Telomere Shortening Reduces Regenerative Capacity after Acute Kidney Injury

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ABSTRACT

Telomeres of most somatic cells progressively shorten, compromising the regenerative capacity of human tissues during aging and chronic diseases and after acute injury. Whether telomere shortening reduces renal regeneration after acute injury is unknown. Here, renal ischemia-reperfusion injury led to greater impairment of renal function and increased acute and chronic histopathologic damage in fourth-generation telomerase-deficient mice compared with both wild-type and first-generation telomerase-deficient mice. Critically short telomeres, increased expression of the cell-cycle inhibitor p21, and more apoptotic renal cells accompanied the pronounced damage in fourth-generation telomerase-deficient mice. These mice also demonstrated significantly reduced proliferative capacity in tubular, glomerular, and interstitial cells. These data suggest that critical telomere shortening in the kidney leads to increased senescence and apoptosis, thereby limiting regenerative capacity in response to injury.


As the population ages, the number of elderly patients with chronic kidney disease is expected to grow, making kidney aging an issue of great clinical importance. Old kidneys show a decline in function as reflected by increased renal vascular resistance, reduced renal plasma flow, and increased filtration fraction.1 Morphologic changes include cortical thinning2 and a histology indicating deterioration, such as tubular atrophy, interstitial fibrosis, and glomerulosclerosis.3 In the aging population, acute kidney injury is significantly more common, the clinical course is more severe, and kidney function is less likely to recover.4 The elderly are more likely to develop end-stage renal failure than the young.5 Donor age is one of the most important predictors of long-term graft survival, and older donor kidneys are more likely to fail when they experience rejection, even when the rejection is relatively mild.6 Taken together, the phenotype of renal aging is described not only by the loss of function and mass but also by the loss of an appropriate response toward injury.

Telomere shortening and dysfunction are crucial determinants for human lifespan and the re-
diseases associated with aging, such as cardiovascular disease, ulcerative colitis, liver cirrhosis, and infections, show accelerated telomere shortening. A correlation between telomere length and risk for death from heart disease or infections has been reported. Laboratory rodents have long telomeres relative to humans. Murine telomere length can be reduced to a “human length” by serial intercrossing of Terc−/− mice. Late-generation Terc−/− mice with critically short telomeres show defects in homeostasis of proliferative organs, but these are not the only organ systems that are affected by premature aging phenotypes. Liver regeneration after acute and chronic stresses is diminished in late-generation Terc−/− mice with short telomeres (G4) compared with G1 Terc−/− and wild-type Terc+/+ mice. Greater damage in G4 Terc−/− is accompanied by an increase in critically shortened telomeres, higher expression of the downstream mediator p21, increased apoptosis, and reduced cellular regeneration measured by proliferation marker Ki-67.

RESULTS

Histopathology of the Kidney after IRI

Acute tubular damage assessed for the cortical and corticomedullary region was highest on days 1 and 3 in Terc+/+, G1 Terc−/−, and G4 Terc−/− (Figure 1, A and B). On day 3, G4 Terc−/− showed significantly more damage when compared with Terc+/+ and G1 Terc−/−. On days 7 and 30, acute tubular damage decreased, ranging approximately 10%, without significant differences between the groups.

Histopathologic alterations classified as chronic tubular damage were observed starting from day 7 and significantly increased by day 30 in Terc+/+, G1 Terc−/−, and G4 Terc−/− (Figure 1C). There was a tendency toward a higher incidence in G4 Terc−/−, but this was significant only when the corticomedullary junction was assessed separately (P = 0.01 for the comparison of G4 Terc−/− with Terc+/+ on day 30; data not shown).

Interstitial fibrosis was seen only on day 30 in Terc+/+, G1 Terc−/−, and G4 Terc−/− with significantly higher levels in G4...
Terc−/− kidneys (Figure 1, D and E). Sham-operated and nonoperated controls showed neither acute or chronic tubular damage nor any interstitial fibrosis (data not shown).

Renal Expression of Connective Tissue Growth Factor after IRI

Connective tissue growth factor (Ctgf) is recognized as an important player in fibrogenic pathways. Because of the significant increase in interstitial fibrosis in G4 Terc−/− kidneys, we performed Western analysis for Ctgf in a subgroup of animals on days 7 and 30 after IRI (Figure 2). For day 7, we saw no significant differences among the three groups (Figure 2, A and B). Highest values for Ctgf were seen for G1 Terc−/− and G4 Terc−/− kidneys on day 30, but the differences between groups were NS (Figure 2, A and C).

