Administration of poly-D-glutamic acid induces proliferation of erythropoietin-producing peritubular cells in rat kidney

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Kishore BK, Isaac J, Westenfelder C. Administration of poly-D-glutamic acid induces proliferation of erythropoietin-producing peritubular cells in rat kidney. Am J Physiol Renal Physiol 292: F749–F761, 2007. First published October 3, 2006; doi:10.1152/ajprenal.00034.2006.—Erythropoietin (EPO), a 34-kDa glycoprotein, is produced predominantly by peritubular interstitial cells (PIC) in the renal cortex and is physiologically released when ambient oxygen pressure falls. However, the exact nature of EPO-producing cells in the kidney is not well understood. We discovered that brief administration of a low-molecular-weight synthetic peptide, poly-D-glutamic acid (PDG), induced prompt and robust expansion of EPO-producing PIC in rat kidney, without evidence of tubular cell necrosis/apoptosis or fibrotic reaction. Proliferating PIC in PDG-treated rats were noninflammatory, α-smooth muscle actin negative, and specifically expressed CD73 (ecto-5′-nucleotidase), EPO mRNA, and protein. Increased numbers of EPO-positive PIC persisted even after the cessation of PDG treatment. No erythropoietic effects of EPO were detected, potentially suggesting maintained physiological control of EPO secretion in this normoxic model. We showed previously that PDG is readily filtered at the glomeruli and is rapidly taken up by proximal tubular cells, where it produces an apparently non-toxic, acute lysosomal storage condition by virtue of its non-hydrolyzable nature (Kishore BK, Maldague P, Tulkens PM, Courtoy PJ. Lab Invest 74: 1013–1023, 1996). Based on these findings, we suggest that unknown signaling molecules, produced by PTC in response to lysosomal PDG storage, appear to specifically stimulate the proliferation of EPO-producing PIC. We conclude that this model is uniquely suited to investigate the biology of EPO production by PIC and may thus facilitate the development of novel and more economical therapies of anemias and other EPO-responsive conditions.

anemia; proximal tubular cell; fibroblasts; immunohistochemistry; in situ hybridization

IN A NORMAL INDIVIDUAL, THE HEMATOcrit IS REMARKABLY MAINTAINED AT A CONSTANT VALUE. CENTRAL TO THIS ELEGANT, OXYGEN PRESSURE-SENSITIVE CONTROL MECHANISM IS THE GLYCOPROTEIN HORMONE ERYTHROPOIETIN (EPO), PRODUCED PROMINENTLY BY PERITUBULAR INTERSTITIAL CELLS OF THE RENAL CORTEX, FROM WHERE IT IS TRANSPORTED VIA THE CIRCULATION TO THE BONE MARROW. HERE, IT REGULATES SURVIVAL, PROLIFERATION, AND MATURATION OF ERYTHROID PROGENITOR CELLS INTO MATURE RED BLOOD CELLS (15, 26, 49). DECREASED PRODUCTION OF EPO RESULTS IN ANEMIA. THE LATTER IS OFTEN ASSOCIATED WITH ACUTE AND CHRONIC RENAL FAILURE, END-STAGE RENAL DISEASE (ESRD), MALIGNANCIES, MYELOMA, HIV INFECTION AND AIDS, RHEUMATOID ARTHRITIS, MYELOPLASTIC SYNDROMES, AND OTHER CHRONIC DISEASES (10).

While physiological EPO production is stimulated by hypoxia, the administration of angiotensin II, the iron chelator desferoxamine (DFO), or cobalt is also known to upregulate EPO production. However, because of adverse effects, none of the latter can be used therapeutically. Hence the only currently available treatment option for patients with EPO-responsive anemias is the administration of recombinant human EPO (rHuEpo), a drug that has remained very expensive.

EPO IS DISTINCT AMONG THE HEMATOPOIETIC GROWTH FACTORS, BECAUSE IT IS PRODUCED PRIMARILY IN THE KIDNEY RATHER THAN THE BONE MARROW. THE KIDNEY APPARENTLY FUNCTIONS AS A CRITMETER IN THAT IT SENSES PO2 AND EFFECTIVE ARTERIAL BLOOD VOLUME (5). BY REGULATING THE RED CELL MASS THROUGH EPO AND PLASMA VOLUME THROUGH EXCRETION OF SALT AND WATER, THE KIDNEY SETS THE HEMATOCRIT AT A NORMAL AVERAGE VALUE OF 45%, WHICH OPTIMIZES OXYGEN DELIVERY TO TISSUES (15, 26, 49). THUS FACTORS THAT AFFECT TISSUE PO2 (E.G., HYPOXIA) OR RENAL BLOOD FLOW AND SODIUM REABSORPTION (E.G., ANGIOTENSIN II) ARE KNOWN TO INDUCE EPO PRODUCTION IN THE KIDNEY.

