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Renal response to repetitive exposure to heme proteins: Chronic injury induced by an acute insult

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Renal response to repetitive exposure to heme proteins: Chronic injury induced by an acute insult.

Background. Renal diseases are conventionally classified into acute and chronic disorders. We questioned whether acute, reversible, renal insults may be induced to incite a chronic scarring process, employing as an acute insult the glycerol model of heme protein-induced renal injury.

Methods. Rats were subjected to weekly injections of hypertonic glycerol for up to six months. Renal function was serially determined, and the effect of such insults on renal histology and renal expression of collagen and fibrogenic cytokines was assessed.

Results. After the first injection of glycerol, which, expectedly, induced a prompt fall in the glomerular filtration rate (GFR), subsequent injections encountered a remarkable renal resistance in that the fall in GFR was markedly blunted. This resistance to acute decline in renal function in rats subjected to repetitive injections of glycerol was accompanied by less necrosis and apoptosis of renal tubular epithelial cells after such injections. The attenuation in the fall in GFR in response to repetitive exposure to glycerol-induced heme protein injury was maintained for up to six months. A progressive decline in GFR appeared after three months and was accompanied by histologic tubulointerstitial injury, the latter assessed at six months. These kidneys demonstrated up-regulation of collagen I, III, and IV in conjunction with increased expression of the oxidant-inducible, chemotactic cytokine, monocyte chemoattractant protein-1 (MCP-1), and the oxidant-inducible, fibrogenic cytokine, transforming growth factor-β1 (TGF-β1). The exposure of the kidney to a single injection of hypertonic glycerol increased the expression of both cytokines some three to five days following this exposure, while the exposure of NRK 49F cells in culture to an iron-dependent model of oxidative stress also increased expression of TGF-β1 and collagen mRNAs.

Conclusions. We conclude that this nephrotoxic insult, repetitively administered, encounters a resistance in the kidney such that the expected fall in GFR does not occur. However, with time, such resistance is accompanied by a decrease in GFR, the latter associated with chronic tubulointerstitial disease. Thus, a long-term cost is exacted, either along with, or as a consequence of, such resistance. We suggest that chronic up-regulation of such oxidant-inducible genes such as TGF-β1 and MCP-1 contributes to tubulointerstitial disease, and iron-mediated oxidative stress may directly induce TGF-β1.

Key words: renal resistance, kidney scarring, nephrotoxicity, tubulointerstitial disease, iron toxicity, oxidative stress.

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The classification of renal insufficiency into acute or chronic disorders is based on such differentiating features as the nature and duration of the offending insult, the rapidity of onset of such insults, the pathologic changes instigated in the kidney in such settings, and finally, the reversibility of changes so induced. Acute renal insufficiency arising in the setting of ischemic or nephrotoxic insults occurs relatively rapidly, commonly involves sublethal and lethal injury to tubular epithelial cells, and is usually a reversible condition [1–4]. Recovery from such acute insults requires, in part, reparative responses that restore vitality to sublethally injured cells in conjunction with regenerative mechanisms that replenish cells lost by necrosis or apoptosis [1–4].

Chronic renal insufficiency arises in fundamentally different settings from those that precipitate acute renal failure, arising as it does from diabetic nephropathy, assorted glomerulopathies, vasculitides, interstitial nephritides, and sclerosing vascular lesions [5]. The histologic appearance of chronic renal insufficiency is dominated by elaboration of extracellular matrix and interstitial cellular infiltration; concomitantly, the tubular epithelial compartment undergoes dystrophic and atrophic changes, while sclerosis and other architectural alterations envelope the glomerular tuft.

These distinct features of acute and chronic renal insufficiency would seem to argue that the pathogenesis of these disorders would be similarly distinct, as these disorders originate from and are sustained by different underlying mechanisms. But how immutable are the boundaries that demarcate the respective origins of these two renal syndromes? Can mechanisms conventionally incriminated in the pathogenesis of either one of these seemingly disparate forms of renal disease, in a given circumstance, be induced to provoke the other?

We approached these questions from the perspective of insults that characteristically induce acute renal fail-

ure, specifically questioning whether repetitive exposure to such insults can convert or transform an acute reversible lesion to one that is chronic and associated with fibrogenesis, matrix expansion, and scarring. There is a growing sense in the study of progressive renal disease that certain forms of chronic renal injury may be a consequence of repeated exposure to acute insults to the kidney. For example, ischemic nephropathy, which may account for as much as 15% of end-stage renal disease, may reflect, at least in some instances, recurrent acute ischemic insults to the kidney [6]; repeated episodes of acute cellular rejection provide one of the strongest predictors for chronic allograft dysfunction [7]. The progression of certain glomerulopathies such as IgA nephropathy, lupus nephropathy, and membranoproliferative glomerulonephritis may reflect repeated deposition of immune reactants in the glomeruli, and attendant recruitment of an inflammatory and ultimately fibrogenic response [8]. Acute intermittent urinary tract obstruction may trigger repeated episodes of inflammation in the kidney that incite a chronic fibrosing interstitial response [9].

As a method of exposing the kidney to an acute insult and in part because of our ongoing interest in determining the response to injury induced by heme proteins, we used the glycerol model of acute renal failure. This model is an established, well-characterized model of acute renal failure that reflects the nephrotoxicity of a defined insult, namely, heme protein-instigated, oxidant-mediated injury [10, 11]. Iron-dependent, oxidative stress provides a pathogenetic pathway incriminated in both acute and chronic renal disease [10–13]. Moreover, repetitive application of this acute insult may provide a useful model for studying hematuric conditions in rodents and for which there are few, if any, satisfactory models. Finally, repetitive application of this acute insult is germane, in general, to the phenomenon of acquired resistance to renal injury [14–20], and in particular, it allows the exploration of the nature of renal responses following repetitive exposure to heme proteins.

METHODS

The model of repetitive administration of glycerol

Glycerol-induced renal injury was induced by the weekly administration of 7.5 mL/kg body wt of a 50% solution of glycerol and water, half of the volume injected into each anterior thigh muscle under ether anesthetic [21]. The control rats received no injection. Both groups were deprived of water overnight for 16 hours but were allowed free access to Purina rat chow (Ralston Purina Co., St. Louis, MO, USA). All injections were administered at weekly intervals over six months, except for the second and third injections, the second injection being nine days from the first, and the third being nine days from the second. This was done since it was found in preliminary studies that such intervals, in the initial stages of repetitively administering glycerol, were optimal in allowing the expression of resistance without incurring appreciable mortality between these injections.