Telomere Quantitative Fluorescence In Situ Hybridization Analysis

We analyzed telomere length in a subgroup of animals by quantitative fluorescence in situ hybridization (Q-FISH) analysis (Figure 3, A and B). In nonoperated controls, mean telomere fluorescence intensity (TFI) in tubular cells from Terc+/+ kidneys was significantly higher when compared with G4 Terc−/− kidneys (99.7 versus 73.8; P < 0.05). Analysis of kidney sections from day 30 after IRI revealed that in both groups, the injury led to significant telomere shortening (TFI at d30 53.8 [in Terc+/+] versus 44.5 [in G4 Terc−/−]). Even though the difference in mean TFI was no longer significant, the percentage of critically shortened telomeres, defined as a TFI ≤40, was significantly higher in G4 Terc−/− kidneys (48.6 versus 14.0% in Terc+/+; P < 0.001).

p21, p16INK4a, and p53 mRNA Expression in the Kidney after IRI

There was a significant increase in cell-cycle inhibitor p21 mRNA expression in Terc+/+, G1 Terc−/−, and G4 Terc−/− kidneys for all time points after IRI when compared with sham-operated and nonoperated controls (Figure 3C). Apart from day 1, G4 Terc−/− showed significantly higher levels of p21 when compared with either Terc+/+ or G1 Terc−/−. This was also true for G4 Terc−/− controls that were sham-operated, and the same tendency was seen in nonoperated animals.

Cell-cycle inhibitor p16INK4a showed a continuous increase after IRI in Terc+/+, G1 Terc−/−, and G4 Terc−/− kidneys with significantly higher levels on day 30 compared with sham-operated and nonoperated controls (Figure 3D). Apart from day 30, significantly higher values of p16INK4a were seen in G4 Terc−/− kidneys when compared with either Terc+/+ or G1 Terc−/−. Even though the higher values seen now for G4 Terc−/− and G1 Terc−/− kidneys compared with Terc+/+ animals were no longer significant after post hoc testing with Bonferroni correction (P = 0.07 and P = 0.09, respectively).

p53 mRNA levels were significantly upregulated after IRI on days 1 and 3 in Terc+/+, G1 Terc−/−, and G4 Terc−/− mice compared with sham-operated controls (Supplemental Figure 1) and continuously decreased with time. Apart from day 3, for which G4 Terc−/− kidneys showed higher values when compared with Terc+/+ and G1 Terc−/−, there were no statistically significant difference among the three groups.

Proliferation and Apoptosis after IRI

Proliferation was measured using Ki-67 immunostaining. Quantification of positively stained nuclei revealed significantly lower proliferation rates in tubular and interstitial cells of G4 Terc−/− kidneys on day 3 (Figure 4A) and in tubular, glomerular, and interstitial cells of G4 Terc−/− kidneys on day 30 (Figure 4B). As previously shown by our group20 and other investigators,21 we confirmed very low proliferation rates in renal cells of sham-operated (Figure 4C) and nonoperated controls (data not shown), without significant differences among the three groups. Representative stainings are shown in Figure 4D.

Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (Figure 4E). On days 3 and 30 after IRI, G4 Terc−/− kidneys showed a significantly higher number
of TUNEL-positive tubular and interstitial cells compared with Terc\(^{+/+}\). The same was true for the comparison of G4 Terc\(^{-/-}\) with G1 Terc\(^{-/-}\) kidneys, with the exception of interstitial cells 3 d after IRI (Figure 4, F and G). As expected, sham-operated controls showed very low numbers of apoptotic tubular and interstitial cells, and no apoptotic cells were seen in glomeruli (Figure 4H). No statistical differences were found between controls.