In view of the above, attempts to induce production of EPO safely in the normal or diseased kidney are highly relevant with potential exploitation for clinical use. In this context, we serendipitously discovered a method whereby EPO-producing peritubular cells in rat kidney were stimulated to proliferate. Specifically, the administration of a low-molecular-weight synthetic peptide, poly-D-glutamic acid (PDG), induced an extensive proliferation of EPO-producing peritubular interstitial cells in rat kidney. We previously demonstrated that PDG is readily filtered at the glomeruli and is rapidly taken up by proximal tubular cells, where it produces an apparently non-toxic, acute lysosomal storage condition by virtue of its non-hydrolyzable nature (18, 23). To the best of our knowledge, this is the first animal model where such a striking proliferation of EPO-producing peritubular interstitial cells has been achieved. We also demonstrated that poly-D-glutamic acid, which is rapidly hydrolyzed by lysosomal hydrolases, produced neither a lysosomal storage condition nor a proliferation of peritubular interstitial cells (21, 22). Hence, we hypothesized that this hitherto undefined molecules produced and/or released by proximal tubular cells in response to lysosomal PDG storage specifically stimulate the proliferation of EPO-producing peritubular interstitial cells. This novel model, we posit, promises to have significant physiological, cell biological, biotechnological, and clinical applications. In this study, we...
present detailed cellular and molecular characteristics of our model.

MATERIALS AND METHODS

PDG. PDG, as sodium salt, with a mean molecular mass of 13,000 or 20,500 Da was purchased from Sigma (St. Louis, MO).

Experimental animals and treatment. The animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committees of the Veterans Affairs (VA) Salt Lake City Health Care System and the University of Utah. Specific pathogen-free male Sprague-Dawley rats (Harlan, Madison, WI) were housed two or three per cage in the Veterinary Medical Unit of the VA Salt Lake City Health Care System, which is an Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited and US Department of Agriculture- and Public Health Service-approved animal facility. Rats were maintained in a pathogen-free state and fed ad libitum a commercial rodent diet and had free access to drinking water. All animals were acclimated to the housing conditions for about 1 wk before experiments were conducted.

In the first series of experiments, groups of rats (n = 4/group) were injected subcutaneously with either PDG (molecular mass 13,000 Da) solution at a dose of 250 mg/kg body wt or sterile normal saline daily for 4 consecutive days and were euthanized 1 day after the cessation of treatment. This series of rats weighed 273 ± 5 g (means ± SE).

In the second series of experiments, groups of rats (n = 5/group) were injected subcutaneously with either PDG (molecular mass 20,500 Da) at a dose of 250 mg/kg body wt or sterile saline for 4 consecutive days and were euthanized 1 day after the cessation of treatment. This series of rats weighed 175 ± 3 g.

In the third series of experiments, groups of rats were subcutaneously injected with sterile saline (n = 4/group) or with PDG (molecular mass 20,500 Da) at a dose of 250 (n = 3) or 150 mg/kg body wt (n = 3) for 4 consecutive days and were euthanized 6 days after the cessation of PDG treatment. This series of rats weighed 193 ± 4 g.

PDG was dissolved in the same sterile saline solution (0.9% NaCl, USP) that was injected as the vehicle to control rats. All injections were made subcutaneously on the back of the trunk in a volume of 0.4–0.7 ml depending on the weight of the animal. For antemortem determination of microhematocrits, free flowing tail blood was collected in heparinized capillary tubes.

Euthanasia and collection of blood and tissue samples. All rats were euthanized by pentobarbital sodium overdose. Preterminal blood samples were collected by cardiac puncture, and serum was separated after clotting. Serum samples were assayed for EPO levels by using a commercial ELISA kit (Alpco Diagnostics, Windham, NH). Kidneys were rapidly removed, and tissue samples from the cortical region and medullary junction of a normal rat kidney, using gene-specific primer pairs. After the specificity of the PCR amplifications by sequencing the PCR product was verified, the cDNA fragment was subcloned into pGEM-T Easy Vector (Promega, Madison, WI). Competent cells were transformed with the pGEM-T-EPO-cDNA, grown, and plasmid DNA was harvested and purified by standard methods. The insert was cut with NcoI and Ndel restriction enzymes and purified. Sense and antisense DIG-labeled riboprobes were synthesized using T7 and SP6 transcription initiation sites in the insert, respectively. The yield of cRNA and efficiency of DIG labeling were assessed by the measurement of absorbance at 260 nm in a spectrophotometer and by DIG Quantification Strips (Roche Diagnostics, Indianapolis, IN), respectively.

Hybridization of EPO mRNA in kidney sections with DIG-labeled riboprobes was performed as described previously (19). After deparaffinization, sections were postfixed in 4% formalin for 30 min, washed, and quenched in 1.5% hydrogen peroxide for 25 min. Sections were then treated with proteinase K (10 μg/ml) at 37°C for 30 min, followed by acetylation. After being washed with PBS, sections were prehybridized in the hybridization mixture at 42°C for 40 min. Sections were then hybridized with DIG-labeled riboprobes (100–200 ng/ml) in the hybridization mixture overnight at 42°C. The hybridization mixture contained 250 μg/ml tRNA, 500 μg/ml herring sperm DNA, 2 μM EDTA, 1× Denhardt’s reagent, 10% dextran sulfate, 50% formamide, and 10 mM DTT in 4× SSC. After hybridization, sections were washed with 1× SSC at 60°C and blocked with maleic acid buffer. Sections were then overlaid with 1:100 or 1:200 dilution of anti-DIG-peroxidase-labeled antibody in blocking buffer at room temperature for 1–2 h. After washing, a color reaction was performed using a DAB substrate kit for 5 min. Sections were then counterstained with hematoxylin, dipped in 95% ethanol for 5 min, air

Immunochemistry staining for specific proteins. Kidney and spleen sections were stained for the immunohistochemical detection of specific proteins by the methods established in our laboratories (19). Briefly, deparaffinized and rehydrated sections were digested for 10 min at room temperature with protease 2 (Ventana Medical Systems, Tucson, AZ). Then, the sections were incubated with primary antibody at room temperature overnight. Appropriate peroxidase-conjugated secondary antibody was used at a dilution of 1:300 for 8 min. Antigen-antibody reaction sites were detected with a DAB basic kit (Ventana Medical Systems). Sections were counterstained with hematoxylin for 2 min. Except for the incubation with the primary antibody, sections were processed on a Ventana automated instrument at 40°C.

