Telomerase deficiency delays renal recovery in mice after ischemia–reperfusion injury by impairing autophagy

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The aged population suffers increased morbidity and higher mortality in response to episodes of acute kidney injury (AKI). Aging is associated with telomere shortening, and both telomerase reverse transcriptase (TerT) and RNA (TerC) are essential to maintain telomere length. To define a role of telomerase deficiency in susceptibility to AKI, we used ischemia/reperfusion injury in wild-type mice or mice with either TerC or TerT deletion. Injury induced similar renal impairment at day 1 in each genotype, as assessed by azotemia, proteinuria, acute tubular injury score, and apoptotic tubular epithelial cell index. However, either TerC or TerT knockout significantly delayed recovery compared with wild-type mice. Electron microscopy showed increased autophagosome formation in renal tubular epithelial cells in wild-type mice but a significant delay of their development in TerC and TerT knockout mice. There were also impeded increases in the expression of the autophagosome marker LC3 II, prolonged accumulation of the autophagosome protein P62, an increase of the cell cycle regulator p16, and greater activation of the mammalian target of rapamycin (mTOR) pathway. The mTORC1 inhibitor, rapamycin, partially restored the ischemia/reperfusion-induced autophagy response, without a significant effect on either p16 induction or tubule epithelial cell proliferation. Thus, muting the maintenance of normal telomere length in mice impaired recovery from AKI, owing to an increase in tubule cell senescence and impairment of mTOR-mediated autophagy.

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AKI frequently results from tubule injury by acute ischemic or toxic exposure to the kidney,[1,2,3] with higher morbidity and increasing mortality seen in aged patients (⩾65 years).[4–7] Age-related renal morphological changes, functional alterations, and accompanying comorbidities may all contribute to the vulnerability of the aged population to either acute or chronic renal injury.[8–10] However, intrinsic underlying predisposing molecular and genetic mechanisms related to aging *per se* remain incompletely studied.

Telomeres become shorter each time a cell divides and are shortened in an age-dependent manner in human kidney, particularly in the renal cortex,[11] and telomere shortening reduces regenerative capacity after renal injury.[12] Telomerase is a reverse-transcriptase enzyme complex that adds DNA-sequence repeats (TTAGGG) to the 3′ end of DNA strands in the telomere regions at the ends of the eukaryotic chromosomes. There are two major components in the transcriptase ribonucleoprotein complex: the RNA-directed DNA polymerase, TerT, and the RNA template, TerC. TerC or TerT gene mutations are invariably associated with marked telomere shortening, resulting in dyskeratosis congenita and inherited bone marrow failure syndromes in humans,[13] and are risk factors for a range of other human telomeric syndromes, including aplastic anemia, idiopathic pulmonary fibrosis, and acute myeloid leukemia.[14] Telomerase participates in chromosomal repair; *de novo* synthesis of telomere repeats may occur at double-stranded breaks.[15]

Absence of telomerase leads to telomere shortening progressively during successive generations of TerC- or TerT-deficient mice.[16–19] To investigate the impact of telomerase on renal tubular injury and regeneration, we induced acute renal damage by clamping both renal pedicles in G4 mice with either TerT or TerC deficiency to compare tubular injury and regeneration with wild-type mice and to explore underlying mechanisms. As nontelomerase functions have also been linked to TerT,[20] we used both TerC and TerT knockout (KO) mice in the current study to clarify the role of telomerase deficiency. Short telomeres in those mice were confirmed by our coauthor, Dr Lawson, and colleagues previously.[21]

RESULTS

I/R led to renal injury in each genetic group, but with delayed recovery in mice with TerC/TerT KO

I/R-induced elevation of BUN with a peak at day 1 in mice from each genetic group (Figure 1a). BUN quickly returned to normal within 14 days in the wild-type mice but was delayed in both the TerC or TerT KO mice (Figure 1a). Serum creatinine levels were consistent with the BUN results (Supplementary Figure 1a online). As sham operation did not cause significant differences in renal function (data not shown), subsequent studies only investigated mice subjected to I/R. I/R induced both increased albuminuria (Supplementary Figure 1b online) and low-molecular-weight proteinuria (so-called tubular proteinuria)[22] (Supplementary Figure 1c online); albuminuria and total proteinuria persisted longer in mice with either TerC KO or TerT KO.