Inflammatory Response after IRI
We characterized the infiltrating cell populations using CD3 (T lymphocytes) and CD68 (macrophages) immunostaining at days 3, 7, and 30 (Table 1). For CD3\(^{+}\) cells, we found no differences among the three groups at days 3 and 7; however, on day 30, the amount of CD3\(^{+}\) cells was significantly lower in G4 Terc\(^{-/-}\) when compared with G1 Terc\(^{-/-}\) kidneys. The

Increases in CD3\(^{+}\) cells over time, which were seen for all three groups, were significant only for Terc\(^{+/+}\) (ANOVA \(P = 0.009\)) and G1 Terc\(^{-/-}\) (ANOVA \(P < 0.001\)) animals. The number of CD68\(^{+}\) cells was higher when compared with CD3\(^{+}\) cells. Comparing the three groups, we found differences only at day 7, with less CD68\(^{+}\) cells in G1 Terc\(^{-/-}\) when compared with G4 Terc\(^{-/-}\). This difference was no longer seen at day 30. There seemed to be no tendency for CD68 infiltrating cells over time, even though ANOVA became significant for the group of G1 Terc\(^{-/-}\) (\(P = 0.009\)) mice because of the low number of cells at day 7.

Kidney Function after 15 Min of IRI to the Left Kidney and Right Nephrectomy
We performed additional IRI experiments using 15 min of ischemia to the left kidney followed by nephrectomy of the
right kidney. Longer ischemia times had led to death in G4 Terc−/− animals. On day 30, creatinine clearances in G4 Terc−/− mice were significantly lower compared with Terc+/+ (Table 2). A similar, almost significant tendency had been seen for the comparison at day 7. No significant differences were seen for nonoperated or sham-operated control animals.

**DISCUSSION**

Critically short telomeres in kidneys of late-generation Terc−/− mice increase the susceptibility to acute cell death and reduce long-term regenerative capacity of the kidney after IRI. Telomere attrition is observed with increasing age in human tissues, including the kidney, and is accelerated in various age-associated human diseases (for review, see reference[2]). Our study provides a mechanistic approach by investigating the effect of critically short telomeres on kidney regeneration after acute replicative stress. Reduced long-term regeneration in G4 Terc−/− mice was reflected by more chronic deterioration and reduced organ function. Significantly lower creatinine clearances in G4 Terc−/− compared with Terc+/+ were seen 30 days after IRI, and G4 Terc−/− mice did not show compensatory hypertrophy of the right kidney after unilateral clamping without contralateral nephrectomy. The excess in acute damage after IRI in late-generation Terc−/− reflects the reduced ability of cells with critically short telomeres to respond adequately toward stresses that under normal circumstances result in increased proliferation. The pronounced chronic deterioration found in G4 Terc−/− with increased interstitial fibrosis on day 30 supports this and argues for a loss of parenchyma as a result of absent tubular regeneration during early days, resulting in replacement of lost cells by fibrotic tissue. Our results are in line with studies in liver from late-generation Terc−/− mice showing reduced regenerative capacity after acute stress through genetic, surgical, and chemical ablation of liver cells.[22]

DNA damage signaling in response to telomere dysfunction induces transient cell-cycle arrest, senescence, or apoptosis, depending on cell type and severity of stress.[23] In late-generation Terc−/− mice, apoptosis is the dominant phenotype in thymic lymphocytes and male germ line,[13,24] whereas cell-cycle arrest/senescence is the major checkpoint limiting liver regeneration.[15] In our model of renal IRI, G4 Terc−/− kidneys show significantly higher numbers of TUNEL-positive cells, but with a maximum of only 1 to 3% apoptotic cells, it seems that senescence pathways are driving the failure of kidney regeneration seen in G4 Terc−/−. Most senescent cells remain present and are not simply lost. The phenotype of senescent fibroblasts in culture is associated with a typical expression pattern: Increases in cell-cycle regulators such as p16INK4a and p21 and extracellular matrix proteins (e.g., collagen, fibronectin) as well as decreases in genes that are involved in cell-cycle progression. Senescent cells secrete degradative proteins, inflammatory cytokines, and growth factors (e.g., Ctgf[25]) that may promote tissue aging and possibly possess a complex role in chronic disease processes.[26] We previously showed that senescence features correlate with the presence of interstitial fibrosis in aged human kidneys, and it is conceivable that interstitial fibrosis simply reflects the accumulation of fibroblasts that have turned senescent.
Significantly higher levels of p21 in G4 Terc−/− controls are in line with the concept of an increased induction of senescence as a result of dysfunctional telomeres even before any injury.27,28 Telomere dysfunction leads to formation of DNA-damage foci that activate ATM/ATR signaling,29 and a downstream cascade stabilizes p53 and, beside others, upregulates p21.6 The cyclin-dependent kinase inhibitor p21 mediates p53-dependent and -independent induction of senescence.30 p21 deletion can prolong the lifespan of human cells31 and of mice with dysfunctional telomeres.8 Our finding of increased p21 expression after IRI reinforces observations showing p21 induction after IRI.30 The upregulation of p21 is more pronounced in G4 Terc−/− mice probably as a result of more telomeres becoming critically shortened. The concept of an increase in senescent cells in G4 Terc−/− mice is further confirmed by reduced proliferation 3 and 30 days after IRI. p53 has been shown to be upregulated in some cell types after IRI, but 30 days after IRI, there was no longer a difference between expression levels in kidneys from both generations of Terc−/− mice. p16INK4a is the key mediator for STASIS (stress or aberrant signaling induced senescence), a pathway that can induce senescence independent of telomere dysfunction.34 The interplay between replicative senescence and STASIS is not completely unraveled yet, but there are data suggesting connections under certain circumstances. As primary cells divide and telomeres shorten, p16INK4a levels progressively increase.35 p16INK4a can enforce a G1/S arrest in response to DNA damage,36 and telomere damage can also elicit a G1/S arrest through p16INK4a, especially in cells lacking p53 function.37 Our results may be explained by the observation that even the first generation of telomerase-deficient mice show a shortened lifespan,38 and one could speculate that telomere shortening per se, even in the absence of critically short telomeres, has an effect on regulation of p16INK4a. A reason for this could be the missing protection of telomeres through telomerase.7