Polyclonal antibodies to CD68, CD45, CD20, CD3, vimentin, and α-smooth muscle actin used in this study have been verified in the clinical immunopathology laboratory of the Associated Regional University Pathologists Laboratories, where they are used routinely (www.aruplab.com). Monoclonal mouse anti-rat PCNA and Ki-67 antibodies were purchased from Dako Cytomation (Carpinteria, CA). The former has been verified by us previously (39). Goat polyclonal IgG to e-cadherin (sc-14682), and EPO (sc-1310), as well as the specific blocking peptide to EPO (sc-1310P), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Despite prior characterization and routine use, where appropriate negative controls for immunohistochemistry were run in parallel by either omitting the primary antibody or substituting the primary antibody with a preimmune IgG fraction or by preadsorption of the primary antibody by the corresponding blocking peptide. Where applicable, spleen or uterus sections were used as positive controls.

In situ hybridization for the cellular localization of EPO mRNA. Paraffin sections of kidneys were labeled for the cellular expression of EPO mRNA by in situ hybridization. Generation of digoxigenin (DIG)-labeled riboprobes was performed by the methods established in our laboratories (19, 40). A 340-bp cDNA probe specific for rat EPO was generated by RT-PCR on RNA extracted from the cortico-medullary junction of a normal rat kidney, using gene-specific primer pairs. After the specificity of the PCR amplifications by sequencing the PCR product was verified, the cDNA fragment was subcloned into pGEM-T Easy Vector (Promega, Madison, WI). Competent cells were transformed with the pGEM-T-EPO-cDNA, grown, and plasmid DNA was harvested and purified by standard methods. The insert was cut with NcoI and Ndel restriction enzymes and purified. Sense and antisense DIG-labeled riboprobes were synthesized using T7 and SP6 transcription initiation sites in the insert, respectively. The yield of cRNA and efficiency of DIG labeling were assessed by the measurement of absorbance at 260 nm in a spectrophotometer and by DIG Quantification Strips (Roche Diagnostics, Indianapolis, IN), respectively.

Hybridization of EPO mRNA in kidney sections with DIG-labeled riboprobes was performed as described previously (19). After deparaffinization, sections were postfixed in 4% formalin for 50 min, washed, and quenched in 1.5% hydrogen peroxide for 25 min. Sections were then treated with proteinase K (10 μg/ml) at 37°C for 30 min, followed by acetylation. After being washed with PBS, sections were prehybridized in the hybridization mixture at 42°C for 40 min. Sections were then hybridized with DIG-labeled riboprobes (100–200 ng/ml) in the hybridization mixture overnight at 42°C. The hybridization mixture contained 250 μg/ml tRNA, 500 μg/ml herring sperm DNA, 2 μM EDTA, 1× Denhardt’s reagent, 10% dextran sulfate, 50% formamide, and 10 mM DTT in 4× SSC. After hybridization, sections were washed with 1× SSC at 60°C and blocked with maleic acid buffer. Sections were then overlaid with 1:100 or 1:200 dilution of anti-DIG-peroxidase-labeled antibody in blocking buffer at room temperature for 1–2 h. After washing, a color reaction was performed using a DAB substrate kit for 5 min. Sections were then counterstained with hematoxylin, dipped in 95% ethanol for 5 min, air
dried, and mounted with coverslips using Vecta Mount (Vector Laboratories).

Examination of sections. Processed tissue sections were examined under a Reichert or Nikon light microscope, and digital pictures were taken with a Nikon 995 Coolpix camera.

Quantitative RT-PCR for EPO mRNA. Quantification of EPO mRNA expression in the renal cortex or corticomedullary junction was achieved by real-time RT-PCR by methods established in our laboratories (20, 39, 40). Total RNA was extracted from the tissues using the TRIzol method (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. The extracted total RNA was further purified using RNeasy Mini Columns with DNase I treatment (Qiagen, Valencia, CA) to remove traces of genomic DNA present in the samples. cDNA was prepared by reverse transcribing total RNA using oligo (dT) priming and Superscript Reverse Transcriptase II (Invitrogen) according to the manufacturer’s instructions. cDNA was amplified using TaKaRa Ex Taq R-PCR Version in the Smart Cycler II System (Cepheid, Sunnyvale, CA), and SYBR Green was used for detection. cDNA was amplified according to the following steps: 1) 95°C for 2 min; 2) 95°C for 10 s; 3) 66°C for 10 s; 4) 72°C for 17 s; and 5) 84°C for 6 s to detect SYBR Green (nonspecific products melt at <85°C and are therefore not detected). Steps 2–5 were repeated for an additional 39 cycles, while at the end of the last cycle the temperature was increased from 60 to 95°C (0.2°C/s) to produce a melt curve. PCR reactions were considered valid only if the amplification was linear and the amplifications efficiency was ≥95%. Parallel amplifications were performed using primer pairs specific for β-actin, a housekeeping gene. Relative expression values were computed by normalizing EPO expression to that of β-actin in the samples, and plotted as the percentage of mean values obtained in rats treated with saline. The primer pairs used were the following: for EPO cDNA: AGTCGCCTCTGGAGAGA (forward), AGGATGGCTTCTGAGAGCAG (reverse), amplicon 200 bp, annealing temperature 66°C; and for β-actin cDNA: AGAGGAAATCGTGCGTGACA (forward), CACTGTGTTGGCATAGAGGTC (reverse), amplicon 275 bp, annealing temperature 66°C.