Renal histopathology assessed with periodic acid–Schiff (PAS) staining further confirmed I/R-induced renal injury, predominantly in proximal tubules. All mice subjected to I/R demonstrated significant tubular damage, including loss of brush border, shedding of both necrotic and viable epithelial cells into the tubular lumen, tubular dilation, cast formation, and cell lysis (Figure 1b). A semiquantitative acute tubular injury score indicated that in wild-type mice morphology began to recover from day 3 and had almost returned to normal at day 14, whereas slower histologic evidence of recovery was seen in either TerC or TerT KO mice (Figure 1c). Only mild fibrosis by Masson's trichrome stain was detected within the observation period in all groups (data not shown). KIM-1 immunohistology was increased in all groups post I/R, but had returned to baseline levels by day 14 in wild-type mice while remaining elevated in TerC or TerT KO mice (data not shown).

Telomerase deficiency was linked to more severe apoptosis, lower proliferation, and upregulated cell cycle inhibitor, p16, in TECs after I/R

I/R induced significant apoptosis from day 1 in each group, but there was evidence for prolonged apoptosis in the TerC KO and TerT KO mice (Figure 1d and e); significant differences were seen at days 5 and 7 (Figure 1d and e). Apoptosis was predominantly detected in proximal tubules (Figure 1d). Determination of active cleaved caspase-3 further confirmed the extended apoptosis in TerC/TerT KO mice following I/R (Figure 1f). Cell proliferation evaluated by Ki67 staining indicated that Ki67 positivity, predominantly in proximal tubule, appeared as early as day 1, with a peak at day 3 in wild-type mice. In contrast, there was delayed and blunted Ki67 positivity in TerC and TerT KO mice (Figure 2a and b).

Previous studies have indicated that in the presence of dysfunctional telomeres environmental stress leads to induction of the cell cycle regulator p16.[23] Few positive p16 cells were detected in kidneys from wild-type mice at baseline, and I/R induced only moderate upregulation at day 1, especially in proximal tubule epithelial cells (Figure 3a). There was higher basal expression and stronger I/R stimulation of p16 in both TerC and TerT KO mice, and increased p16 expression was still present 7 days post I/R (Figure 3b and c).

Telomerase deficiency blunted autophagic responses to I/R

Autophagy is an important cellular housekeeping process and has been proposed to be renoprotective[24,25] and influenced by age.[26,27] Autophagosomes, detected by electron microscopy, increased in wild-type mice, beginning as early as on day 3. In contrast, there was a delay in the onset of autophagosome development in either TerC or TerT KO mice, and increased autophagosomes were detected as late as day 14 after I/R injury (Figure 4a). Consistent with the delayed appearance of autophagosomes, the LC3 II/I ratio significantly increased by day 3 after I/R in wild-type mice, but only at day 14 after I/R TerC or TerT KO mice (Figure 4b and d). There was also prolonged accumulation of P62 following I/R injury in mice with telomerase deficiency (Figure 4c and d).

mTOR Activation was associated with autophagy impairment in telomerase-deficient mice during I/R

mTOR signaling is crucial for cellular functions and has important roles in aging.[28] mTOR activation has been reported in genetic telomerase-deficient (K5TRF2/TerC$^{-/-}$) mice.[29] Following I/R injury, phospho-mTOR expression persisted in either TerC or TerT KO mice compared with wild-type mice (Figure 5a and d). In isolated tubular epithelial cells (TECs), there was also increased phosphorylation of mTOR, along with its downstream targets p70-S6 kinase and 4E-BP1, in response to an anoxic insult (Supplementary Figure 2a online).

To determine the role of mTOR in autophagy impairment, we inhibited mTORC1 activity in response to anoxia by administration of rapamycin in primary cultured TECs. In TECs from wild-type mice, moderate increases in mTOR activation were detected at 24 hrs, which returned to normal at 48 hrs, but mTOR activation was still detected in TerC KO mice at 48 hrs after the anoxic insult (Supplementary Figure 2b online). There was also delayed conversion of the autophagic marker LC3 I to II and persistent P62 in TECs with TerC deletion (Figure 6a and b), both of which were reversed by rapamycin (Figure 6c and d). Anoxia induced conversion of LC3 as early as 6 h post I/R in wild-type mice and reached peak levels at 24 h. In the wild-type mice, the response to rapamycin was more significant at 6 h instead of 24 h following transient anoxia (Supplementary Figure 3 online).

To examine further the effect of mTOR in telomerase-deficient kidney during I/R, we treated TerC mice with the mTORC1 inhibitor rapamycin. Rapamycin promoted conversion of LC3 from I to II in TerC KO mice, which was most obvious on days 3 to 5 following I/R (Figure 7), when LC3 II was still very low without treatment. As I/R-induced LC3 conversion earlier in wild-type mice, a stimulation response by rapamycin in these mice was seen on day 1 (Figure 7). Correspondingly, rapamycin reduced P62 accumulation in