antigen-1, Wilms’ tumor 1 (WT-1), and CD34, suggesting active, healing tissue. Furthermore, kidney extracts from experimental rats showed increases in expression levels of growth factors involved in renal repair, the number of proliferating cells, especially at the injured edge, the number of WT-1–positive cells in the glomeruli, and WT-1 gene expression. These results suggest that contact between the omentum and injured kidney slows the progression of CKD in the remnant organ, and this effect appears to be mediated by the presence of omental stem cells and their secretory products.


The omentum has long been known to have the capacity to migrate to injured organs and facilitate their healing.1–3 Surgeons have taken advantage of this property to repair organs by extending and attaching the omentum to fractured bones, severed spinal cords, and even ischemic hearts.4–8 We previously showed that fusing the omentum to an injured liver caused it to nearly double in size,9,10 and that pieces of diabetic pancreas placed in the omentum induced new insulin-producing cells.11 These healing properties of the omentum have been attributed to its high content of progenitor cells as well as growth and angiogenic factors.12,13

Although adult stem cells have shown promise in treating AKI,14–18 whether they could alleviate CKD has not been fully explored. One technical problem encountered in experimental investigation of this issue is the fact that adult stem cells do not survive in the body for more than a few days after injection19–21; therefore, stem cells will have to be injected every few days for several weeks to see an observable effect. There are currently no technologies by which adult stem cells could be made to survive in the body for long periods. Therefore, the concept
that adult stem cells could be beneficial in treating CKD remains an untested hypothesis.

In this study, we overcame this difficulty by allowing the activated omentum, a rich source of adult stem cells, to fuse to the 5/6 nephrectomized kidney (a model of CKD in rats). This maneuver allowed us to permanently lodge adult stem cells in the vicinity of the injured kidney. We report that by doing so, the progression of CKD was slowed, likely due to the presence of stem cells and their secretory paracrine factors in the vicinity of the injured kidney.

RESULTS

Fusion of Omentum to the Injured Kidney Attenuated Progression of CKD

In experimental rats in which 5/6 nephrectomy was performed and the omentum was mobilized by polydextran, we observed fusion between the omentum and the two poles of the remnant left kidney (Figure 1, A–C). In control 5/6 nephrectomized and omentectomized rats, there was absence of fusion of omentum to the remnant kidney but an adventitious tissue adhered to the kidney at the injured edge (Figure 1D).

Compared with control rats, experimental rats showed plasma creatinine levels that were 20% lower at week 6 and 30% lower at week 12 (Figure 2A). In addition, plasma urea nitrogen levels in the experimental group were 30% lower compared with the control group at week 6 and 50% lower at week 12 (Figure 2B) (weekly plasma creatinine and urea nitrogen levels are presented in Supplemental Table 1). Creatinine clearance was higher in experimental rats compared with controls at week 6 (0.22 ± 0.01 versus 0.16 ± 0.01 ml/min per 100 g; P < 0.05) and week 12 (0.18 ± 0.01 versus 0.10 ± 0.01 ml/min per 100 g; P < 0.05). The average body weight, kidney weight/body weight ratio, urine output, and proteinuria were not significantly different between experimental and control groups (see Supplemental Results, Supplemental Table 1).

Histologic Examination of the Injured Edge of Kidney

Histologically, the injured edge of the experimental kidney was demarcated by the omental tissue showing empty spaces occupied by the polydextran particles (hematoxylin and eosin staining in Figure 3B and trichrome staining in Figure 4B). In contrast, the injured edge of control kidney was devoid of omentum but associated with an adhering tissue (Figure 3A). Compared with control kidneys that showed lack of perfusion and greater scarring of the glomeruli and tubules at the injured edge (Figures 3A and 4A), the experimental kidneys appeared to be well perfused and better preserved (Figures 3B and 4B). In contrast to experimental kidneys, tubules at the injured edge of control kidneys appeared to be dedifferentiated based on the presence of vimentin (a marker of epithelial to myofibroblast transformation) (Figure 5) and absence of Phaseolus vulgaris agglutinin, a tubular differentiation marker (Figure 6), suggesting that the tubules in control kidneys were damaged to a greater extent than in the experimental kidneys.

Histologic and Biochemical Changes in Different Regions of the Remnant Kidney Parenchyma

Preliminary histologic examination of the kidney tissues suggested that the changes in the experimental kidney were regionally distributed, whereas they appeared to be uniformly distributed in the control kidneys. Therefore, further histologic and biochemical studies were performed on different regions of the experimental remnant kidney parenchyma (near omentum, farther from omentum, and farthest from omentum) as described in Concise Methods and depicted in Supplemental Figure 1. For biochemical studies, the remnant kidney was surgically separated into the three zones and the tissue extracts were used for various analyses.