Statistical analysis. Quantitative data are expressed as means ± SE. Differences among the means of multiple groups were analyzed by ANOVA followed by Tukey-Kramer’s multiple comparison test. Differences between the means of two groups were analyzed by unpaired t-test with or without Welch correction. P < 0.05 was considered significant.

RESULTS

Proliferation of peritubular interstitial cells in the renal cortex induced by PDG treatment. In our previous report we documented that PDG treatment, which causes an acute lyso-

Fig. 1. Immunohistochemical labeling of interstitial cells with proliferating cell nuclear antigen (PCNA). Rats were injected with either poly-D-glutamic acid (PDG; 250 mg·kg⁻¹·day⁻¹) or saline for 4 consecutive days and euthanized on day 1 or 6 after the cessation of the treatment. Formalin-fixed and paraffin-embedded tissue sections were immunohistochemically labeled with a specific antibody to PCNA. A and B: representative profiles of cortical region in a normal rat kidney. D and E: representative profiles of cortical regions in PDG-treated rat kidneys 1 day after the cessation of treatment. F and G: representative profiles of cortical regions in PDG-treated rat kidneys 6 days after the cessation of treatment. Proliferating cell nuclei are labeled in brown (arrows). However, in these profiles there are interstitial cells that did not label with the PCNA antibody (arrowheads). C: negative control for immunohistochemical labeling on a PDG-treated rat kidney (day 1), where the primary antibody was omitted from the incubation.
tubular cells in PDG-treated rat kidneys.

We further confirmed the proliferative nature of peritubular interstitial cells in PDG-treated rat kidneys by immunohistochemical labeling for Ki-67 protein. Unlike PCNA, Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis) but is absent from resting cells (G0) (36). Figure 2, A–C, shows labeling of renal cortical sections in rats treated with saline or with PDG for 4 consecutive days and euthanized 1 day after the treatment. As shown in Fig. 2A, Ki-67 antibody did not label either the tubular cells or peritubular interstitial cells in saline-treated rats except a sporadic one (arrow in panel A). However, in PDG-treated rat kidneys, several intensely labeled nuclei could be seen in the peritubular regions (thin arrows in Fig. 2, B and C). As with PCNA antibody, the Ki-67 antibody did not label all the cells in the peritubular interstitial cells (thick arrows in Fig. 2C). Labeling of tubular cells, if any, in PDG-treated rats was occasional or sporadic, as shown in Fig. 2B (thick arrow).

Immunohistochemical staining for different cell surface markers (CDs) specific for inflammatory cells. Studies showed that one of the hallmarks of a proteinuria-induced and lysosomal-mediated tubulointerstitial reaction and fibrosis is interstitial infiltration of inflammatory cells (6, 9, 50). Therefore, we examined whether the PDG-induced increase in the peritubular interstitial cell population was caused by infiltrating inflammatory cells. To screen for various inflammatory cell types, we used specific antibodies to cell surface markers (CDs). Sections of normal rat spleen were used as positive controls for immunohistochemical labeling. As shown in Fig. 3, the peritubular interstitial cells in PDG-treated rats did not stain for CD68 (marker for macrophage/monocyte lineage), CD45 (leukocyte common antigen), CD20 (marker for B lymphocytes), or CD3 (marker for T lymphocytes). In contrast, sections of spleen showed distinct and strong labeling for all these markers. Although there was some nonspecific labeling of proximal tubular epithelial cells with CD3 antibody in both saline- and PDG-treated rat kidneys (arrows), the interstitial cells in both groups did not show any labeling.

Immunohistochemical labeling of peritubular cells for CD73 or ecto-5′-nucleotidase. After confirming that proliferating peritubular interstitial cells in the PDG-treated rat kidney were not inflammatory cells, we examined whether these were...
resident fibroblast-like cells of the cortical region. To identify and characterize these cells further, we performed immunohistochemical labeling using a specific antibody to CD73 or ecto-5'-nucleotidase, a marker for renal cortical resident fibroblast-like cells (12, 17, 29). As shown in Fig. 4B, the proliferating peritubular cells in the PDG-treated rat kidneys immunolabeled with CD73 antibody, similar to the peritubular interstitial cells in a normal kidney (Fig. 4A). The additional expression of CD73 in proximal tubular brush border is well known (4, 12, 38, 48). Together, these observations confirm that the proliferating peritubular cells in PDG-treated rat kidneys were in fact resident fibroblast-like cells.