Measurement of creatinine concentrations and urinary protein excretion

Serum and urine creatinine concentrations were determined by the Jaffe reaction using a Beckman Creatinine Analyzer II (Beckman Instruments, Inc., Fullerton, CA, USA). Urinary protein excretion was performed using the Coomassie method. Creatinine clearances were determined five days after the intramuscular injection of glycerol. This time point was chosen so as to obtain a "steady-state" assessment of serial changes of kidney function over a protracted period of time without the confounding effect of acute renal hemodynamic and other reversible effects of heme proteins that exist in the period immediately following the administration of glycerol. However, to provide a complete characterization of changes in renal function after repetitive administration of glycerol, additional studies were undertaken in which serum creatinine was determined the day after the injection of glycerol.

Determination of creatine kinase activity, plasma hemoglobin concentration, and lactate dehydrogenase

Plasma creatine kinase (CK) activity was measured by a colorimetric method based on the production of phosphorus using a Sigma Diagnostics Creatine Phosphokinase kit. Plasma hemoglobin concentrations were assayed by the method described by Winterbourn [22], while lactate dehydrogenase (LDH) activity was assayed by determining the rate of formation of nicotinamide adenine dinucleotide (NADH).

Histologic and morphometric studies

The kidney was subjected to perfusion fixation in formalin and sections stained with hematoxylin and eosin [23]. The extent of acute cellular injury, as assessed by the severity of tubular epithelial cell necrosis, was evaluated 24 hours after glycerol in rats subjected to one injection of glycerol or three sequential injections of glycerol, according to the protocol described previously in this article [12]. Apoptosis involving both proximal and distal tubules was assessed by the TUNEL technique using the Apoptag method (Intergen Co., Purchase, NY, USA), as described previously [12].

Chronic tubulointerstitial injury was assessed by histologic evaluation of the extent of tubular atrophy and by quantitation of interstitial collagen in cortex and medulla, the latter undertaken using the Bioquant Imaging and Morphometric System (R and M Biometrics Inc., Nashville, TN, USA). Kidney sections were stained with Masson Trichrome, which detects collagen in the kidney as the blue-staining area. Twenty fields were randomly

Table 1. Characteristics of rats subjected to repetitive glycerol injections and control rats after six months

	Control	Glycerol	*P* value
Body weight *g*	497 ± 7	418 ± 9	<0.05
Hematocrit %	52 ± 1	41 ± 1	<0.01
Systolic blood pressure *mm Hg*	139 ± 6	137 ± 6	NS
Kidney weight *g*	2.04 ± 0.05	1.95 ± 0.11	NS
Kidney weight/body weight	0.41 ± 0.01	0.47 ± 0.02	0.05
Urinary flow rate *mL/24 h*	28 ± 3	45 ± 4	<0.05
Urinary protein excretion *mg/24 h*	73 ± 13	143 ± 24	<0.05

The control and glycerol-treated rats comprised $N = 5$ and $N = 5$, respectively, for all parameters except for kidney weights where $N = 5$ and $N = 4$, respectively.

selected in the cortex and again in the medulla, and mean scores were calculated for interstitial collagen present in the cortex and medulla.

RNA extraction and Northern blot hybridization

Total RNA from rat kidney and from cultured cells was isolated using a modification of the guanidinium-isothiocyanate/cesium chloride method, and Northern blot analysis was performed, as previously described [23]. Aliquots (20 μg) of total RNA were separated by electrophoresis. Autoradiograms were quantitated by computer-assisted videodensitometry, and the results were standardized by the method of Correa-Rotter, Mariash, and Rosenberg [24]. This established method of standardization corrects for any variability due to loading and transfer, and factors the optical density of the message for the given gene with the optical density of the 18S rRNA, the latter obtained on a negative of the ethidium bromide-stained nylon membrane. Probes for rat collagen α1(I), α1(III), and α1(IV), and transforming growth factor-β1 (TGF-β1) were employed, as described previously [23].

Exposure of NRK 49F cells to an iron-based oxidant system

Rat kidney fibroblast (NRK 49F) cells were maintained in culture as previously described in Dulbecco's modified Eagle's medium (DMEM) with 5% newborn calf serum and supplemented with 0.1 mmol/L nonessential amino acids [23]. Following growth to near confluency, the serum-containing DMEM was replaced by a similar medium containing 1% insulin-transferrin-selenium instead of serum, after which these cells were incubated for six hours. The cells were then washed twice with Hank's balance salt solution (HBSS) and incubated for two hours in either HBSS (control) or HBSS containing an iron-driven, oxidant-generating system consisting of $FeCl_3$ (20 μmol/L)/ethylenediaminetetraacetic acid (EDTA; 200 μmol/L)/ascorbate (500 μmol/L) [25]. The cells were then washed once in HBSS and maintained for 18 hours in the presence of DMEM containing 1% insulin-transferrin-selenium, after which RNA was extracted.

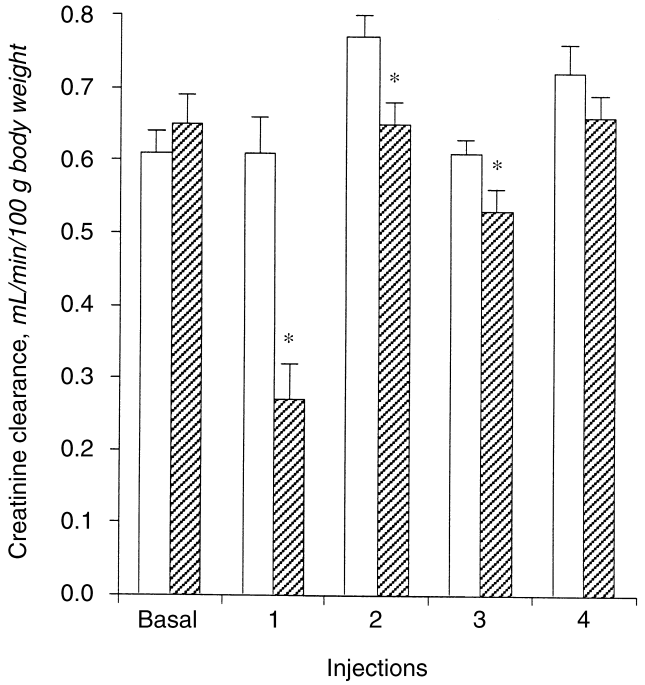

Fig. 1. Sequential determinations of creatinine clearance in rats subjected to repetitive injections of glycerol over four to five weeks. Symbols are: (□) control rats; (▨) glycerol-treated rats ($N = 5$ in control rats and $N = 5$ in glycerol-treated rats, except at baseline and at first injection when $N = 7$ and $N = 6$, respectively, in glycerol-treated rats). *$P < 0.05$ vs. control at that time point.