Glomerulosclerosis and Tubular Injury Were Reduced in Experimental Rats, Especially in Regions Near the Omentum

A gradient of glomerular as well as tubular injury was seen in the three regions of the experimental kidneys; minimal injury

Figure 1. Fusion of omentum to the remnant kidney. (A) A normal adult rat kidney. (B) The remnant rat kidney after 5/6 nephrectomy. (C) Omental attachment at the upper pole (black arrow) and lower pole (blue arrow) of the remnant kidney 2 weeks after 5/6 nephrectomy. The omentum remains tightly attached to the remnant kidney for up to 12 weeks. (D) Injured upper pole (black arrow) and lower pole (blue arrow) of the remnant kidney of a control omentectomized rats 2 weeks after 5/6 nephrectomy. Note the absence of omentum in D but an adventitious tissue adhering to the injured edge.
in the kidney tissue juxtaposed to the omentum compared with the deeper areas of kidney farther from the omentum (Figures 7 and 8). In experimental rats compared with controls, the glomerulosclerosis index was 70% lower in regions near the omentum, 5% lower in areas farther from the omentum, and had no change in areas farthest from the omentum (Figure 7). Similarly, the tubular injury index was 46% lower in regions near the omentum, 19% lower in areas farther from the omentum, and had no change in areas farthest from the omentum (Figure 8). Of note, regional differences in glomerulosclerosis as well as tubular injury indices in the control kidneys were not significant and therefore were measured as a composite index by scoring of random fields (see Supplemental Results).

The percentage of glomeruli that exhibited different degrees of glomerulosclerosis in each of the three zones (Figure 9) was consistent with the regional differences seen in the glomerulosclerosis indices in Figure 7. Glomeruli near the omentum mostly showed milder scarring (0–2+) whereas glomeruli farther and farthest from the omentum showed a greater degree of scarring (1+ to 4+).

In contrast to the regional differences seen in glomerular and tubular injury, such differences were not seen when the kidney sections were immunostained for early markers of fibrosis (α-smooth muscle actin [α-SMA] and type IV collagen). Experimental kidneys uniformly showed significantly less accumulation of α-SMA in the tubulointerstitial areas compared with controls (Figures 10, A–C). Experimental kidneys also showed significantly less accumulation of type IV collagen in the glomerular and tubular basement membranes compared with controls (Figures 10, D–F). Quantitative data of α-SMA and type IV collagen staining are presented in the Supplemental Results.

High Cellular Proliferation at the Injured Edge of Experimental Kidneys

At day 3, 5-bromodeoxyuridine (BrdU)–labeled cells in high numbers were visible at the injured edge adjacent to the omentum (Figure 11A). Fewer BrdU-labeled cells were present in the injured kidney tissue farther from the omentum (Figure 11B) and in minimal numbers (background levels) in the kidney tissue farthest from the omentum (Figure 11C). The staining was predominantly present on tubular cells with a modest number of interstitial as well as glomerular cells. Control kidneys, in contrast, were minimally labeled (as in Figure 11C) and no regional differences in labeling were observed.

Increase of WT-1–Positive Cells in the Glomeruli of Experimental Kidneys

Proliferation of WT-1–positive podocytes in the glomeruli is considered a sign of glomerular repair. Compared with control or normal healthy rat kidneys, the experimental kidneys showed a 1.6- to 1.8-fold increase in the number of WT-1 cells...
in the glomeruli at both the 2- and 4-week time points (Figures 12 and 13). Note that regional differences in WT-1 staining were absent; therefore, the regional data were pooled for comparison with controls. Measured glomerular areas and the number of WT-1–positive cells in the Bowman’s capsule (parietal epithelial cells) at weeks 2 and 4 are shown in Supplemental Figures 2 and 3.

mRNA expression for the WT-1 gene (by RT-PCR) was performed to substantiate the histochemical results. In the absence of regional differences, the regional data were pooled for comparison with controls. Five- and 10-fold increases were seen in the mRNA expression of the WT-1 gene at 2 and 4 weeks, respectively, in experimental kidneys compared with control kidneys (Figure 12D), confirming the histochemical results shown in Figure 12C.

Levels of Growth Factors in the Kidney Tissues

Three growth factors, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and IGF-1, known to be important in repair of injured kidney,23–25 were measured in kidney extracts from the three zones of experimental kidneys. In the absence of regional differences, the regional data were pooled for comparison with controls. VEGF progressively increased by 3- to 5-fold in the experimental kidneys compared with the control kidneys during the 4 weeks after subtotal nephrectomy (Figure 14A). HGF decreased slightly in the first week, but increased by 40% above the respective control and normal kidney by 4 weeks (Figure 14B). IGF-1 increased by 6-fold in the first week after nephrectomy compared with controls but then decreased significantly below control and normal kidney levels after 1 week (Figure 14C).