**Immunohistochemical labeling of peritubular cells for EPO.** Since cortical peritubular interstitial cells are known to synthesize EPO, we examined whether the CD73-positive, proliferating peritubular interstitial cells also expressed EPO protein. As shown in Fig. 5C, immunohistochemical labeling with a specific antibody to rat EPO clearly documented that these cells were rich in EPO protein. Furthermore, as depicted in Fig. 5D, occasional mitotic figures were seen among the EPO-positive peritubular interstitial cells. Although this EPO antibody has been in use in our laboratory, we performed negative controls to document the specificity of labeling (Fig. 5, E and F).

**Expression of EPO mRNA in the renal cortex of PDG-treated rats.** To examine whether PDG treatment also induced increased expression of EPO mRNA, we performed quantitative real-time RT-PCR using specific primers for rat EPO. As shown in Fig. 6, the relative expression of EPO mRNA, normalized to the expression of β-actin mRNA, was increased by about threefold in the renal cortex of PDG-treated rats compared with saline-treated rats.
Cellular localization of EPO mRNA in peritubular interstitial cells. To localize the increased EPO mRNA at the cellular level in PDG-treated rat kidneys, we performed in situ hybridization using DIG-labeled riboprobes specific for EPO mRNA. Figure 7 shows the results of the in situ hybridization studies. In both saline- and PDG-treated rat kidneys, the sense riboprobe did not label any structure, indicating the specificity of our technique (Fig. 7, A and C). The antisense riboprobe labeled some cells in the peritubular interstitial spaces in the corticomedullary junction of normal rat kidney (Fig. 7B). However, in PDG-treated rat kidneys, the labeling of peritubular cells was intense in several places, as shown in Fig. 7D, although regions of lesser intensity were observed (not shown). The labeling was restricted to cytoplasm in the perinuclear region. Adjacent tubular cells were not labeled with the antisense riboprobe, indicating as well that the labeling was specific for EPO mRNA.

Persistence of proliferating peritubular cells in PDG-treated rat kidneys. To further elucidate the nature of the proliferative response seen with PDG treatment, we carried out time-lag studies. We treated rats with saline or PDG for 4 consecutive days and euthanized them 6 days after the cessation of treatment. Figure 1, F and G, shows kidney sections from these PDG-treated rats immunolabeled with PCNA antibody. As shown in these profiles, the peritubular region was studded with a large number of cells even 6 days after the cessation of PDG treatment. PCNA antibody clearly labeled several (thin arrows) but not all (thick arrow) peritubular interstitial cells. There were no discernible differences in the proportion of proliferating peritubular cells in rats treated with PDG either at 250 or 150 mg/kg body wt (not shown). Furthermore, the profiles of these cells in time-lag studies are comparable to the ones seen in rats euthanized 1 day after a 4-day treatment with PDG (Fig. 1, D and E). Labeling for Ki-67 protein in the kidneys of rats that were euthanized 6 days after the cessation of a 4-day treatment with PDG also revealed proliferating peritubular interstitial cells, although the fraction of labeled cells appeared to be much smaller compared with the PCNA (Fig. 2D).

Persistent expression of EPO protein and mRNA by proliferating peritubular cells in PDG-treated rat kidneys. To assess whether peritubular cells continue to express EPO protein and mRNA after PDG administration ceased, we performed immunohistochemistry and real-time RT-PCR experiments, respectively. As shown in Fig. 8, immunohistochemistry revealed that even 6 days after the cessation of PDG treatment, substantial numbers of EPO-positive peritubular cells were detected. This picture was very similar to the one seen in PDG-treated rats which were euthanized 1 day after the cessation of PDG treatment for 4 consecutive days (Fig. 5 vs. Fig. 8). In parallel, real-time RT-PCR studies showed a threefold increase in EPO mRNA in the renal corticomedullary tissue (Fig. 9), which was similar to that seen in rats that were euthanized 1 day after the cessation of PDG treatment (Fig. 6 vs. Fig. 9).

Effect of PDG treatment on fibrotic reaction and epithelial mesenchymal transition in the kidney. Recent reports demonstrated that proteinuria-induced tubulointerstitial reaction or fibrosis is mediated through lysosomal injury of proximal tubular cells (6–8). Since PDG treatment causes proximal tubular lysosomal overload, we examined whether PDG treatment induces a fibrotic reaction and/or epithelial mesenchymal transition in the kidney. A fibrotic reaction was assessed by Trichrome stain. As shown in Fig. 10B, no fibrotic reaction could be seen in PDG-treated rats, except the expected staining of collagen fibers around the vasculature. We also examined the effect of PDG treatment on epithelial mesenchymal transition, by immunohistochemical labeling for vimentin. Vimentin is an intermediate filament protein expressed in cells of mesenchymal origin. When epithelial cells transform into mesenchyme, they express vimentin (13). As shown in Fig. 11, PDG treatment caused minimal and patchy epithelial mesenchymal transition in the interstitium, but not in the tubules.