Impaired regeneration in late-generation telomerase-deficient mice is also seen with regard to the immune system. Immunosenescence mainly affects the specific immune responses with a reduction of germinal center reactivity upon immunization and a reduced proliferative capacity of T and B cells.7,15 The increase in CD3+ cells that was significantly less pronounced in G4 Terc−/− mice confirms these observations; however, the unspecific immune response, as reflected by CD68-infiltrating cells, seemed to be conserved in G4 Terc−/− mice.

Another major regulator of senescence, the cell-cycle inhibitor p16INK4a, is progressively induced after IRI. p16INK4a expression was higher in G4 Terc−/− kidneys for the early days after IRI, but 30 days after IRI, there was no longer a difference between expression levels in kidneys from both generations of Terc−/− mice. p16INK4a is the key mediator for STASIS (stress or aberrant signaling induced senescence), a pathway that can induce senescence independent of telomere dysfunction.34 The interplay between replicative senescence and STASIS is not completely unraveled yet, but there are data suggesting connections under certain circumstances. As primary cells divide and telomeres shorten, p16INK4a levels progressively increase.35 p16INK4a can enforce a G1/S arrest in response to DNA damage,36 and telomere damage can also elicit a G1/S arrest through p16INK4a, especially in cells lacking p53 function.37 Our results may be explained by the observation that even the first generation of telomerase-deficient mice show a shortened lifespan,38 and one could speculate that telomere shortening per se, even in the absence of critically short telomeres, has an effect on regulation of p16INK4a. A reason for this could be the missing protection of telomeres through telomerase.7

### Table 1. Characterization of infiltrating cells after IRI in Terc mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Terc+/+</th>
<th>G1 Terc−/−</th>
<th>G4 Terc−/−</th>
<th>P, ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>9.1 ± 1.6</td>
<td>8.1 ± 0.9</td>
<td>6.8 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td>CD68</td>
<td>76.7 ± 18.9</td>
<td>129.5 ± 12.4</td>
<td>106.7 ± 8.1</td>
<td>NS</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>34.2 ± 9.4</td>
<td>25.6 ± 5.4</td>
<td>11.8 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>CD68</td>
<td>99.4 ± 5.5</td>
<td>70.6 ± 2.4</td>
<td>120.0 ± 11.3*</td>
<td>0.002</td>
</tr>
<tr>
<td>Day 30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>46.8 ± 8.0</td>
<td>62.1 ± 5.1</td>
<td>20.0 ± 7.6b</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>CD68</td>
<td>107.2 ± 14.8</td>
<td>121.4 ± 16.1</td>
<td>103.1 ± 17.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

*p = 0.001 for the comparison G1 Terc−/− and G4 Terc−/−. | |
| p = 0.003 for the comparison with G1 Terc−/− and P = 0.06 for the comparison with Terc+/+.