Statistical analysis

Data are presented as means ± SEM. For comparisons of two groups, the unpaired or paired Student *t*-test or the nonparametric Mann–Whitney test was used as appropriate. For analyses involving more than two groups, analysis of variance (ANOVA) and the Student–Neumann–Keuls test were employed. The results are considered significant for $P < 0.05$.

RESULTS

Sequential changes in creatinine clearance and other functional parameters in rats subjected to repetitive glycerol injections

Over the six months of observation, the rate of increase of body weight in glycerol-treated rats was lower than in the control rats, and by six months, the mean body weight in the glycerol-treated rats was significantly less as compared with the untreated controls (Table 1). Creatinine clearance data were thus factored for 100 g body wt. Figure 1 shows sequential creatinine clearance data for the first four injections of glycerol. As demonstrated and expected, there was a significant and promi-

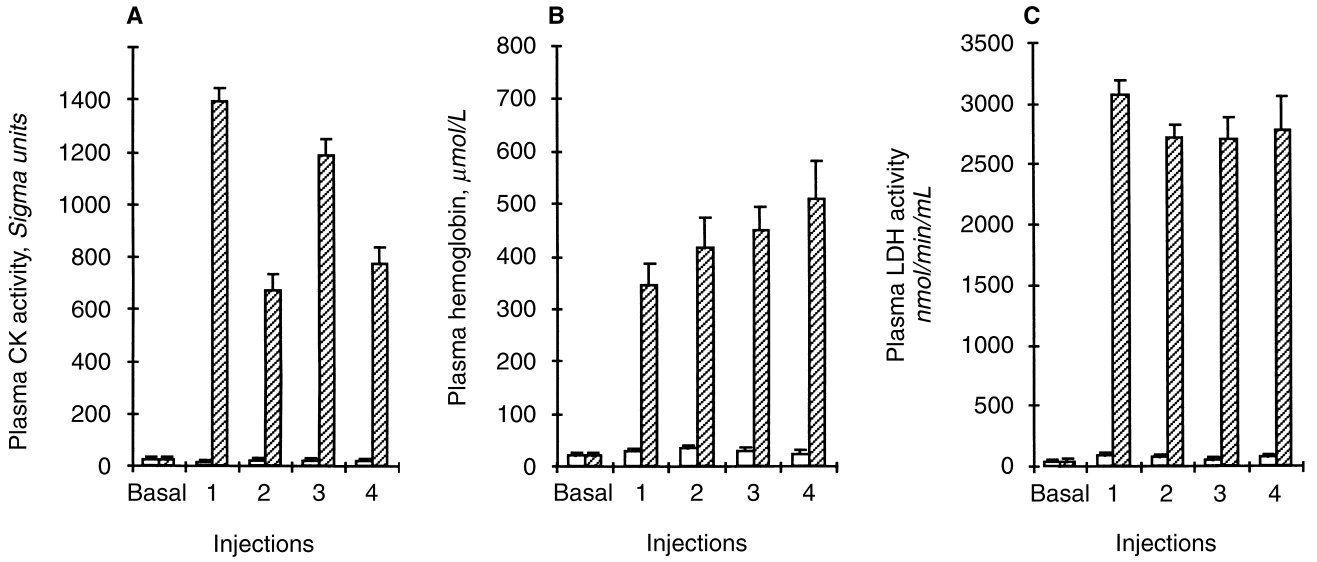

Fig. 2. Sequential determinations of plasma creatine kinase (CK; *A*), hemoglobin (*B*), and lactate dehydrogenase (LDH; *C*) in rats subjected to repetitive injections of glycerol over four to five weeks. Symbols are: (□) control rats; (▨) glycerol-treated rats ($N = 5$ in control rats and $N = 10$ in glycerol-treated rats).

nent fall in creatinine clearance after the first injection of glycerol. However, with second and third injections, creatinine clearances were less impaired in the glycerol-injected rats when compared with these differences after the first injection. The differences between the control and glycerol-treated rats at these time points (0.12 ± 0.03 at second injection and 0.08 ± 0.03 mL/min/100 g body wt at third injection) were significantly less than the difference observed between the control and glycerol-treated rats after the first injection (0.38 ± 0.06 mL/min/100 g body wt, ANOVA). By the fourth injection, creatinine clearances were not significantly different between the control and glycerol-injected rats.

To determine whether this attenuation in the fall in glomerular filtration rate (GFR) was due to less muscle injury or less hemolysis, we measured an enzyme released from muscle (CK), a product released from erythrocytes (hemoglobin), and an enzyme released from both muscle and erythrocytes (LDH). As shown in Figure 2, CK fell with subsequent injections, while hemoglobin tended to rise; LDH remained relatively constant with successive injections. It is unlikely that resistance observed from the second dose onward can be ascribed simply to lesser amounts of heme protein delivered to the kidney. For example, at the time of the third injection, while CK was diminished by 15%, hemoglobin was increased by 30%, and LDH was decreased by 12% compared with the first injection (Fig. 2).

The profile of changes in creatinine clearance (serially measured 5 days after the glycerol injection) over the six months of observation are shown in Figure 3. The fall in creatinine clearance observed after the first injection was attenuated and then disappeared (Fig. 3). By the fourth month, a reduction in creatinine clearance appeared in the glycerol-treated rats, and this reduction persisted during the fifth and sixth months of observation (Fig. 3).

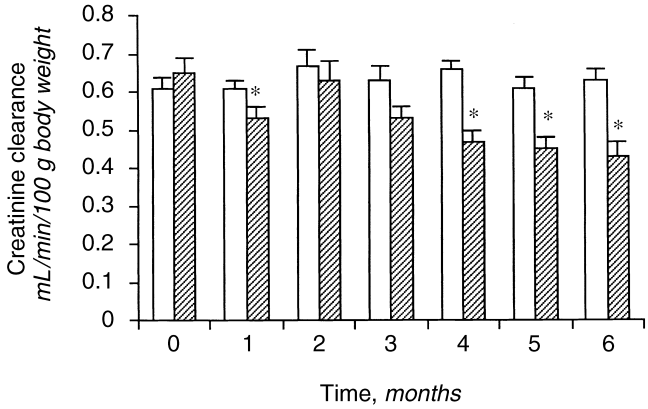

Fig. 3. Sequential determinations of creatinine clearance in rats subjected to repetitive injections of glycerol over six months. Symbols are: (□) control rats; (▨) glycerol-treated rats ($N = 5$ in control rats and $N = 5$ in glycerol-treated rats, except at the baseline when $N = 7$ in the glycerol-treated rats). *$P < 0.05$ vs. control at that time point.

The general characteristics of these rats sacrificed after six months are summarized in Table 1. Systolic blood pressure was not different from the control rats, while, not unexpectedly, hematocrit was lower than the control rats. Urinary protein excretion and urinary flow rates were significantly greater in the glycerol-treated rats.