Immunohistochemical localization of α-smooth muscle actin. Using a specific antibody to α-smooth muscle actin, we examined whether the proliferating peritubular cells show evidence of transformation into myofibroblasts. As shown in Fig. 12, transformation of proliferating peritubular cells into myofibroblasts was not observed in PDG-treated rat kidneys.
myofibroblasts was not observed in rats euthanized either on day 1 or day 6 after the cessation of PDG treatment. The expected labeling of the smooth muscle layer of blood vessels in Fig. 12B confirms the efficiency of our labeling method for α-smooth muscle actin.

Studies on the biological effects of PDG-induced increase in EPO production. Since increased EPO production and secretion in the kidney should result in erythropoietic effects, we measured serum EPO levels and hematocrits in control and PDG-treated animals. Serum EPO levels in PDG-treated groups were similar to the ones seen in saline-treated control animals [6.4 ± 0.4 mU/ml in saline-treated controls (n = 6) vs. 5.7 ± 0.7 in 4-day model (n = 6), and 4.7 ± 1.4 mU/ml in time-lag model (n = 5); not significantly different from each other]. In line with these data, the hematocrit values in PDG-treated group did not show any increase [42 ± 2 controls (n = 4) vs. 43 ± 0.3 PDG-treated group (n = 3)]. These data suggest that the EPO expressed by the increased numbers of interstitial cells in the kidneys of PDG-treated rats was not released into the systemic circulation or that it was in a biologically inactive form that was not detected by the utilized assay.

Effect of PDG treatment on the viability of proximal tubular cells. In our previous reports, we documented that PDG treatment did not cause proximal tubular cell necrosis. In those studies, we assessed proximal tubular cell necrosis directly by conventional morphological examination and indirectly by determining the incorporation of [3H]thymidine into nuclear DNA of proliferating S-phase cells in vivo (18). The indirect assessment was based on the observation that necrosed tubular cells are promptly replaced by adjacent regenerating cells (28). However, it is possible that PDG treatment induces only apoptotic cell loss and not necrosis of proximal tubular cells. Hence, we examined the kidney sections from saline- or
PDG-treated rats for signs of apoptotic cell death using the TUNEL assay. As shown in the Fig. 13, similar to saline-treated control rats, PDG-treated rats did not show any apoptotic cell death of proximal tubular cells. On the other hand, apoptotic cells were clearly seen in the positive controls (cisplatin-treated rat kidneys) (37).

DISCUSSION

How to induce production of EPO safely in the normal or diseased kidney has been a therapeutic dilemma. In this study, we present an apparently safe method whereby EPO-producing peritubular cells in rat kidney were specifically induced to proliferate extensively with preserved expression of EPO.

EPO is a 34-kDa glycoprotein which regulates hematopoiesis through its actions as a survival factor (antiapoptotic), mitogen, and differentiation factor in erythroid progenitor cells (15, 26, 49). EPO also acts as a mitogen and survival factor in several nonhematopoietic organs and stimulates angiogenesis (3, 33, 42, 43, 45). We recently reported that tubular and other renal cells express authentic EPO receptors through which EPO elicits its antiapoptotic, motogenic, chemokinetic, and mitogenic responses (42–46).

The kidney is the predominant (>80%) and the liver the secondary source of EPO in the adult. EPO synthesis and secretion increase when intrarenal oxygen pressure is reduced by hypoxia or anemia (15, 49). EPO is normally produced in outer medullary interstitial cells of the cortical labyrinth, and upon hypoxic stimuli, EPO-producing cells are seen in the interstitium of mid- and outer cortex and outer stripe of the medulla as well as the medullary rays. The rate of EPO synthesis, a 165-amino acid glycoprotein, is primarily under transcriptional, and to a lesser extent, posttranscriptional control (27). The EPO gene is activated by low PO2 or iron depletion via a cellular “oxygen-sensor.” Hypoxia-inducible factor (HIF) is a transcriptional complex that plays a central

Fig. 6. Quantification of EPO mRNA in the renal cortex. Rats were injected with either PDG (250 mg·kg⁻¹·day⁻¹) or saline for 4 consecutive days and euthanized 1 day after the cessation of treatment. Total RNA was extracted from the renal cortex and reverse transcribed. Using specific primer pairs for EPO and β-actin, PCR amplifications were performed on the cDNA using SYBR Green for detection. Relative expression values were computed by normalizing the EPO expression to that of β-actin in the samples and plotted as the percentage of mean values obtained in rats treated with saline. Statistical significance between the means of the 2 groups was assessed by unpaired t-test.

PDG-treated rats for signs of apoptotic cell death using the TUNEL assay. As shown in the Fig. 13, similar to saline-treated control rats, PDG-treated rats did not show any apoptotic cell death of proximal tubular cells. On the other hand, apoptotic cells were clearly seen in the positive controls (cisplatin-treated rat kidneys) (37).

Fig. 7. Cellular localization of EPO mRNA in peritubular interstitial cells by in situ hybridization. Rats were injected with either PDG (250 mg·kg⁻¹·day⁻¹) or saline for 4 consecutive days and euthanized 1 day after the cessation of treatment. Formalin-fixed, paraffin-embedded kidneys were processed for in situ hybridization. mRNA in the sections was hybridized using DIG-labeled riboprobes that correspond to a small portion of the EPO gene sequence. Sites of hybridization were visualized by incubating with peroxidase-labeled anti-digoxigenin antibody followed by color reaction with diaminobenzidine (DAB). A and B: profiles from salinetreated rats. C and D: profiles from PDG-treated rats. A and C: profiles where the sections were probed with sense riboprobe (negative controls). B and D: profiles where the sections were probed with antisense riboprobe. The sites of hybridization are seen as a reddish brown coloration in the peritubular region in B and D (arrows). Intensity of labeling in PDG-treated rats varied from strong, as shown here, to much lighter (not shown).
regulatory role in the expression of oxygen-sensitive genes. Mature EPO is not stored before release, but secretion coincides with the simultaneous removal of a terminal arginine residue.