Attachment of Omentum to the Kidney Induced Progenitor Cells at the Injured Edge

We have previously shown that the omentum has resident stem cells, which are activated after the omentum attaches to an injured site.12 Consistent with this, we found that CD34 cells (progenitors of blood vessels) were induced after the omentum fused to the injured kidney, resulting in abundant blood vessels in the fusion zone (Figure 15A). Also induced at the injured edge were WT-1 cells, which are kidney progenitor cells that form glomeruli and nephrons during embryogenesis. These cells appeared to be originating in the omental tissue and infiltrating into the kidney parenchyma at the fusion zone (Figure 15B). In addition, cells bearing stem cell antigen-1 (Sca-1), a well known marker of very early murine hematopoietic stem cells, expanded in the omentum and concentrated around the polydextran particles embedded in the omentum (Figure 15C). Whereas the CD34 and WT-1 cells were seen in the kidney parenchyma at the injured edge, the Sca-1 cells were limited to the omental tissue and were absent in the kidney parenchyma. By contrast, there was an absence of CD34+, WT-1+, and Sca-1–positive cells at the injured edge in control rats (not shown).
DISCUSSION

We show that attaching the omentum to a 5/6 nephrectomized kidney improved residual renal function in the remnant kidney. By 6 weeks, the control omentectomized rats developed CKD, manifested by elevated plasma creatinine/urea nitrogen and reduced creatinine clearance. In contrast, fusing the omentum to the remnant kidney resulted in higher creatinine clearance and lower plasma creatinine/urea nitrogen levels. There was also reduced glomerulosclerosis and tubulointerstitial injury compared with controls, more markedly in the region adjacent to the omentum and less in areas farther from the omentum. Furthermore, experimental kidneys showed fewer myofibroblasts (by α-SMA immunostaining) in the interstitial area and reduced thickening of the glomerular and tubular basement membranes (by type IV collagen immunostaining).

To further understand the salutary effect of omental fusion, we studied the tissue at the fusion zone. The fusion zone showed absence of inflammatory cells, minimal fibrosis, good vascularization, and structural preservation of the parenchyma. Importantly, the fusion zone contained hematopoietic (Sca-1-positive), kidney-specific (WT-1-positive), and angiogenic progenitor (CD34-positive) cells. In contrast, the injured edge of control kidneys that lacked the omentum showed extensive fibrosis, sparse blood vessels, dedifferentiation of tubules, and absence of stem cells.

Consistent with the presence of progenitor cells adjacent to the injured kidney; we found high levels of paracrine factors VEGF, HGF, and IGF-1 in the experimental kidney. Whereas VEGF and HGF levels were consistently high, IGF-1 levels were 3-fold higher in the experimental kidney initially (1 week), but 2- to 3-fold lower than controls at later time points. Although IGF-1 has been shown to be a mitogenic factor, important for tissue repair, it has also been implicated in renal fibrosis in sustained high levels.26,27 Our finding of low levels of IGF-1 later in the experiment would seem to be in accordance with the reduced fibrosis that we observed in the experimental kidneys. The high paracrine growth factor levels in experimental kidneys also seemed to be responsible for the high level of cellular proliferation observed at the fusion zone. Of note, the growth factor levels were uniformly high in all parts of the subtotal nephrectomized kidney, although there was a gradient of cellular proliferation, high in the parenchyma near the omentum and lower away from the omentum. This was
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Indeed, experimental kidneys by the omentum compared with the rapid revascularization and hemostasis of the injured edge of the injured kidney. It is possible that this may be due to a progression of CKD by mostly rescuing the nephrons at the vicinity of the diseased kidney, have the potential to treat CKD. On the basis of these results, we speculate that the recovery of function in our model depended not only on the proximity of the kidney parenchyma to the omentum but also on the diffusible factors from the omental stem cells.

In summary, slowing the progression of CKD by fusing activated omentum to the diseased kidney supports the concept that adult stem cells, especially when placed in the vicinity of the diseased kidney, have the potential to treat CKD. Clearly, omental fusion in humans as a surgical maneuver is not a practical solution to treat CKD. However, our study does suggest an alternative source of adult stem cells derivable from the omentum as being effective in treating CKD. On the basis of our findings, we are also tempted to recommend that after

Figure 8. Kidney histology showing different grades of tubular injury at week 12 after inducing CKD in rats (trichrome staining). Arrows in A–C show fields of interstitial expansion, fibrosis, and tubular dilation. (A) Representative tubulointerstitial area showing a histology score of 1+ (area of interstitial expansion, fibrosis, and tubular dilation involving <25% area of the total field). Arrows show areas of interstitial expansion. (B) Representative tubulointerstitial area showing a histology score of 2+ (lesion area between 25% and 50% of the total field). Left arrow shows interstitial fibrosis and the right arrow shows a dilated tubule. (C) Representative tubulointerstitial area showing a histology score of 3+ (lesions involving 50%–75% of the total field). Arrows show dilated tubules. (D) Tubular injury index at week 12 in experimental rats compared with control rats. Compared with controls, experimental rats showed 46% less tubulointerstitial injury in the areas near the omentum, 19% less in areas farther from the omentum, and no effect in areas farthest from the omentum. However, none of these differences were statistically significant. Data are expressed as the mean ± SEM. n=8 in each group. Scale bar, 100 μm.