### Table 2. Kidney function in Terc+/+, G1 Terc−/−, and G4 Terc−/− mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Creatinine Clearance (ml/min)</th>
<th>P, ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nonoperated</td>
<td>0.145 ± 0.051 (n=6)</td>
<td>0.220 ± 0.024 (n=5)</td>
</tr>
<tr>
<td>sham-operated</td>
<td>0.197 ± 0.024 (n=3)</td>
<td>0.240 ± 0.031 (n=3)</td>
</tr>
<tr>
<td>Mice after IRI (15 min of ischemia to left kidney, right nephrectomy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>0.130 ± 0.012 (n=6)</td>
<td>0.110 ± 0.023 (n=7)</td>
</tr>
<tr>
<td>day 30</td>
<td>0.157 ± 0.017 (n=6)</td>
<td>0.151 ± 0.017 (n=7)</td>
</tr>
</tbody>
</table>

*p = 0.06 for the comparison G4 Terc−/− vs. Terc+/+. | |
| p < 0.05 for the comparison G4 Terc−/− vs. Terc+/+. |
to injury. We provide evidence that a reason for this restriction of old kidneys could be telomere shortening and dysfunction, leading to impaired replication and regeneration. The changes induced through the activation of certain senescence proteins contribute to the collapse of tissue integrity, leading to a disturbed organ homeostasis. On the basis of this study, a pathogenic role of telomere dysfunction for the development of renal insufficiency through structural and functional impairment is likely.

CONCISE METHODS

Animals
All procedures performed on animals were done in accordance with institutional guidelines for animal research and were approved by the local government authorities.

Generation and Genotyping of G4 Terc−/− Mice
Terc+/- mice on C57BL/6j background were purchased from Jackson Laboratories.10 G1 Terc−/− and Terc+/+ control animals were derived from heterozygous intercrosses according to previously published breeding strategies.16 Mating of G1 Terc−/− animals to each other generated G2 Terc−/− animals. Following this mating scheme, Terc−/− animals up to the fourth generation were bred. G4 Terc−/− mice displayed the typical phenotype that has been described before,16 including decreased survival, reduced fertility, premature hair graying, alopecia, and ulcerative skin lesions.

Ischemia-Reperfusion Injury
Ischemia-reperfusion surgery was performed in 3- to 4-mo-old age-matched male Terc+/+ , G1 Terc−/− , and G4 Terc−/− mice according to current standard protocols. Briefly, mice were anesthetized with inhalational isoflurane and were kept on a heating pad to maintain body temperature during surgery. A midline laparotomy was made, and the bowel was gently placed aside by retractors. The renal pedicles were exposed, and adjacent fat tissue was removed carefully. The left renal pedicle (artery and vein) was clamped for 30 min, using non-traumatic microsurgical vascular clips (Aesculap, Tuttingen, Germany). Occlusion of blood flow was confirmed by visual inspection of the kidneys. The right kidney was left in situ to reduce otherwise high mortality rates. After removal of the clip, the kidneys were observed for approximately 5 min to ensure blood reflow, and then fascia and skin were sutured in two layers with 6-0 silk and polyethylene. All animals received the same volume of warm saline instilled in the peritoneal cavity during the surgical procedure and were allowed to recover with ad libitum access to food and water. Mice for the group with 30 min of renal ischemia were killed after 7 (Terc+/+, n = 6; G1 Terc−/−, n = 7; G4 Terc−/−, n = 6) or 30 (Terc+/+, n = 6; G1 Terc−/−, n = 7; G4 Terc−/−, n = 6) days.

Controls consisted of nonoperated (Terc+/+, n = 8; G1 Terc−/−, n = 7; G4 Terc−/−, n = 8) and sham-operated animals (Terc+/+, n = 3; G1 Terc−/−, n = 3; G4 Terc−/−, n = 3). Sham-operated animals underwent anesthesia, laparotomy, and renal pedicle dissection only. They were killed 7 days after surgery.

After the mice were killed, the kidneys were excised, adjacent tissue was carefully removed, kidneys were decapsulated, and weight was determined. Part of each kidney was immediately snap-frozen in liquid nitrogen for later RNA extraction; a second part was fixed in 4% formaldehyde and paraffin-embedded.

Creatinine Clearance Determination
Except for day 1, all mice were kept in metabolic cages for the last 24 h before being killed. Twenty-four-hour urine was collected, urine volume was recorded, and aliquots were stored at −80°C for subsequent analysis. Blood was obtained by cardiac puncture under deep anesthesia right before the mice were killed. Plasma and urinary creatinine were determined using an enzymatic method that has been validated in rodents.99 Creatinine clearance (ml/min) was derived from the following formula: Urinary creatinine × urine volume × 1440 min⁻¹ × plasma creatinine⁻¹.