Fibroblast-like cells in the corticomedullary interstitium of the kidney synthesize EPO, while tubular cells do not (1, 24, 25, 31, 32, 34). The former specialized cells express ecto-5’-nucleotidase or CD73 on their surface and are strategically located so as to facilitate EPO secretion into peritubular capillaries, which, in turn, carry EPO into the systemic circulation. Significantly, EPO-producing cells are in direct contact with the basolateral aspects of proximal tubular and outer medullary cells. This anatomic relationship facilitates bidirectional signaling between tubular and fibroblast-like peritubular interstitial cells (32). This is exemplified by paracrine EPO signaling reported by us previously (42, 43, 45, 46), as well as paracrine signaling via an IGF-1 axis reported by others (16), where signals that originate in peritubular fibroblast-like cells affect the function and proliferative response of adjacent tubular cells. In contrast to the above observations, the current study on PDG suggests potential paracrine signaling originating from the proximal tubular cells, which likely induces proliferation of EPO-producing peritubular cells. Specifically, we demonstrate that a PDG-induced lysosomal storage condition in proximal tubular cells is associated with extensive proliferation of EPO-producing peritubular fibroblast-like cells with sustained EPO expression. Considering the facts that, unlike proximal tubular cells, proliferating peritubular cells do not show PDG-induced lysosomal alterations (23), administered PDG is rapidly cleared from body fluids (18), and there is the persistence of proliferating EPO-producing cells even 6 days after the cessation of PDG treatment (the current study), the signal for the proliferation of EPO-producing peritubular cells seems to be paracrine in nature, most probably originating from PDG-loaded proximal tubular cells.

PDG is a synthetic polymer of n-glutamic acid units. Peptides containing d-amino acid moieties are resistant to hydrolysis in mammals. During our attempts to inhibit the nephrotoxicity of aminoglycoside antibiotics by polyanionic peptides that mimic polyaspartic acid (18, 23), we discovered that a low-molecular-weight PDG (±20 kDa) causes an acute lysosomal storage condition in proximal tubular cells associated with an extensive proliferation of peritubular fibroblast-like cells in rat kidney. Studies conducted by others on rat models of experimental proteinuria indicated that lysosome-mediated tubular cell injury brought about by protein overload of these digestive organelles can elicit tubulointerstitial reactions, which may play a role in the pathogenesis of tubulointerstitial nephritis and fibrosis (2, 6–9, 41, 50). The hallmark of these models of massive proteinuria-induced tubulointerstitial reaction and fibrosis is the infiltration of inflammatory and/or proinflammatory cells with the production and release of inflammatory cytokines, chemokines, oxygen radicals, complement deposition, matrix protein overproduction, inadequate matrix degradation, matrix remodeling, etc., ultimately leading to interstitial fibrosis and loss in kidney function (2, 7, 41, 50). Thus, in both clinical and experimental proteinuric conditions,

Fig. 8. Persistently proliferating peritubular interstitial cells in PDG-treated rat kidneys express EPO protein. Rats were treated with saline or with PDG (250 or 125 mg kg⁻¹ day⁻¹) for 4 consecutive days and euthanized 6 days after the cessation of treatment. Formalin-fixed and paraffin-embedded tissue sections were immunohistochemically labeled with a specific antibody to EPO. A: labeling of peritubular cells in a saline-treated rat kidney. B and C: representative profiles from PDG-treated rat kidneys. Positive labeling for EPO is seen as brown coloration all over the peritubular interstitial cells (arrows).

Fig. 9. Persistently proliferating peritubular interstitial cells in PDG-treated rat kidneys express EPO mRNA. Rats were treated with saline or with PDG (250 or 125 mg kg⁻¹ day⁻¹) for 4 consecutive days and euthanized 6 days after the cessation of treatment. Total RNA was extracted from the corticomedullary regions and reverse transcribed. Using specific primer pairs for EPO and β-actin, PCR amplifications were performed on the cDNA employing SYBR Green for detection. Relative expression values were computed by normalizing the EPO expression to that of β-actin in the samples and plotted as the percentage of mean values obtained in rats treated with saline. Since both groups of PDG-treated rats gave comparable results, the data from them were pooled and plotted as one bar. Statistical significance between the means of the saline- or PDG-treated groups was assessed by unpaired t-test with the Welch correction.
tubulointerstitial reactions are initiated by glomerular protein leaks. In contrast to these published models, as documented here, our model of a proximal tubular cell lysosomal storage condition induced by low doses of a low-molecular-weight PDG does not elicit any inflammatory reaction. On the other hand, in our model we observe an extensive proliferation of EPO-producing peritubular fibroblast-like cells. In fact, it is well known that inflammatory cytokines, such as IL-1β and TNF-α, inhibit EPO expression (14, 46). Thus our PDG model, as documented here, is distinctly different from other published models of massive proteinuria-induced and tubular lysosomal injury-mediated interstitial reactions.