possibly because the injured tissue in the fusion zone could be more responsive to the growth factors than the more remote parenchyma that did not suffer as much damage. There was additional evidence that the injured kidney was responding to a milieu of growth factors in that the WT-1 gene was upregulated 5- to 10-fold and WT-1–positive podocytes in the glomeruli were upregulated 2-fold in the experimental kidney. Conceivably, the high growth factor levels in the kidney were due to direct diffusion from the attached omental tissue or to induction of such factors in the kidney by diffusion of other bioactive proteins from the omentum.

From our results of the regional differences in the glomerular and tubular injury in the experimental kidneys, it appears that the omental fusion may have been instrumental in slowing the progression of CKD by mostly rescuing the nephrons at the edge of the injured kidney. It is possible that this may be due to a rapid revascularization and hemostasis of the injured edge of experimental kidneys by the omentum compared with the injured edge of control kidneys lacking the omentum. Indeed, it is well known that the omentum, in addition to being a source of growth factors and stem cells, brings about tissue repair by readily vascularizing the injured organs with which it fuses.

There has been a growing recognition that podocytes, among the glomerular cells (mesangial and endothelial cells being others), are the key to maintaining the health of the glomerulus. This concept is based on experimental data showing that podocytes cannot be replaced after injury in normal adults, whereas endothelial and mesangial cells are able to regenerate. It is possible that the rescue of podocytes in our model, as reflected by the increase in WT-1–positive cells in the glomeruli, may have contributed to the preservation of kidney function. In accordance with the podocyte theory of glomerular health, several glomerular diseases have been associated with podocyte damage or depletion.

In our experiments, among other changes, omental fusion also resulted in repopulation of such podocytes that are so crucial for glomerular health.

It should be emphasized that among the many changes we noted in the experimental kidney, some of the changes (glomerulosclerosis, tubular injury, and BrdU) were regionally distributed, whereas others (α-SMA, type IV collagen, and changes in WT-1 protein and gene expression as well as growth factor levels) were uniformly distributed throughout the entire kidney parenchyma. On the basis of these results, we speculate that the recovery of function in our model depended not only on the proximity of the kidney parenchyma to the omentum but also on the diffusible factors from the omental stem cells.

In summary, slowing the progression of CKD by fusing activated omentum to the diseased kidney supports the concept that adult stem cells, especially when placed in the vicinity of the diseased kidney, have the potential to treat CKD. Clearly, omental fusion in humans as a surgical maneuver is not a practical solution to treat CKD. However, our study does suggest an alternative source of adult stem cells derivable from the omentum as being effective in treating CKD. On the basis of our findings, we are also tempted to recommend that after
partial nephrectomy in humans, the omentum could be brought in contact with the remnant kidney to preserve and possibly improve its function.

CONCISE METHODS

Induction of CKD in Rats and Mobilization of Omentum

All animal experiments were conducted according to the protocol approved by the Institutional Animal Care and Use Committee of the John H. Stroger Jr. Hospital of Cook County. Briefly, 12-week-old male Sprague-Dawley rats (body weight 300–350 g) were laparotomized under general anesthesia and underwent right nephrectomy and surgical excision of the upper and lower poles of the left kidney (5/6 nephrectomy). The removed kidney and the excised poles were weighed to ascertain the removal of 5/6 of the total kidney mass. This is a well established model in rats to induce progressive CKD without causing systemic hypertension.37,38 The nephrectomized rats were then divided into two groups. In the omentum group (experimental), before suturing the rats, 5 ml of polydextran particle slurry (Sephadex G-25 course, particle size \( \leq 100 \) \( \mu \)M; Sigma-Aldrich, St. Louis, MO) (1:1 in normal saline) was introduced into the abdominal cavity to mobilize the omentum and to facilitate its fusion to the injured kidney as well as to aid us in histologically identifying the injured edge of the kidney. The omentectomized group consisted of rats that were subjected to 5/6 nephrectomy and simultaneous complete omentectomy to prevent omentum from fusing to the injured kidney. Note that this group did not receive the polydextran particle slurry. The rats were maintained on normal rat chow and water ad libitum. At weekly intervals, rats were weighed and bled via tail vein to obtain blood samples. Rats were euthanized at day 3 and weeks 6 and 12 after inducing CKD. At euthanasia, depending on the experiment, the remnant kidney was either cleared of the fused omentum and weighed before further biochemical processing or the attached omentum was left intact with the kidney tissue for histologic examination.