Body and Kidney Weight in Terc+/+ and Terc−/− Mice
G4 Terc+/+ mice showed reduced body weights compared with Terc+/+ and G1 Terc−/−, as has been described before.16 The mean weight differences between Terc+/+ and G4 Terc−/− per group for each day ranged between 18 and 26%. The body weights of G1 Terc−/− mice were comparable to those of Terc+/+ mice (data not shown). Weight of the clamped left kidney after IRI related to body weight did not show differences among Terc+/+, G1 Terc−/−, and G4 Terc−/−; however, the weight of the right kidney increased significantly as a result of compensatory hypertrophy only in Terc+/+ and G1 Terc−/− mice, but not in G4 Terc−/− mice (Supplemental Table 1) probably also as a result of the diminished regenerative capacity in these mice.

Histopathology of Kidney
Tissue sections were cut in 3-μm sections with a Leica RM 2165 microtome (Leica Instruments, Nussloch, Germany) and stained with hematoxylin and eosin, periodic acid-Schiff, or Masson Trichrome. High-power field (HPF) pictures (×200 magnification) of the whole cortex (>20) and corticomedullary junction (>10) were taken of each mouse kidney using a Leica DM LB2 digitizing microscope and a Leica DFC 320 camera (Leica Instruments) with QWin V3 software (Leica). Both cortex and corticomedullary junction were analyzed, and results (“total damage”) as shown in the Results section were obtained on a prorata basis.

Acute and chronic renal damage was quantified using ImageJ 1.37c software (National Institutes of Health, Bethesda, MD). Briefly, damaged tubular area and total tubular area were marked on each HPF screen, and ratio of damaged tubules to total tubular area was determined. Acute damage was mainly reflected by tubular necrosis,
whereas chronic damage consisted of tubular deterioration including reduced tubular diameter, thickened tubular basement membrane, and loss of tubular nuclei.

The degree of renal interstitial fibrosis was measured using Mas- tron Trichrome staining and a semiquantitative scoring system. The area of blue-stained interstitial fibrosis was detected using QWin 3 software package and related to total tubulointerstitial area. Fibrosis was evaluated by analysis of 20 HPFs (×200 magnification) for each animal.

**Telomere Q-FISH Analysis**

Q-FISH was performed as described. Nuclei were stained with DAPI, and image z stacks were taken using a Leica DM5500B (Leica Instruments) with a ×100 objective, capturing fluorescence images with a DFC360FX camera using LASAF software (Leica). Untreated mice (Terc+/−, n = 6; G4 Terc−/−, n = 6) and mice 30 d after renal ischemia (Terc−/−, n = 6; G4 Terc−/−, n = 7) were analyzed with three images per animal. Images were generated as projected, deconvolved z stacks (Huygens, SVI). TFI per nucleus in tubular cells (100 nuclei per mouse) were quantitatively analyzed using ImageJ 1.42 software (National Institutes of Health). Results are given as frequency histograms.

**Real-Time Reverse Transcriptase–PCR**

Total RNA was extracted from murine renal tissue samples using Trizol reagent (Invitrogen, Karlsruhe, Germany). DNA integrity was verified by agarose gel electrophoresis, and cDNA was obtained by reverse transcription of 1 μg total using MMLV reverse transcriptase and random primers (Invitrogen). For quantitative PCR of p16, p21, CD3, CD68, CD3, and CD68, one subgroup of animals for days 3 (Terc+/−, n = 7; G1 Terc−/−, n = 6; G4 Terc−/−, n = 6) and 30 (Terc+/−, n = 8; G1 Terc−/−, n = 9; G4 Terc−/−, n = 7) as well as sham-operated controls (Terc+/−, n = 3; G1 Terc−/−, n = 3; G4 Terc−/−, n = 3) and nonoperated controls (Terc+/−, n = 6; G1 Terc−/−, n = 7; G4 Terc−/−, n = 7) were evaluated.