In accordance with the above conclusion, the proliferating peritubular cells in PDG-treated rat kidneys did not stain for CD68-, CD45-, CD20-, and CD3-specific markers for macrophage/monocyte lineage, leukocytes, and B and T lymphocytes, respectively. This ruled out the possibility that the proliferating interstitial cells in PDG-treated rat kidneys are inflammatory cells. On the other hand, immunohistochemical labeling with specific CD73 (ecto-5′-nucleotidase) antibody, a marker enzyme for renal cortical interstitial fibroblasts (12, 17, 29), established the nature of these proliferating cells as fibroblasts. Since cortical peritubular fibroblast-like cells synthesize EPO, we examined by immunohistochemistry, real-time RT-PCR, and in situ hybridization whether CD73-positive proliferating interstitial cells also express EPO protein and mRNA. Our results confirmed that the proliferating peritubular cells in PDG-treated rat kidneys do synthesize EPO.

Furthermore, we document that even 6 days after the cessation of a 4-day course of PDG, increased numbers of EPO-producing peritubular cells were persistently present in the rat kidney, which, in addition, appeared qualitatively and quantitatively similar to the ones that were observed immediately after a 4-day treatment with PDG. The presence of large numbers of EPO-producing peritubular cells in PDG-treated rats after a time lag may be due to persistent proliferation or continued survival of already proliferated cells. In our previous study, we did not observe a significant proliferative response of peritubular cells 7 days after the cessation of PDG treatment as assessed by [3H]thymidine incorporation into nuclear DNA of S-phase cells (23). The method of [3H]thymidine incorporation assesses the proliferative response only in the shorter S phase of the cell cycle, whereas immunohistochemical labeling for PCNA and Ki-67, although less specific, monitors a given proliferative response over a relatively longer window of expression during the cell cycle. In addition or alternatively, it is conceivable that PDG administration alters the phenotype of the proliferating peritubular cells, making them constitutively produce EPO without further need for PDG-induced paracrine signaling. Obviously, more extensive studies by approaches which are beyond the scope of this communication are needed to reach a sound conclusion in this respect.

Fig. 11. Effect of PDG treatment on epithelial mesenchymal transition in the kidney. Rats were injected with either PDG (250 mg·kg⁻¹·day⁻¹) or saline for 4 consecutive days and euthanized 1 day after the cessation of treatment. Formalin-fixed and paraffin-embedded kidney sections were stained with trichrome reagent. A: representative profile of cortical region in a saline-treated rat. B: representative profile of cortical region in a PDG-treated rat. Note the minimal and patchy labeling for vimentin in the interstitial region, but not over the tubules (arrows in B). C: positive control for vimentin where smooth muscles of uterine myometrium are labeled for vimentin.
Another important aspect of our study is the finding of a large increase in the number of interstitial fibroblasts without any evidence of fibrosis or epithelial mesenchymal transformation, as shown by both absent Trichrome and immunohistochemical labeling for vimentin. In addition, these cells were negative for α-smooth muscle actin, indicating that there was no apparent transformation into myofibroblasts. These findings underscore the fact that tubular cell-mediated proliferation of fibroblasts can apparently occur independently of epithelial mesenchymal transformation, without transformation into myofibroblasts, and without fibrosis.

The mitogenic signal that specifically stimulates the proliferation of EPO-producing peritubular cells in PDG-treated rat kidneys is unknown at present. A complex cytokine network is recognized in the tubulointerstitial microenvironment of the normal kidney, which can be perturbed in disease conditions (35, 47). In fact, progression of chronic kidney disease has been ascribed to the profibrotic actions of released cytokines such as TGF-β (11). Hence, we hypothesize that the noncytotoxic lysosomal storage of PDG in proximal tubular cells results in the elaboration and release of signaling molecules, one or more of which specifically stimulate the robust proliferation of EPO-producing peritubular cells. Obviously, the molecular identification of such paracrine signals, currently pursued by us, will greatly advance our knowledge of the biology of EPO-producing cells and EPO production in the kidney.

Finally, in the current study we were unable to detect a PDG-induced rise in serum EPO and hematocrit levels. This unexpected finding may be due to a number of reasons. First, the EPO produced de novo by increased numbers of interstitial fibroblast-like cells may not be released into the circulation because these animals are normoxic or because these cells have changed their phenotype and unphysiologically store EPO, possibilities that await examination. Second, the synthesized EPO in PDG-treated rats may be a bioinactive variant of native EPO that lacks erythropoietic activity and is not detected by standard assays. However, preliminary observations suggest that the EPO produced by increased numbers of interstitial cells may be biologically active locally, because we found evidence for angiogenesis in the kidneys of PDG-treated rats (not shown here).

In conclusion, we present a novel method whereby the number of EPO-producing cells in the kidney can be rapidly expanded. The administration of PDG appears safe and lacks cytotoxicity and proinflammatory or profibrotic effects. We
expect that this unique method will help to identify intrarenal signals that specifically induce proliferation of EPO-producing cells, facilitate the study of renal EPO biology, and aid in the development of novel and more economical approaches in the treatment of patients with EPO-sensitive anemias and other EPO-responsive conditions. (44)

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DISCLOSURES


REFERENCES