In these studies, creatinine clearance was measured five days after the injection of glycerol so as to avoid

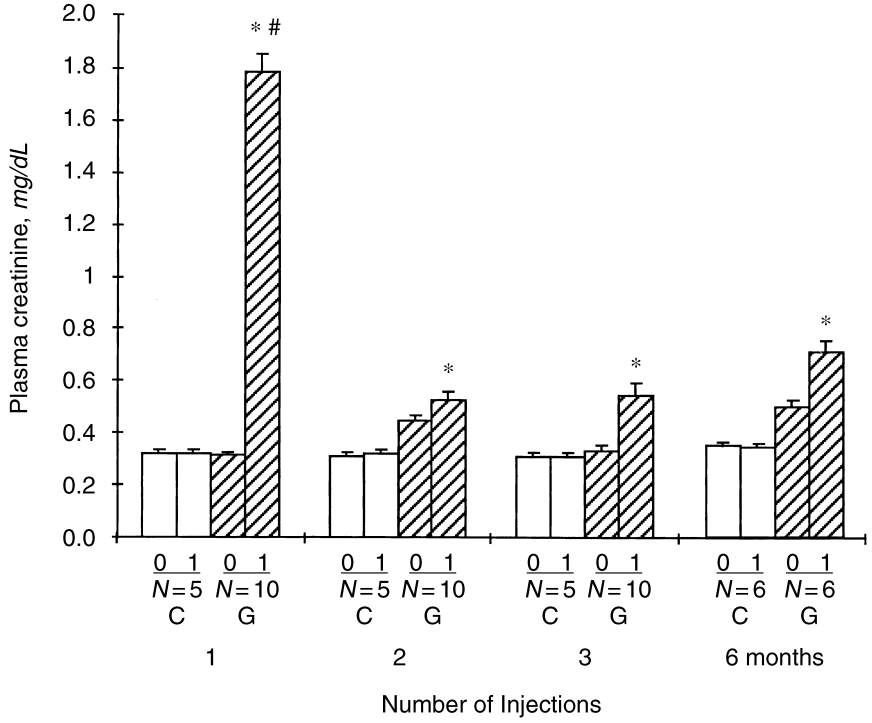

Fig. 4. Serum creatinine measured one day after the administration of glycerol in rats subjected to one, two, and three injections of glycerol, and rats repetitively injected with glycerol for six months. The numbers "0" and "1" indicate serum creatinine measurements made just prior to and one day after the injection of glycerol, respectively. Symbols are: (□) control rats; (▨) glycerol-treated rats. $*P <$ 0.05 vs. glycerol-injected rats on day 0. $\#P <$ 0.05 vs. increment in serum creatinine between day 0 and day 1 in all other glycerol-injected groups.

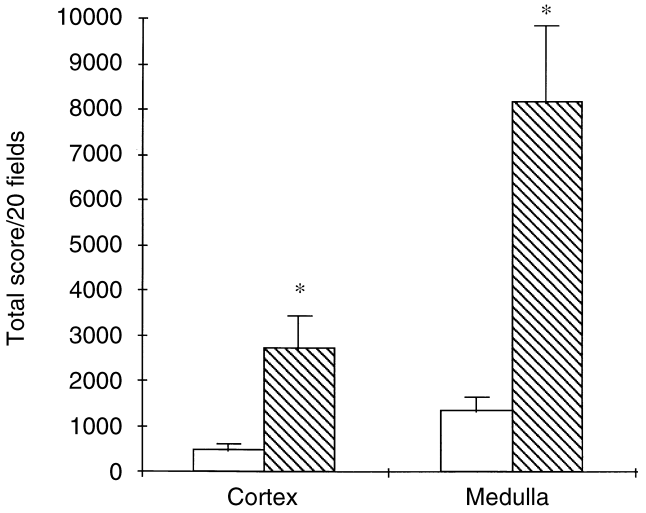

Fig. 6. Morphometric assessment of collagen deposition in cortex and medulla in control rats and glycerol-treated rats after repetitive injections with glycerol for six months. Symbols are: (□) control rats; (▨) glycerol-treated rats ($N = 4$ in control rats and $N = 4$ in glycerol-treated rats). $*P < 0.05$ vs. control.

examining the additive effects on creatinine clearance that arise from acute hemodynamic and other actions of heme proteins. Such an approach allows a serial examination of chronic and persisting changes, which may occur with repetitive administration of glycerol. We also assessed renal function by serum creatinine determined the day after the injection of glycerol in groups of rats subjected to one, two, and three injections of glycerol and groups repetitively injected with glycerol for six months. As shown in Figure 4, rats that were repetitively injected with glycerol for a relatively short-term (2 and 3 injections) or long-term (injected repetitively for 6 months) period were resistant to this insult, as reflected by a markedly blunted rise in serum creatinine the day after the administration of glycerol. The increment in serum creatinine in rats after two injections (0.08 ± 0.02 mg/dL), three injections (0.22 ± 0.05 mg/dL), and injections for six months (0.21 ± 0.03 mg/dL) were all markedly and significantly lower than that observed in rats after one injection of glycerol (1.48 ± 0.07 mg/dL, $P <$ 0.05, ANOVA).

Morphometric and histologic studies in rats subjected to repetitive glycerol injections for six months

Histologic analyses in rats subjected to repetitive administration of glycerol for six months demonstrated significant tubulointerstitial disease, as shown in representative photomicrographs in Figure 5. Interstitial cellular infiltration, tubular atrophy, tubular dilation, and tubular casts were manifested quite prominently in rats subjected to repetitive administration of glycerol. There were no significant glomerular abnormalities observable on light microscopy. Tubulointerstitial disease was quantitated by morphometric determination of deposition of collagen in the interstitium (Fig. 6). Deposition of collagen in the cortical and medullary interstitium was increased