Other Controls

Additional controls were set up as follows. First, we utilized 5/6 nephrectomized rats without polydextran \((n=6)\) to determine whether fused unactivated omentum alters the course of CKD. The results were inconclusive in these rats because the omentum failed to fuse with the injured kidney in 2/6 rats and the omentum only fused to the upper pole and not to the lower pole of the injured kidney in 3/6 rats. Second, rats having normal kidneys (without nephrectomy) but with polydextran-activated omentum \((n=4)\) were utilized to examine whether activated...
omentum itself alters renal function or structure. In these rats, no histologic abnormalities were observed in the kidney and renal function remained normal for up to 6 weeks. Third, 5/6 nephrectomized rats were subjected to omentectomy and given polydextran \((n=4)\). In these rats, we observed the same functional and structural changes by 12 weeks as observed in our main controls without the polydextran. Of note, some of the polydextran particles in these animals appeared to be incorporated in the epididymis and the mesentery.

**Measurement of Renal Function**
Renal functional parameters, plasma creatinine, plasma urea nitrogen, creatinine clearance, urine output, and proteinuria were measured weekly. Plasma and urine creatinine were measured by colorimetric Jaffe reaction (Sigma Diagnostics, Inc., St. Louis, MO). Plasma urea nitrogen was calculated from the measurement of urea, which was assayed using an improved colorimetric Jung method that uses a chromogenic reagent that forms a colored complex with urea, measurable at A520 (BioChain Institute, Inc., Hayward, CA). Proteinuria and urine output were measured in 24-hour urine collection, during which food was withdrawn. Proteinuria was quantified by sulphasalicylic acid method using HSA as a standard. Creatinine clearance was expressed in milliliters per minute per 100 g of body weight.

**Assessment of Cell Proliferation by BrdU Incorporation**
BrdU (100 mg/kg in 1 ml saline) (Sigma-Aldrich) was injected subcutaneously in rats every day for 3 days after subtotal nephrectomy. After termination, kidney tissues were fixed and processed for immunofluorescence as described below.

**Processing of Tissue for Histology and Biochemical Assays**
Harvested kidneys were split transversely into equal halves. One half with the omental attachments on the upper and lower poles was processed for histology. Histologic analyses were performed on three zones of the kidney: zone 1 (near the omentum), which represented approximately 2 mm of kidney tissues from the upper and lower omental fusion points; zone 2 (far from omentum), which represented approximately 2 mm of kidney tissue deeper from the upper and lower zone 1 tissues; and

![Figure 11.](image-url) Immune staining for BrdU in experimental kidney tissue fused to the omentum at day 3 after inducing CKD in rats. (A) BrdU-labeled cells in highest numbers in the part of the kidney near to the omentum (zone 1). Careful examination shows that tubular cells, interstitial area, and glomeruli are labeled (red arrows point to glomeruli on the sections). (B) BrdU-labeled cells in fewer numbers in the kidney farther from the fused omentum (zone 2). (C) BrdU-labeled cells in minimal numbers (background levels) in the kidney farthest from the omentum (zone 3). Control CKD kidney as well as normal adult kidney show a background level of BrdU staining as shown in C (not shown). Scale bar, 100 \(\mu m\).

![Figure 12.](image-url) Expression of WT-1 protein in glomeruli and WT-1 gene expression in kidney extracts. (A) Representative picture of WT-1–positive cells in the glomeruli of a normal rat kidney by immunoperoxidase staining (brown nuclear stain). (B) WT-1–positive cells in the glomeruli of control rats at 2 weeks. (C) WT-1–positive cells in the experimental rat glomeruli 2 weeks after inducing CKD. It is important to point out that no regional differences in WT-1 staining in the three areas of the kidney (near, farther, farthest from omentum) are observed. Also note that the diffuse brown staining of the tubules is a nonspecific reaction. Compared with normal (A) or control rat glomeruli (B), the glomeruli of experimental rats (C) show an increase in WT-1–positive cells. (D) Expression of WT-1 mRNA in control and experimental rat extracts by RT-PCR. It is important to point out that in the initial testing, no regional differences in WT-1 expression are observed among the three regions of the kidney (near, farther, farthest from omentum); therefore, the data from the three regions are pooled for this figure. The top panel shows the WT-1 and \(\beta\)-actin bands and the bottom panel shows the densitometric quantitation of the WT-1 band expressed as a ratio of WT-1/\(\beta\)-actin. Controls are similar at 2 and 4 weeks. The figure shows the 2-week control assumed as 1. WT-1 mRNA is found to increase by 5-fold and 10-fold at the 2-week and 4-week time points, respectively, in experimental rat kidneys \((n=12\) tissues) compared with controls \((n=4\) tissues). *P<0.05 compared with controls; ^P<0.05 compared with the 2-week time point. Scale bar, 100 \(\mu m\).
Figure 13. Number of WT-1 cells in glomeruli of experimental (EXP) and control (Cont) rat kidneys expressed as the number of WT-1–positive cells/unit glomerular area. A unit of glomerular area is taken as 10^5 square pixels. The number of WT-1 cells in 2- and 4-week experimental kidneys is 1.6- to 1.8-fold higher than in controls or normal healthy rats. *P<0.05 compared with normal and control groups; #P<0.05 compared with the 2-week control group. n=60 glomeruli in the normal and control groups and n=80 glomeruli in the experimental groups.

zone 3 (farthest from omentum), which represented the remaining deeper region of the kidney tissue as depicted in Supplemental Figure 1.

