Transition of kidney tubule cells to a senescent phenotype in early experimental diabetes

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Satriano J, Mansoury H, Deng A, Sharma K, Vallon V, Blantz RC, Thomson SC. Transition of kidney tubule cells to a senescent phenotype in early experimental diabetes. Am J Physiol Cell Physiol 299: C374–C380, 2010. First published May 26, 2010; doi:10.1152/ajpcell.00096.2010.—Diabetic nephropathy is the commonest cause of end-stage renal disease. Inordinate kidney growth and glomerular hyperfiltration at the very early stages of diabetes are putative antecedents to this disease. The kidney is the only organ that grows larger with the onset of diabetes mellitus, yet there remains confusion about the mechanism and significance of this growth. Here we show that kidney proximal tubule cells in culture transition to senescence in response to oxidative stress. We further determine the temporal expression of G1 phase cell cycle components in rat kidney cortex at days 4 and 10 of streptozotocin diabetes to evaluate changes in this growth response. In diabetic rats we observe increases in kidney weight-to-body weight ratios correlating with increases in expression of the growth-related proteins in the kidney at day 4 after induction of diabetes. However, at day 10 we find a decrease in this profile in diabetic animals coincident with increased cyclin-dependent kinase inhibitor expressions. We observe no change in caspase-3 expression in the diabetic kidneys at these early time points; however, diabetic animals demonstrate reduced kidney connexin 43 and increased plasminogen activator inhibitor-1 expressions and increased senescence-associated β-galactosidase activity in cortical tubules. In summary, diabetic kidneys exhibit an early temporal induction of growth phase components followed by their suppression concurrent with the induction of cyclin-dependent kinase inhibitors and markers of senescence. These data delineate a phenotypic change in cortical tubules early in the pathogenesis of diabetes that may contribute to further downstream complications of the disease.

proximal tubule; hypertrophy; cyclin-dependent kinase inhibitor; connexin 43

The functional unit of the kidney, the single nephron, shows a particular organization including a tubuloglomerular contact site that contributes to the fine coordination between glomerular filtration and tubular reabsorption through the mechanism of tubuloglomerular feedback (TGF). The TGF mechanism refers to a series of events whereby changes in the Na+, Cl−, and K+ concentrations in the tubular fluid are sensed by the macula densa cells at this contact site. An increase or decrease in late proximal tubular flow rate, and thus in Na+, Cl−, and K+ delivery at the macula densa, elicits inverse changes in glomerular filtration rate (44). There is now convincing evidence for a primary increase of fluid and electrolyte reabsorption in the proximal tubule in rats with streptozotocin (STZ)-induced experimental type 1 diabetes (6, 35, 43, 45, 47) as well as in early type 1 diabetes in humans (9, 23). Increased reabsorption lowers the concentration of Na+, Cl−, and K+ in the tubular fluid at the macula densa thereby eliciting a TGF-dependent increase in nephrin filtration rate (47, 48). Inhibiting the synthesis of polyamines required for growth results in a parallel decrease in proximal tubular hyperreabsorption and glomerular filtration in diabetic rats (43). This illustrates the impact of tubular growth on kidney function in response to STZ-diabetes. Glomerular expansion would also be affected by suppression of kidney growth.

With regard to total volume, growth of the diabetic kidney is attributed primarily to the proximal tubule, where a period of hyperplasia precedes diabetic hypertrophy (25). High glucose treatment of a mesangial cell line stimulates a biphasic early cell proliferation (24 to 48 h) and a later growth inhibitory phase (72 to 96 h) (58). The early proliferative phase is associated with increased expression of the immediate early genes and growth factors (61). The diabetic kidney begins changing from hyperplastic to hypertrophic growth very early in the course of hyperglycemia, at approximately day 4 in the STZ model (25), which matches the time frame of hyperplasia we observed using 5-bromodeoxyuridine (BrdU) incorporation (15). Expression of the cyclin-dependent kinase inhibitor (CKI) p27KIP1 (p27) increases in response to hyperglycemia or diabetes, which can be attributed, in part, to induction by PKC (57) and transforming growth factor-β (TGF-β) (26). Targeted disruption of the p27 gene does not affect hyperglycemia in the STZ diabetic model but does decrease hypertrophy, with resultant reductions in albuminuria, extracellular matrix (ECM) production, glomerulosclerosis, and structural damage (5, 55). TGF-β is thought to contribute to ECM production in this model, so it is curious as p27 is downstream from TGF-β that diabetic p27−/− mice display markedly lower ECM production even though TGF-β levels remain unaltered (5, 55). These data demonstrate a key role of p27 in diabetic hypertrophy, which is an important component of disease progression. This raises the question, is there something unusual about cellular hypertrophy in diabetes?