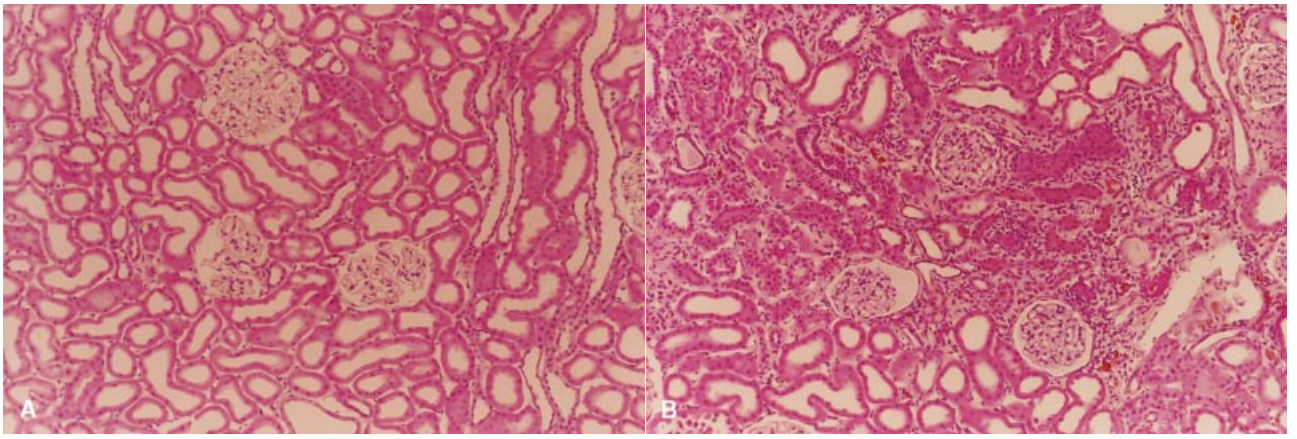

Fig. 5. Representative histologic sections of the kidney stained with hematoxylin and eosin in control (*A*), and glycerol-treated rats after repetitively injected with glycerol for six months (*B*) (original magnification ×100).

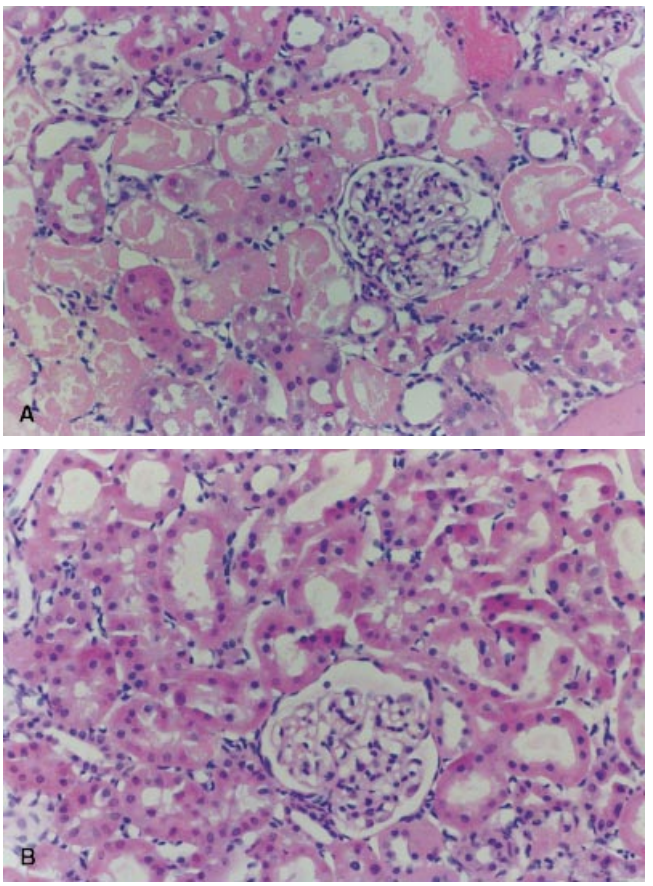

Fig. 7. Representative histologic sections of superficial cortex of the kidney stained with hematoxylin and eosin in rats subjected to one injection of glycerol (*A*) and rats subjected to three injections of glycerol (*B*) (original magnification ×200).

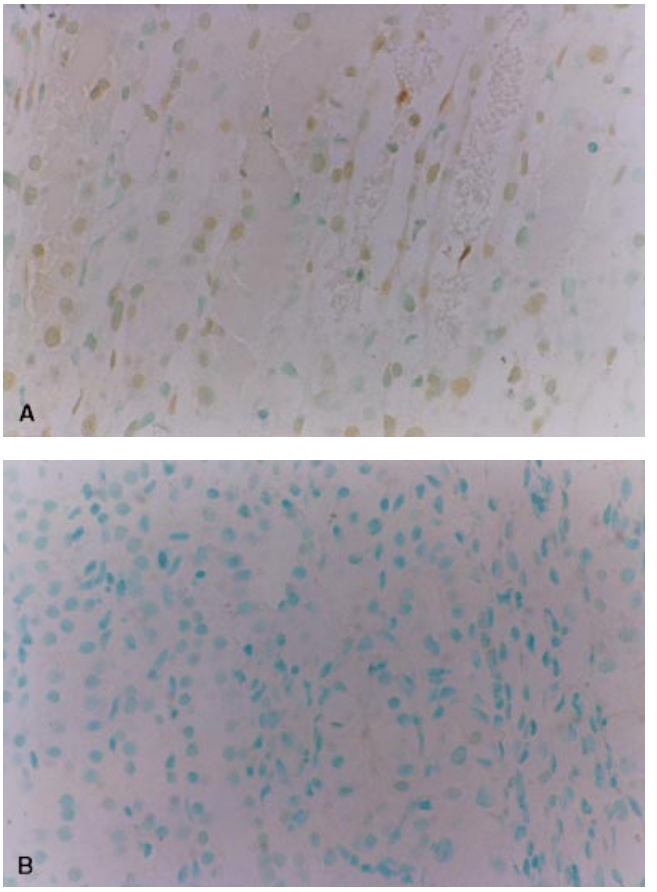

Fig. 8. Representative histologic sections of deep cortex of the kidney stained for apoptosis by the TUNEL technique in rats subjected to one injection of glycerol (*A*) and rats subjected to three injections of glycerol (*B*) (original magnification ×400).

fivefold and sixfold, respectively, in rats subjected to repetitive administration of glycerol.

Comparative studies of renal histologic injury in rats subjected to one injection of glycerol or three injections of glycerol

We also undertook histologic studies for the assessment of necrosis and apoptosis in rats subjected to either a single injection or three injections of glycerol. These studies were undertaken 24 hours after the last injection of glycerol in either group. In rats subjected to one injection of glycerol, as expected, acute tubular necrosis was marked and involved principally the proximal tubule (Fig. 7A), and as described previously, apoptosis was abundant in distal renal tubules (Fig. 8A) [12]. The renal histologic appearance was strikingly ameliorated in rats that were subjected to three injections of glycerol. In this latter group, there was little, if any, cellular necrosis involving the proximal tubules or any other tubular segments (Fig. 7B). Additionally, the distal nephron in this group of rats did not exhibit apoptosis, which was prominent in rats subjected to one injection of glycerol (Fig. 8B). Both groups demonstrated occasional foci of apoptosis in proximal tubular epithelial cells. Thus, reduction in morphologic injury, as assessed by necrosis and apoptosis, accompanies the resistance to renal functional decline observed in rats subjected to three injections of glycerol.