The other half was cleared of the omental attachment from both poles and subsequently resected into five pieces for WT-1 gene activation and growth factor assays as follows: two 2-mm pieces were collected from the poles (near the omentum), two 2-mm pieces from deeper tissue from both upper and lower aspects of the kidney (far from omentum), and the remaining approximately 4-mm middle piece (farthest from omentum) (depicted in Supplemental Figure 1). Note that omental tissue is easily recognized by its white color compared with the reddish-brown color of the remnant kidney and therefore could be completely removed from the remnant kidney.

Histopathology
Kidney tissues were fixed in 4% formalin and embedded in paraffin. Kidney sections 4–6 μM in thickness were stained with hematoxylin and eosin, periodic acid–Schiff, and trichrome stains and were observed under an optical microscope. Assessment of glomerulosclerosis and tubular injury was performed using the semiquantitative scale described by Cao et al.39 Briefly, glomeruli in each kidney tissue were visualized at a magnification of ×200 and graded as follows: 0, normal; 1+, mesangial expansion and slight glomerular damage involving <25% of the glomerulus; 2+, mild sclerosis involving 25%–50% of the glomerulus; 3+, moderate sclerosis involving 50%–75% of the glomerulus; and 4+, severe sclerosis involving >75% of the glomerulus (see Figure 7, A–D, for representative pictures). In control rats, an initial analysis of tubular injury in the three zones (near, farther, farthest from omentum) to arrive at a mean for each region of each rat. The overall tubular injury index (mean±SEM) was computed from the individual means of all of the rats in the group.

Tubular injury was visualized at magnification of ×100 and graded fields as follows: 0, normal; 1+, area of interstitial inflammation, fibrosis, and tubular dilation with cast formation (lesion) involving <25% of the field; 2+, lesion involving 25%–50% of the field; 3+, lesion involving 50%–75% of the field; and 4+, lesion area involving >75% of the field (see Figure 8, A–C, for representative pictures).

In control rats, an initial analysis of tubular injury in the three zones of the kidney showed an absence of regional differences; therefore, the tubular injury index was determined by random scoring of the tubular areas. Tubular injury was scored from 25 random fields per rat and the total score was averaged to arrive at a mean for each rat. In experimental rats (n=8), regional tubular injury was scored from 4–6 random fields per rat from each of the three regions (near, farther, farthest from omentum) to arrive at a mean for each region of each rat. The overall tubular injury score (mean±SEM) was computed from the individual means of all of the rats in the group.

To determine the pattern of glomerulosclerosis in the three regions of the experimental kidneys, the percentage of glomeruli exhibiting different levels of glomerulosclerosis (0, 1+, 2+, 3+, 4+) in each of the three zones (near, farther, farthest from omentum) were computed. For this analysis, a total of 120–160 glomeruli were scored from each region of experimental rats (n=8).

Histochemistry
Both immunofluorescence and immunoperoxidase techniques were utilized in the study using paraffin-embedded kidney sections. The sections were deparaffinized before staining. Sections were then treated with citrate buffer (pH 6) at 100°C for 10 minutes for antigen retrieval. For immunofluorescence, sections were incubated with the primary antibody (4°C overnight) followed by an appropriate secondary antibody conjugated to FITC (room temperature for 1 hour). After washing, the slides were either wet mounted in PBS/glycerol (1:1) or in 4’,6-diamidino-2-phenylindole containing mounting medium (to stain cell nuclei blue) for microscopic examination. For immunoperoxidase staining, the sections were incubated with the respective primary antibody (4°C overnight) followed by incubation with corresponding biotin–conjugated secondary antibodies and finally with the ABC kit (Vector Laboratories, Burlingame, CA). The reaction was detected by incubation of slides in diaminobenzidine-hydrogen peroxide solution (Vector Laboratories). The slides were counterstained with hematoxylin and mounted with Permount mounting medium. Negative controls consisted of sections in which the primary antibodies were omitted. All slides were examined and digitally photographed using an epifluorescent/optical microscope (Nikon, New York, NY). The following primary antibodies were used: mouse anti–α-SMA (tubulointerstitial fibrosis marker), mouse anti-βActin (cell proliferation marker), goat anti-vimentin (a marker of epithelial to myofibroblast transformation) (Sigma-Aldrich); goat anti-type IV collagen (basement membrane marker) (SouthernBiotech, Birmingham, AL); mouse anti–WT-1 (renal mesenchymal stem cell
Figure 14. Levels of growth factors VEGF, HGF and IGF-1 in control and experimental kidney. It is important to point out that in the initial testing, no regional differences in any of the three growth factors are observed among the three regions of the experimental kidneys (near, farther, farthest from omentum); therefore, the data from the three regions are pooled for these figures. (A) VEGF progressively increases by 3- to 5-fold in the experimental kidney during 4 weeks after inducing CKD compared with control as well as to normal healthy kidneys. *P<0.05 compared with normal and all control groups; †P<0.05 compared with the week 1 experimental group. (B) HGF decreases slightly in the experimental group in the first week but increases by 40% above the respective control and normal kidney by 4 weeks. *P<0.05 compared with the week 4 control and with week 1 experimental groups; †P<0.05 compared with normal and week 1 control. (C) IGF-1 increases in the experimental group by 6-fold in the first week compared with its respective control and 3-fold compared with normal kidney but then decreases significantly below control and normal kidney levels after 1 week. *P<0.05 compared with normal, week 2, and week 4 experimental groups and all controls; †P<0.05 compared with normal and week 4 control; ‡P<0.05 to their respective controls. n=6 tissues each in normal, control, and experimental groups. C, control kidney; E, experimental kidney; N, normal healthy kidney.