Characterization of infiltrating cells was done by counting all CD3+ and CD68+ cells, respectively, within the corticomedullary zone of each kidney. Depending on kidney size, 10 to 20 HPFs (×200 magnification) were photographed and analyzed by a blinded observer. For each kidney section, the mean number of CD3+ and CD68+ cells was calculated. Cells counts were done in a subgroup of animals for days 3 (Terc+/−, n = 5; G1 Terc−/−, n = 5; G4 Terc−/−, n = 5), 7 (Terc+/−, n = 5; G1 Terc−/−, n = 5; G4 Terc−/−, n = 5), and 30 (Terc+/−, n = 5; G1 Terc−/−, n = 5; G4 Terc−/−, n = 5).

**TUNEL Assay**

The ApopTag Peroxidase Kit-In situ Apoptosis Detection Kit (Chemicon Europe Ltd., Hofheim, Germany) was used for TUNEL staining. Three-micrometer sections were deparaffinized and rehydrated in ethanol followed by incubation with proteinase K (Sigma-Aldrich, Taufkirchen, Germany). After quenching, equilibration buffer was applied, followed by working-strength enzyme. Cells were regarded as TUNEL-positive when their nuclei were stained brown and displayed typical apoptotic morphology. Ten HPFs at ×200 magnification were evaluated, and the percentage of positively stained nuclei was related to total nuclei number for tubules, glomeruli, and interstitium. For TUNEL, a subgroup of animals for days 3 (Terc+/−, n = 5; G1 Terc−/−, n = 5; G4 Terc−/−, n = 5) and 30 (Terc+/−, n = 7; G1 Terc−/−, n = 7; G4 Terc−/−, n = 7) and sham-operated controls (Terc+/−, n = 3; G1 Terc−/−, n = 3; G4 Terc−/−, n = 3) were evaluated.
Western Immunoblot

Approximately 30 mg of snap-frozen kidney tissue was lysed in 250 μl of ice-cold Triton extraction buffer (1% Triton X 100, 100 mM Tris [pH 7.4], 100 mM Na₃PO₄, 100 mM NaF, 10 mM EDTA, and a cocktail of protease inhibitors [complete mini; Roche, Mannheim Germany]) using a tissue lyser (Qiagen, Hilden, Germany). The homogenized samples were agitated for 30 min at 4°C, vortexed, and centrifuged for 30 min at 30,000 × g. The protein content of the supernatants was measured using the Bradford method (Protein Assay Kit; BioRad, Munich, Germany).

Proteins were separated on polyacrylamide gels and blotted onto nitrocellulose membranes. After blocking for 1 h in TBS-T (10 mM Tris [pH 7.4], 138 mM NaCl, and 0.05% Tween-20) containing 3% BSA, blots were incubated with Ctgf primary antibody (1:200; Santa Cruz Biotechnology, Heidelberg, Germany) in 1% BSA at 4°C overnight. Blots were washed three times for 15 min with TBS-T; and incubated with horseradish peroxidase–conjugated anti-goat secondary antibody (1:50,000; Santa Cruz Biotechnology, Munich, Germany). Blots were exposed to ECL films (GE Healthcare, Munich, Germany). Blots were stripped and reprobed with β-actin antibody (Abcam, Cambridge, UK), which served as a loading control.

Western blot analysis was performed only for a subgroup of animals for days 7 (Terc−/−, n = 3; G1 Terc−/−, n = 3; G4 Terc−/−, n = 3) and 30 (Terc−/+, n = 3; G1 Terc−/−, n = 3; G4 Terc−/−, n = 3). Results are given as fold expression on the basis of the expression of two samples from kidneys of Terc−/+ nonoperated controls.

Statistical Analysis

Data were evaluated using the SPSS 16.0 (SPSS Inc., Chicago, IL). Means among treatment groups were compared using ANOVA, and t tests with Bonferroni correction were applied for multiple pair-wise comparisons. Differences with regard to the number of critically shortened telomeres within the groups were analyzed using χ² test. All data are shown as means ± SEM.

NOTE ADDED IN PROOF

Shortly after the online appearance of this article, the authors were made aware of a discrepancy in the number of lanes for the Western blot shown in Figure 2B. The difference came from a 13th lane on the far right of the gel (shown for β-actin and removed for Ctgf), which contained a sample unrelated to the content of the current article and which should have been removed from both blots. The mistake has been corrected. Figure 2B now only shows the 12 lanes that have been analyzed by densitometry, for which the data are presented in Figure 2A. The authors apologize for the error.

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DISCLOSURES

None.

REFERENCES


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