Expression of collagens, TGF-β1, and monocyte chemoattractant protein-1 in rats subjected to repetitive glycerol injections

To explore mechanisms that may be involved in tubulointerstitial injury in rats subjected to repetitive injections with glycerol for six months, we probed the kidney for expression of a fibrogenic cytokine, TGF-β1, and a chemotactic cytokine, monocyte chemoattractant protein-1 (MCP-1), in addition to interstitial and basement collagens. We selected these fibrogenic and proinflammatory cytokines because both of these cytokines are incriminated in progressive renal injury [26–28]; additionally, both cytokines are inducible by oxidative stress [23, 29], a condition that likely occurs in the kidneys of rats subjected to repetitive exposure to heme proteins. In these kidneys, studied six days after the last dose of glycerol, we found increased expression of collagens collagen α1(I), α1(III), and α1(IV) mRNA, thus indicating that interstitial collagens as well as basement membrane collagens were produced in increased amounts (Fig. 9). The mRNA expression for the fibrogenic cytokine TGF-β1 was increased twofold (Fig. 10), while MCP-1 mRNA was increased 2.5-fold (Fig. 11).

Expression of collagens, TGF-β1, and MCP-1 in rats subjected to a single injection of glycerol

To determine the effect of a single injection of glycerol on renal expression of these genes, we performed Northern analyses at a relatively early time point (6 and 12 h) and a relatively delayed time point (5 days) after the administration of a single dose of glycerol. In the initial hours after the administration of glycerol, expression of these cytokines was suppressed, while at a more delayed time point, the expression of TGF-β1 was increased threefold, and expression of MCP-1 was increased fourfold (Fig. 12).

Expression of collagens, TGF-β1, and MCP-1 in cells exposed to an iron-based oxidant system

Iron content was markedly increased in the glycerol model. Indeed, within 24 hours after the administration of glycerol, the content of bioreactive iron was increased almost fourfold [30]. Since up-regulation of collagen and TGF-β1 occurs in response to oxidative stress [23], we examined the effect of an iron-based oxidative stress on expression of these genes in vitro. NRK 49F cells exposed to such stress displayed increased expression of collagen α1(I) and α1(III) mRNA (Fig. 13) and increased expression of TGF-β1 mRNA (Fig. 14). Under the conditions examined, up-regulation of MCP-1 was not observed (data not shown).

DISCUSSION

Insults to the kidney, whether clinically or experimentally induced, are conventionally classified as acute or chronic in nature. Acute renal failure, as occurs in the syndrome of acute tubular necrosis, is precipitous in onset and is accompanied by sublethal and lethal cell injury. After an obligatory reparative and regenerative phase, acute renal failure eventually resolves without significant residual dysfunction or structural derangement [1–4]. Chronic insults are much slower in tempo and generally progressive, and the sustaining mechanisms may be often attenuated but rarely if ever reversed [5]. In this regard, our findings demonstrate that what is generally regarded as an acute reversible insult from which the kidney fully recovers, namely, glycerol-induced, acute, heme protein-dependent, renal injury, can be converted into a chronic, largely irreversible one in which there are tubulointerstitial scarring and collagen deposition in the kidney.

As expected, an abrupt reduction in GFR, as measured by creatinine clearance, occurred after the first administration of intramuscular glycerol. Thereafter, there was an attenuation in the reduction in GFR in response to repeated doses of glycerol. It seems unlikely that such resistance is due to a lesser burden of heme proteins at least for this early phase since, while CK clearly fell with repeated exposures, the plasma levels of hemoglobin tended to rise and LDH remained relatively unchanged

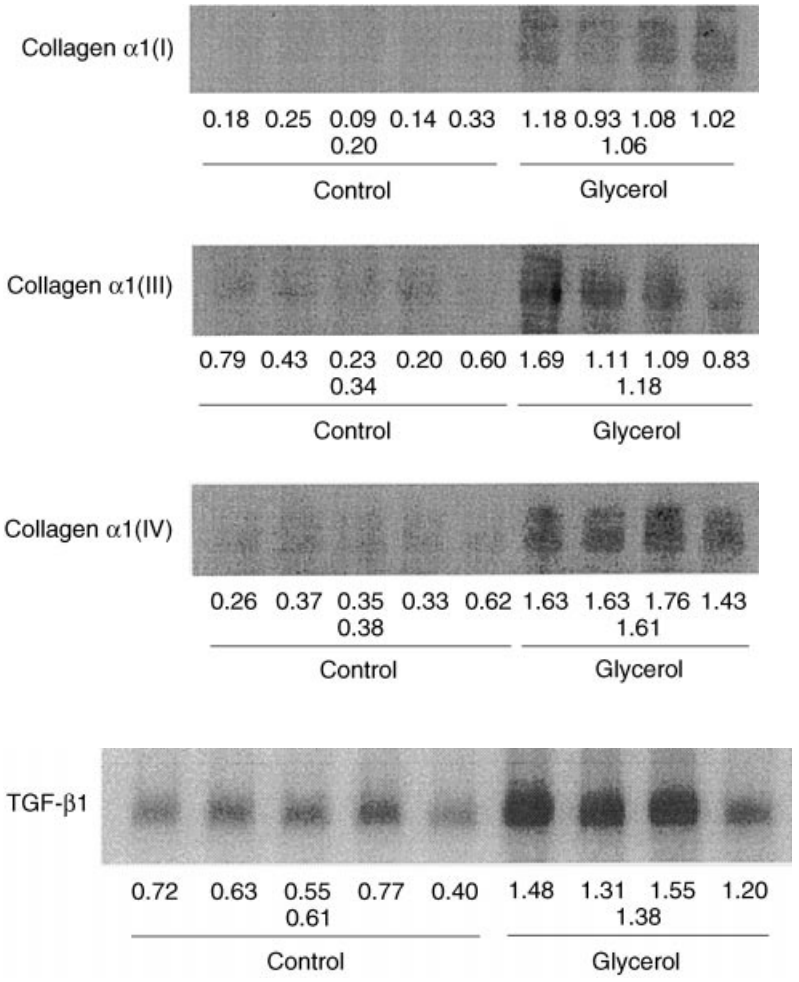

Fig. 9. Renal expression of collagen mRNA for collagen α1(I), α1(III), and α1(IV) in rats subjected to repetitive injections of glycerol and control rats. Each lane represents mRNA extracted from a single kidney from an individual rat. The individual and mean standardized densitometric readings are provided below the Northern analyses.

Fig. 10. Renal expression of transforming growth factor-β1 (TGF-β1) mRNA in rats subjected to repetitive injections of glycerol and control rats. Each lane represents mRNA extracted from a single kidney from an individual rat. The individual and mean standardized densitometric readings are provided below the Northern analyses.