Glomerular WT-1 Cell Counting
After the kidney sections were immunostained for WT-1, glomeruli were digitally photographed at ×200 magnification. WT-1-positive cells in each glomerulus including at the Bowman’s capsule were manually counted. At the same glomerular area (including the Bowman’s capsule) was measured using ImageJ software. The ratio of the count/area (in square pixels) was computed for each glomerulus. The mean of the ratios of WT-1 count/area in each group was used for comparison among various groups.

For statistical comparison, 60 random glomeruli were evaluated from rats in the control group (n=4) and 80 glomeruli from the experimental group (n=4), representing the three regions of the kidney tissue. After an initial statistical analysis on regional differences in experimental rat kidneys showed lack of significant regional differences, the quantitative data of the three regions were pooled for comparison with controls.

Quantification of WT-1 cells in the Bowman’s capsule was similarly determined from the manual counts of the WT-1-positive cells in the Bowman’s capsule and the corresponding linear length of the Bowman’s capsule (in pixels). In the absence of regional differences in the experimental animals, the data of the three regions were pooled for comparison with controls.

Quantification of mRNA for WT-1 by RT-PCR
Different regions of the control kidney tissue (n=4) and experimental tissue (n=4) (after removal of the attached omentum) were used for total RNA isolation and RT-PCR for WT-1 gene and β-actin. The RT-PCR procedure was carried out using the Invitrogen System (Invitrogen, Carlsbad, CA) (amplification: 30 cycles as confirmed to be on a linear slope of product formation). The RT-PCR products were quantified as the ratio of WT-1 gene band density/β-actin band densities performed by image analysis using MIPAV software (JAVA imaging software inspired by the National Institutes of Health). In the absence of regional differences in the control and experimental animals, the data of the three regions were pooled for comparison with controls.

The following forward and reverse primer sequences were used: (1) WT-1: forward (nucleotide 1059) 5’-TGAGAACAATACCGGATGTGAC-3’, reverse (nucleotide 458) 5’-GTAGGTGAGGAAGGAAAATTTC-3’ (predicted size 400 bp; accession number NM_031534; Entrez gene ID 24883); (2) β-actin: forward: (nucleotide 926) 5’-TACGAGTGTGACAGGTGACATCCGT-3’, reverse (nucleotide 1210) 5’-CCTAGAACAGATTCGCGGTGCACGATG-3’.
Measurement of Growth Factors VEGF, HGF, and IGF-1 in Kidney Extracts

Different regions of the control kidney tissue and experimental tissue (after removal of the attached omentum) were placed in ice-cold tubes with PBS and extracted using a sintered glass homogenizer. The extracts were centrifuged at 15,000 × g for 30 minutes and the supernatants were saved at −20°C. Protein concentration was measured in the extracts using Bio-Rad reagent (Bio-Rad Laboratories, Hercules CA) and subsequently adjusted to 1 mg/ml using PBS for the growth factor assays. Assays were carried out using sandwich ELISA kits for murine VEGF, HGF, and IGF-1 supplied by R&D Systems. In initial analysis, no regional differences in growth factor levels were found in the control and experimental animals; therefore, the data of the three regions were pooled for comparison with controls.

Statistical Analyses

The data were expressed as mean ± SEM. Data from the different groups of rats were analyzed by a t test or in case of multiple groups with ANOVA using the Kruskal–Wallis test (with Dunn’s multiple comparisons post test) using GraphPad Instat 3.0. software (GraphPad Software, Inc., La Jolla, CA). The level of statistical significance was set at P < 0.05.