We hypothesize that diabetic hypertrophy is a phenotypic transition to senescence. Senescence is a tumor suppressor mechanism to halt cells from replicating and passing on a potentially damaged genome. Like early diabetic hypertrophy, senescent arrest requires upregulation of CKI (2, 24, 33). Although the prototypical senescent arrest involves the temporal induction of p21WAF1,CIP1 (p21) and p16INK4a (p16) (39, 42), studies demonstrate that induction of p27 can impose a senescent-like growth arrest (3, 22, 60). Oxidative stress produced by exposure of human diploid fibroblasts to hydrogen...
peroxide induces senescence and was attributed to TGF-β induction (19). Furthermore, the active braking of the cell cycle by CKI mediates a G₁ cell cycle arrest in both diabetic hypertrophy and senescence resulting in cells that are unresponsive to mitogenic stimulation. Here we show evidence that in the early STZ diabetic kidney there is an early phenotypic change, primarily in cortical tubule cells. Senescent cells exhibit several aspects of a fairly well-differentiated phenotype; however, this phenotype is skewed in several parameters from that of the normal parental cell type (11). Furthermore, senescent cells would contribute to the oxidative stress, production of growth factors, and ECM observed in diabetes and would be resistant to apoptotic remodeling (4, 40). As such, the transition of proximal tubule cells to a senescent phenotype would offer a mechanism whereby diabetic hypertrophy contributes to disease progression.

It may be that several peculiar aspects of kidney function in early diabetes are not consequences of kidney growth, per se, but are consequences of the mechanism whereby the kidney has grown. Proximal tubular cells that exhibit a senescent arrest may present a chronically altered phenotype that affects transport function, explain unusual phenomena like the salt paradox of the early diabetic kidney (46), and contribute to later diabetic complications (7, 36, 37).

**MATERIALS AND METHODS**

**Materials**

All chemicals were purchased from Sigma unless otherwise stated. Opossum kidney proximal tubule cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle’s media-Ham’s F-12 mix (DMEM/F-12; Cellgro, Herndon, VA) supplemented with 5% FBS (Atlanta Biologicals, Atlanta, GA), 2 mM l-glutamine, and antibiotics, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, in a humidified atmosphere of 95% air-5% CO₂. The media contained sodium pyruvate (110 mg/l) which could dampen the effects of the H₂O₂. Control cells at 3 days were plated at 1.5 × 10⁵ cells per well of a 6-well plate, and both 150 μM and 300 μM concentrations of H₂O₂-treated cells were plated at 3 × 10⁵ cells per well. Cells at 6 days were plated at various densities to compensate for growth starting at 0.5 × 10⁴ for control and 1.5 × 10⁵ for H₂O₂.

**Animals and STZ**

Physiologic parameters. Male adult Sprague-Dawley rats (purchased from Harlan Teklad) were housed and handled in accordance with Veterans Administration and National Institutes of Health guidelines under Institutional Animal Care and Use Committee-approved protocols. Rats were made diabetic by STZ (Sigma Chemical, St. Louis, MO; 65 mg/kg ip) dissolved in sodium citrate buffer (pH 4.2). One day later, the glucose concentration was determined in tail blood samples, and only those animals with blood glucose levels >300 mg/dl were included in further experiments. Vehicle-injected nondiabetic rats served as controls. Blood glucose levels were determined at least four times in every rat after STZ injection, and mean values were calculated. Kidneys were harvested under terminal inactin anesthesia at 4 or 10 days after STZ application. On all left kidneys, kidney dry weight was determined, whereas right kidneys were harvested for Western blot analysis.