(**Results** section). Thus, a period of acquired renal resistance persists for a considerable period after the initial injection. However, after injections over a protracted period, the burden of heme proteins originating from myoglobin would clearly and progressively diminish because of the muscle atrophy that occurs. While a prior study called attention to this acquired resistance [31], such studies examined the response to only one additional exposure after the first exposure and did not examine the severity of muscle or red cell injury induced by either of these two injections [31]. This study did not examine the long-term changes when such acute insults are repeatedly administered [31]. An additional study described chronic interstitial changes in the kidney following repetitive insults, but did not assess functional changes that evolved sequentially from the first dose [32]; and this study did not attempt to uncover a mechanism accounting for the chronic changes [32]. The present study is the first, to our knowledge, to examine sequential functional changes in conjunction with indices of muscle and red cell injury following repeated exposure to glycerol, and it evaluates the functional and structural outcome six months after the initiation of exposure to glycerol.

This resistance to renal injury, as assessed by such functional markers as creatinine clearance and serum creatinine, was accompanied by resistance to acute cell injury. In rats subjected to a single injection of glycerol, as expected, the proximal tubule demonstrated widespread cellular necrosis, and as we previously described [12], the distal nephron demonstrated significant apoptosis. In striking contrast, rats that were subjected to three injections of glycerol demonstrated scant, if any, evidence of cell necrosis involving the proximal tubule and scant evidence of apoptosis involving the distal nephron. These data demonstrate that the resistance to renal functional decline after repeated injections of glycerol is accompanied by a resistance to acute cell injury, the latter assessed by necrosis and apoptosis. Interestingly, occasional foci of apoptosis were observed in the proximal tubule in both groups of rats. We speculate that the presence of apoptosis in the proximal tubule in rats subjected to one or repeated injections may represent differ-

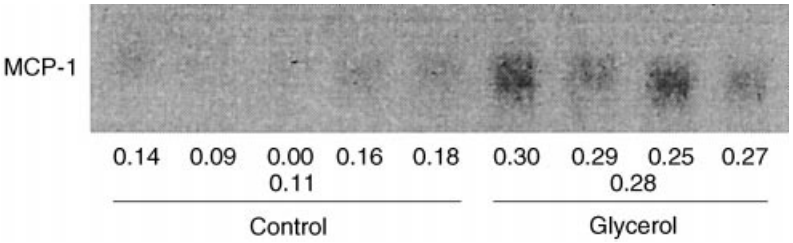

Fig. 11. Renal expression of monocyte chemoattractant protein-1 (MCP-1) mRNA in rats subjected to repetitive injections of glycerol and control rats. Each lane represents mRNA extracted from a single kidney from an individual rat. The individual and mean standardized densitometric readings are provided below the Northern analyses.

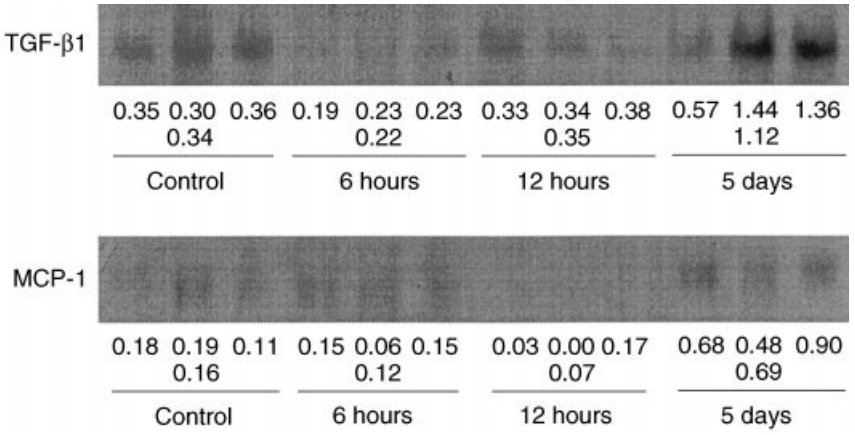

Fig. 12. Time course of TGF-β1 and MCP-1 mRNA expression in control rats and rats subjected to a single injection of glycerol. Each lane represents mRNA extracted from a single kidney from an individual rat. The individual and mean standardized densitometric readings are provided below the Northern analyses.

ent phenomena. In rats subjected to one injection, such apoptosis may represent cell death occurring after the prior injection of glycerol. In rats subjected to three injections, such apoptosis in the proximal tubule may be part of the involutional response in tissues destined to undergo atrophy, as ultimately occurs in the proximal tubule in rats subjected to repeated injections of glycerol.

In our studies, the resistance to the acute reduction in creatinine clearance induced by glycerol was eventually accompanied by decreased GFR and chronic tubulointerstitial disease. Such interstitial disease was attended by up-regulation of interstitial and basement membrane collagens. To explore mechanisms that may contribute to such tubulointerstitial disease, we considered cytokines that are incriminated in chronic inflammation. Additionally, since the administration of glycerol imposes a heme-dependent, oxidative insult, we focused on cytokines that are inducible by oxidative stress, including a fibrogenic cytokine, TGF-β1 [26, 27], and a chemotactic one, MCP-1 [28]. Our attention was particularly drawn to TGF-β1 in view of our prior studies demonstrating that sustained up-regulation of TGF-β1 is induced in the kidney by oxidative stress imposed in in vivo and in vitro settings [23].

Transforming growth factor-β1 is critical in reparative responses to wounding in that it promotes the elaboration of collagen and other extracellular matrix proteins that may temporarily substitute for cells that are lethally damaged and lost [26, 27]. However, persistent up-regulation of this fibrogenic cytokine engenders an aberrant response to injury that is characterized by excessive elaboration of extracellular matrix proteins, and such up-regulation is incriminated in progressive scarring of tissues, as occurs in a number of states, including progressive tubulointerstitial disease [26, 27]. The notion that the repetitive recruitment of TGF-β1–dependent responses is a determinant of scarring was uncovered and substantiated by Border and colleagues in studies involving the anti-Thy 1 model [26, 27]. When this model is induced by a single administration of anti-Thy antibody, acute sublethal and lethal injury to the mesangial cell occurs and is attended by reparative and regenerative responses; the observed transient up-regulation of TGF-β1 assists in the reparative response to this acute insult. However, subsequent administration of anti-Thy leads to sustained up-regulation of TGF-β1 and transforms this model into one of progressive renal injury [26].