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DISCLOSURES

None.

REFERENCES


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Supplementary Results

Body weight, kidney/body weight ratios, urine output and proteinuria

The average body weight gain was slightly higher in experimental rats as compared to controls; however the difference was not statistically significant (not shown). Even though the kidney weight/body weight ratio was 20% higher in the experimental group (33.3 ± 2.8 g/g) than in the control group (27.1 ± 2.4 g/g; N = 8 in each group), the difference did not reach statistical significance. There was also no difference in urine output between the two groups, and both groups showed moderate proteinuria by week 12 (35-40 mg/day; normal rat proteinuria 5-7 mg/day) (weekly proteinuria data is shown in Supplemental Table 1).

Creatinine clearances (comparison between week 6 and week 12 time points)

Between week 6 and 12, creatinine clearance decreased in both groups, although more significantly in the control group (38%; P<0.05) versus only 18% in the experimental group (non-significant).

Glomerulosclerosis injury and tubular injury indices in different regions of control kidneys

Glomerulosclerosis indices

Near omentum: 2.6 ±0.14  
Far from omentum: 2.2 ± 0.3  
Farthest from omentum: 2.3 ± 0.19  
N = 8  
P = non-significant between any two groups.

Tubular injury indices

Near omentum: 1.4 ± 0.3  
Far from omentum: 1.1 ± 0.2  
Farthest from omentum: 0.9 ± 0.3  
N = 8
P = non-significant between any two groups.

**Quantitative data of α- SMA and type IV collagen staining**

α- SMA (percent stained areas): normal 0.07 ± .02; control 10.2 ± 1.6; experimental 1.1 ± 0.08 (n=5; control versus experimental P<0.05).

Type IV collagen (percent stained areas): normal 0.2 ± .01; control 14.4 ± 3.1; experimental 2.8 ± 1.3 (n=5; control versus experimental P<0.05).
Supplemental Table 1

Weekly plasma creatinine, plasma urea nitrogen and proteinuria in control and experimental rats

<table>
<thead>
<tr>
<th>Time (Week)</th>
<th>Plasma creatinine (mg / dL)</th>
<th>Plasma urea nitrogen (mg / dL)</th>
<th>Proteinuria (mg / 24 hours)</th>
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<tr>
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<td>Control</td>
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<td>12</td>
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<td>1.6 ± 0.04</td>
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</table>

Values are expressed as mean ± standard error of mean (SEM); N = 8 in each group.
Supplemental Fig. 1. Processing of the kidney tissue for the purpose of regionally analyzing histological and biochemical findings. The kidney with the attached omentum at the upper and lower poles was longitudinally sectioned into two equal halves. One half with the two attached omental tissues was processed for histology. The tissue sections were histologically evaluated according to the three zones representing distance from the omentum (near, farther, farthest). The other half was first cleared of the omental tissue completely (omental tissue is identifiable as a white tissue in contrast to the red/brown color of the kidney tissue). Subsequently the kidney tissue was excised into the five pieces as shown. The two pieces of zones 1 (as well as of zone 2) were pooled to finally obtain three tissue extracts corresponding to regions ‘near omentum’ (zone 1), ‘farther from omentum’ (zone 2) and ‘farthest from omentum’ (zone 3). The three extracts were used for the WT-1 gene expression and growth factor assays.
**Supplemental Fig. 2.** Glomerular areas measured in the control (Cont) and experimental tissues (EXP) . Note that the glomerular areas measured in the experimental kidneys were found not to be regionally different and therefore the data were pooled for presentation in this figure. They are presented at week 2 and week 4 time points at which the Wt-1 cell data (shown in Fig. 13) was collected. Data are expressed as mean ± SEM. * denotes statistical significance at P<0.05 compared to all other groups. N = 60 glomeruli in normal and control groups and N = 80 glomeruli in the experimental groups. No particular trend in changes of glomerular areas was observed. For example, at week 2 the experimental glomeruli were smaller than the control glomeruli, while at week 4 the experimental glomeruli did not differ significantly from the control glomeruli.
Supplemental Fig. 3. Wt-1 positive cells in the Bowman’s capsule (parietal epithelial cells) in the control (Cont) and experimental tissues (Exp). Note that the data of the experimental kidneys were found not to be regionally different and therefore the regional data were pooled for presentation in this figure. They are presented at week 2 and week 4 time points at which the Wt-1 cell data (shown in Fig. 13) was collected. Data are expressed as mean ± SEM. N = 60 glomeruli in normal and control groups and N = 80 glomeruli in the experimental groups. Data are presented as Number of Wt-1 positive cells/linear length of the Bowman’s capsule. A unit of linear length was taken as 1000 pixels. Results showed that even though there is a slight increase in the number of Wt-1 positive parietal epithelial cells in the Bowman’s capsule in the experimental group at week 2 and week 4 the difference between experimental and control groups did not attain statistical significance.