**Western blot analysis**. Kidneys were harvested and homogenized in lysis buffer [lysis buffer: 1% Triton X-100, 0.5% deoxycholic acid, 1 mM EDTA, 0.1% SDS, 4 mM NaF, Complete protease cocktail (Roche Molecular Biochemicals, Mannheim, Germany), and 1 mM NaVO₄ in PBS]. Lysates at 50 μg/fane were resolved on NuPAGE gels in MOPS buffer (Invitrogen, Carlsbad, CA). Gel proteins were transferred to nitrocellulose membranes and immunoblotted with the appropriate primary antibody, as indicated. The secondary antibody was horseradish peroxidase-conjugated (Santa Cruz Biotechnology, Santa Cruz, CA) for autoradiographic detection by ECL Plus (Amer- sham Pharmacia, Piscataway, NJ), with densitometric analysis by ImageJ software (National Institutes of Health, Bethesda, MD).

**Senescence-Associated β-Galactosidase Activity**

Senescence-associated β-galactosidase (SA-β-Gal) enzymatic assays were performed as per Dimri et al. (16), modified to be run at pH 6.3 rather than 6.0 because as we find lower background in our samples. A similar shift in pH to minimize background interference from β-galactosidase present in all cells was previously reported (28). In brief, kidneys were harvested, frozen in OCT compound, and sectioned at 10 μM. Sections were fixed in 0.5% glacialdehyde for 5 min, washed and placed in X-gal solution (1 mg/ml X-gal, 5 mM K₃Fe[CN]₆, 5 mM K₄Fe[CN]₆, 2 mM MgCl₂, and 150 mM NaCl, in 40 mM citric acid, phosphate at pH 6.3) overnight at 37°C. Diabetic animals in this group were treated with STZ as above, but for 14 days.

**Statistical Evaluation**

Variations between samples within groups were analyzed by ANOVA, with significance determined by Fisher's protected least-significant difference post-hoc test. KaleidaGraph software (version 4.04, Synergy Software) was used for these analyses.

**RESULTS**

**SA-β-Gal Response of Kidney Proximal Tubule Cells to Oxidative Stress**

We first determined whether proximal tubule cells transition to a senescent phenotype upon exposure to oxidative stress. Opossum kidney proximal tubule cells were exposed to 150 μM or 300 μM H₂O₂ for 3 h and then left to incubate at 37°C in fresh media for 3 or 6 days. Figure 1 demonstrates a time and dose response to H₂O₂ as demonstrated by increased...
SA-β-Gal activity. Time after exposure to H₂O₂ is required as late-stage senescence takes time to evolve. This 3- to 6-day incubation period, it should be remembered, allows the nonaffected cells to continue growing, whereas the senescent arrested cells do not. Furthermore, twice as many cells were seeded for the H₂O₂ group to reach equal confluence with the nontreated controls. We assume from this delay that some cells exposed to H₂O₂ repaired and reentered growth phase while others progressed to senescent arrest. Nonetheless, we do see some minor staining in the control cells, yet, if any, cells are observed with a senescent morphology, i.e., larger (G₁ arrested) cells with a “sunny side up egg” appearance. This aberrant morphology and SA-β-Gal staining are clearly more evident in the H₂O₂-treated cells (Fig. 1). We did not formulate DMEM/F-12 without pyruvate for these experiments so the cells may be more sensitive to oxidative stress than observed here.

Physiology of STZ-Treated Rats

STZ increased blood glucose levels compared with control rats (439 ± 19 vs. 112 ± 3 mg/dl); the levels were similar at 4 or 10 days after STZ. STZ increased absolute as well as relative kidney weight at 4 or 10 days after STZ (Table 1). These measurements imply that kidney growth occurs largely by day 4.

Temporal Effects of Diabetes on the Growth Response

Physiology measurements display diabetic kidney growth by the increased kidney weight-to-body weight ratio, as shown in Table 1. Here we evaluated expression of four proteins that are associated with growth and evaluate their changes in expression with diabetes over two time points. The first, phosphatidylinositol 3-kinase-Akt, comprises a coordinated network of pathways supporting antiapoptotic yet progrowth and angiogenic responses that is upregulated by a variety of growth factors in the early stages of kidney growth in diabetes. Activation of Akt requires phosphorylation. The antibody used in these studies detects Thr308-phosphorylated Akt, and thus activated form, of the enzyme (Akt-p; Santa Cruz Biotechnology). The second is cyclin A; its levels increase late in G₁ in response to transactivation of E2F. Cyclin A binds with cyclin-dependent kinase-2 (cdk2) to form an active complex necessary for G₁ to S phase transition (21) and S phase transit (20). The third, proliferating cell nuclear antigen (PCNA), belongs to the DNA family of sliding clamps that encircles DNA and tethers polymerases firmly to it during DNA synthesis. The fourth, Ki67, represents another common marker of cellular proliferation. In accord with the measured kidney growth we observe an upregulation of these growth-associated proteins at day 4. At day 10 these differences are no longer significant. Akt-p, cyclin A, PCNA, and Ki67 expressions at 4 and 10 days are shown in Fig. 2. A–D, respectively.