In the present studies, TGF-β1 was increased in rats subjected to repetitive administration of glycerol. That increased expression of TGF-β1 occurs in response to the glycerol model, independent of chronic scarring, was demonstrated by our studies undertaken following a single exposure to the glycerol model. In the reparative phase of this model, TGF-β1 is increased threefold. Since increased amounts of iron are present in the kidney after a single exposure to glycerol [30] and since iron is an instigator of oxidant-dependent tissue injury, we examined whether the exposure of renal fibroblasts to an iron-based oxidant system would induce TGF-β1. Indeed, in such a cell culture model, TGF-β1, along with collagen

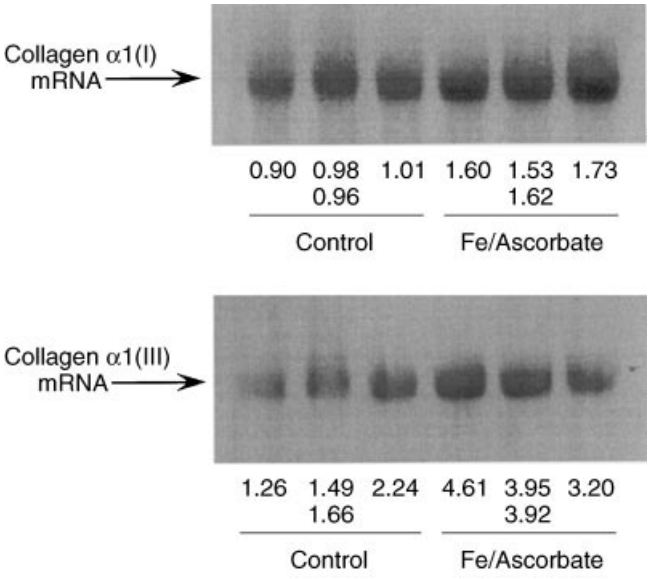

Fig. 13. Effect of an iron-based, oxidant-generating system on expression of collagen α1(I), α1(III) mRNA in NRK 49F cells. The individual and mean standardized densitometric readings are provided below the Northern analyses.

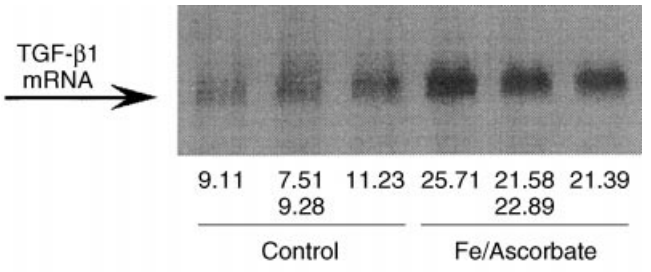

Fig. 14. Effect of an iron-based oxidant generating system on expression of TGF-β1 mRNA in NRK 49F cells. The individual and mean standardized densitometric readings are provided below the Northern analyses.

I and III, was induced in response to iron-based oxidant system. Thus, up-regulation of TGF-β1 occurs in the kidney in vivo following repetitive administration of glycerol in vivo, following a single injection of glycerol in vivo, and in renal fibroblasts exposed to iron-based oxidative stress. Based on these findings, we suggest that sustained up-regulation of TGF-β1, which occurs in this model, reflects, in part, repetitive and cumulative effects of iron-driven oxidative stress. The chemotactic peptide MCP-1, which is also induced by oxidative stress [29], was up-regulated in the kidney subjected to repetitive and single administration of glycerol but not under the conditions we tested in vitro. It is possible that up-regulation of this chemotactic peptide may contribute to the inflammatory infiltrate observed in rats subjected to repeated administration of glycerol. The mechanisms accounting for the lack of expression of MCP-1 in the in vitro model as compared with the in vivo model are uncertain. While the in vitro model reproduces one of the key elements found in the in vivo circumstance, namely, increased amounts of redox-active iron, other characteristics of the in vivo setting—the duration and type of exposure to increased amounts of tissue iron, other redox changes besides bioactive iron, alterations in renal and interstitial hemodynamics, alterations in tissue oxygenation, the cytokine milieu of the chronically inflamed kidney—may be important factors accounting for the up-regulation of MCP-1 in vivo.

We also suggest that our findings may be relevant to progressive renal disease occurring in the setting of hematuria. Glomerulopathies associated with hematuria impose a burden of heme proteins on the proximal tubules [33, 34]. Erythrocytes are engulfed and destroyed by proximal tubules, with the consequence that the proximal tubule is exposed to large amounts of heme proteins. Such heme proteins, by virtue of released heme or iron, can injure cells through a multiplicity of mechanisms. Based on our findings, we suggest that proximal tubular and other renal cells, exposed to a large amount of heme and iron, can recruit an iron-inducible, fibrogenic cytokine such as TGF-β1. Interestingly, intracellular iron is increased in human progressive nephropathies as well as in models of progressive renal injury [35–37] by mechanisms that do not necessitate hematuria and subsequent cellular uptake of erythrocytes. For example, in proteinuric states, iron-bearing proteins such as transferrin are taken up by the proximal tubule. Iron is released from transferrin within the intracellular compartment, in part, by the reduced pH environment of the endosomal-lysosomal pathway [35–37]. In this way, the cellular content of iron can be increased in progressive renal disease through mechanisms that do not involve hematuria. Increased amounts of cellular iron, whether accruing from hematuria-dependent or hematuria-independent pathways, may sustain progressive renal disease by up-regulating fibrogenic cytokines such as TGF-β1, as well as through other mechanisms.

We speculate that these findings may be germane to sickle cell nephropathy [38, 39]. A subset of these patients demonstrated progressive renal disease characterized, in part, by the presence of tubulointerstitial disease and iron deposition in the renal tubular epithelium. The kidneys in these patients are subjected over a protracted period of time to recurrent exposure to heme proteins as a consequence of sickling and episodic breakdown of erythrocytes. Similar changes are observed in patients with paroxysmal nocturnal hemoglobinuria [40]. Based on our present findings, we suggest that such interstitial disease and fibrosis may reflect iron-driven, TGF-β1–dependent processes.

In summary, renal responses to repetitive exposure to heme proteins, as induced by the glycerol model, in-

cludes, initially, sensitivity to the first insult and, subsequently, an acquired resistance to subsequent insults. However, renal resistance is attended by a chronic sclerosing change, which ultimately compromises renal function. Thus, a triphasic response occurs in the kidney repetitively exposed to heme proteins: initial sensitivity, acquired resistance, and chronic inflammation. We suggest that up-regulation of TGF-β1 and MCP-1, which occurs in this model, may contribute to the chronic inflammatory changes observed in the kidney, and at least for TGF-β1, such up-regulation may be dependent on increased amounts of iron in the kidney. The mechanisms accounting for acquired resistance and the relationship between such resistance and ensuing scarring merit further attention. It is possible that these phenomena, acquired resistance and chronic scarring, originate and evolve as independent, separate processes. Alternatively, it is conceivable that processes that render the kidney resistant to subsequent insults may ultimately exact a long-term cost and one that involves chronic tubulointerstitial scarring.

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