Temporal Effects of Diabetes on the CKI Profile

Much of what is known about the G₁ phase of the cell cycle concerns the phosphorylation and inactivation of the restriction

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Values are means ± SE. STZ, streptozotocin; IB, initial body weight; FB, final body weight; KW, kidney weight.
point guardian, the retinoblastoma protein (Rb). Hyperphosphorylation of Rb is carried out in G1 by cyclin D/cdk4/6 and cyclin E/cdk2 (18). Human diploid fibroblasts that reach generational senescence demonstrate a failure to hyperphosphorylate Rb. Senescent cells temporally induce the expression of CKI, particularly p16 (2, 24) and p21 (33). p21 is a universal cyclin/CDK complex inhibitor, whereas p16 binds monomeric cdk4 and cdk6, preventing their association with cyclin D. The switch from hyperplastic to hypertrophic growth of the diabetic kidney is attributed to induction of p27 via TGF-β (25, 26, 30, 56). Increased p27 expression has been associated with senescence (8). Although there is little expression of these CKIs in response to diabetes at day 4, where the growth phase is prevalent, there is a marked response by day 10 (Fig. 3). Interestingly, the negative regulation of Forkhead box, class O (FOXO) on cell cycle progression and proliferation is dependent on p27, which it transcriptionally activates (32). Active Akt phosphorylates and inactivates both FOXO and p27, translocating them out of the nucleus (27). Here we show a temporal decrease in Akt-p (Fig. 2A) that corresponds with an increase in p27 expression (Fig. 3C).

Cyclin A is associated with S phase entry and G1 to S transition. Its expression is regulated by E2F. In senescence, CKI activation of Rb inhibits E2F and thus suppresses cyclin A levels (17). This would correlate these CKI results with the above results of Fig. 2B.

Effects of Diabetes on Apoptotic and Senescent Markers

Both the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway converge at the execution caspase, caspase-3. Cleavage of caspase-3 initiates a series of apoptotic events including protein modification, DNA fragmentation, apoptotic body formation, and externalization of membrane phosphatidylserine for phagocytic recognition. Expression of caspase-3 is observed in all samples, yet there is no significant variation between control and diabetic animals at either 4 or 10 days, implying that we do not observe overt apoptosis at this early-onset stage of diabetes (Fig. 4A). Connexin 43 (Cx43) is a member of a family of hexameric gap proteins important in cell-cell communication, differentiation, and growth control. Reduced expression of Cx43 in aging and its correlation with senescence suggest Cx43 as a senescent biomarker (41). Cx43 tends to decrease in diabetic animals relative to their nondiabetic controls at day 4 and becomes significant by day 10 (Fig. 4B). The remodeling of ECM is a common event in aging. Senescent fibroblasts overexpress plasminogen activator inhibitor type-1 (PAI-1), which regulates ECM fibrolytic activity. We observe an increase in PAI-1 in diabetic animals at day 10 (Fig. 4C). Variations in ECM proteins occur late in the development of senescence.

SA-β-Gal activity increases in response to diabetes by this 14-day time point, predominantly in the cortical tubules (Fig. 5). It should be noted that SA-β-Gal activity, although obvious in the diabetic animals, was not evenly distributed throughout the cortex. Some areas were more prominent than others, which would be in accord with the paracrine effects noted of senescent cells (7). Endothelial cells, glomerular components, and medulla do not demonstrate discernable staining at this early time point.

DISCUSSION

Diabetes mellitus affects the kidney in stages, with sclerosis and kidney failure occurring many years after onset. Here we evaluate the very early stages of disease where the kidney initiates growth, hypertrophy, and hyperfiltration. We find that the molecular signature of senescence develops during this early phase, with SA-β-Gal staining localized principally to the cortical, proximal tubules.

Biopsies from diabetic patients display decreased Cx43 in podocytes (38) and increases in p16 in podocytes and proximal tubule cells (50), with increased SA-β-Gal activity in the tubular component (50). It is unclear from these studies alone whether diabetes induces senescence or whether patients with a higher complement of senescent cells are more susceptible to
disease progression. It is also unclear whether senescence is involved in the pathogenesis of the disease, or if it is a late phase downstream effect. Diabetes produces a vascular aging effect, and high glucose (59) or advanced glycosylation end-products induce senescence in endothelial cells. Treatment with the peroxynitrite inhibitor ebselen suppresses this latter effect (10, 12). Other in vitro studies with high glucose demonstrate induction of senescence in kidney mesangial cells (62) and proximal tubule cells (49) in culture.

Conversion of cortical tubules to a senescent-like phenotype could constitute a significant factor in the progression of diabetic kidney complications. Proximal tubules comprise the major fraction of diabetic hypertrophic growth. Recent work shows that proliferation of proximal tubules is primarily from dedifferentiated cells, not resident stem cells or hematopoietic stem cells (52, 53). These studies further demonstrate that a large population of proximal tubules resides in a prolonged G1 phase, which would suggest a rapid proliferative capacity in response to an emergency (54). Terminally differentiated cells cannot progress to senescence (51), but dedifferentiated proximal tubule cells responding to mitogenic factors would lend themselves to senescent arrest. As stated previously, hypertrophic kidney growth alone may not set in motion the spiraling complications leading to end-stage renal disease. Overexpression of fibronectin and PAI-1 is observed in senescence and diabetes. Thus, the senescent phenotype may contribute to diabetic fibrosis. Senescent cells display a reduction in proteasomal mediated protein degradation (14, 40), which would be a factor for the increased hypertrophy due to decreased proteolysis and progression in later stage diabetes. Patients with overt type 2 diabetic nephropathy display a decrease in Cx43 expression, as observed in senescence (41), on podocytes relative to patients with minor glomerular abnormalities (38). These changes directly correlated with a decline in renal function. In addition, senescence leads to increased TGF-β expression (19), protein oxidation (14, 40), and cellular oxidant production of both superoxide radical and hydrogen peroxide (4). Suppression of autophagy leads to increased oxidative stress with damage to membranes, proteins and DNA, and genomic instability (31), further promoting senescence. Senescent cells perpetuate inflammatory cytokine feedback loops that promote autocrine and paracrine effects (1, 29). These factors all support an inflammatory milieu and increased oxidative stress. The contribution of oxidative stress to complications in diabetes is a complex and controversial issue. Evidence supports a compelling role of glucose-mediated metabolic imbalances in the production of free radicals and the progression of diabetic complications (34). All the factors associated with senescent cells are apparent in the progression of diabetic complications. This hypothesis is in accord with data from p27

differentiation.
knockout mice that exhibit markedly reduced glucose-mediated hypertrophy (5, 55). Importantly, although blood glucose levels were comparable in diabetic p27−/− and p27+/− animals, parameters of downstream complications of diabetes including albuminuria, structural damage, and ECM production were all markedly attenuated in the p27−/− animals. Overall, a single cell blocked from passing on a damaged genome will not affect the organism, yet an accumulation of senescent cells may contribute to the disruption of local tissue integrity and potentiate future declines in function. With aging comes a natural increase in senescent cells and decreases in autophagy. These cells would complement the stress that the diabetes-induced premature senescent cells generate, and along with further decreases in autophagy we hypothesize would place older individuals at a higher risk of developing diabetic nephropathy. However, although the overall senescent load increases with age, it can vary greatly even within the same age group, as observed in rat kidneys (13). A question then is, can the heterogeneity of senescence explain or correlate with the heterogeneity observed in the progression of diabetes? Further advances in the field of senescence will help us to better address this question. Determining that proximal tubule cells convert to a senescent phenotype very early in response to diabetes is an important first step toward defining the components that comprise and underlie the pathogenesis of the disease. Targeting these senescent-like cells has the potential to relieve or possibly and underlie the pathogenesis of the disease. Targeting these senescent-like cells has the potential to relieve or possibly and underlie the pathogenesis of the disease. Targeting these senescent-like cells has the potential to relieve or possibly and underlie the pathogenesis of the disease. Targeting these senescent-like cells has the potential to relieve or possibly and underlie the pathogenesis of the disease. Targeting these senescent-like cells has the potential to relieve or possibly and underlie the pathogenesis of the disease. Targeting these senescent-like cells has the potential to relieve or possibly and underlie the pathogenesis of the disease.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


