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Kidney International, Vol. 50 (1996), pp. 796–804

Myoglobin inhibits proliferation of cultured human proximal tubular (HK-2) cells

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Myoglobin inhibits proliferation of cultured human proximal tubular (HK-2) cells. Following nephrotoxic injury, renal repair is dependent on tubular regeneration. In the case of myoglobinuric acute renal failure (ARF), persistence of myoglobin within tubular cells, or sublethal injury sustained at the height of exposure to it, might retard this process. To test this hypothesis, a human proximal tubular cell line (HK-2) was cultured for 24 hours in the absence or presence of clinically relevant myoglobin concentrations (0.5, 1, 2, 4 mg/ml). Immediately following myoglobin removal, lethal cell injury (vital dye uptake), lipid peroxidation, and DNA damage (alkaline unwinding assay) were assessed. The extent of cell proliferation was estimated over the next four days by a tetrazolium based (MTT) assay and by determining total intracellular LDH. Myoglobin's effects on protein and DNA synthesis were also assessed (^{35}S-methionine and bromodeoxyuridine incorporation, respectively). Myoglobin induced dose-dependent lipid peroxidation (malondialdehyde generation) and cell death (up to 80% vital dye uptake with the 4 mg/ml challenge). Although 1 mg/ml myoglobin caused no cell death, it induced nearly complete growth arrest. This lasted for approximately three days following myoglobin removal from the media. Neither of two control proteins (albumin; lysozyme) nor a second nephrotoxin (gentamicin; 1 mg/ml) reproduced this effect. The 1 mg/ml myoglobin challenge caused an 80 to 90% depression in protein and DNA synthesis. It also induced significant DNA damage, as assessed by the alkaline unwinding assay ($P < 0.01$). Iron chelation therapy (deferoxamine) mitigated myoglobin-induced cell killing. However, its addition following myoglobin loading worsened HK-2 outgrowth by exerting a direct anti-proliferative effect. These results indicate that: (1) sublethal myoglobin toxicity can induce transient proximal tubular cell growth arrest, potentially slowing recovery from ARF; (2) this effect correlates with, and could result from, heme-induced DNA damage and a blockade in DNA/protein synthesis; and (3) deferoxamine can inhibit proximal tubular cell proliferation. This possibility needs to be considered in designing clinical trials with DFO for myohemoglobinuric ARF.

Renal functional recovery following the establishment of acute tubular necrosis (ATN) is dependent on resolution of intratubular obstruction and repair of the damaged tubular epithelium. The latter process requires proximal tubular cell regeneration to re-establish functional and structural tubular integrity [1–3]. Following experimental ischemic renal injury, the tubule presumably can undergo relatively unimpeded repair due to the transient nature of the original insult [4, 5]. However, following nephrotoxic injury, persistence of the toxin within sublethally damaged tubular cells may exert ongoing effects, potentially altering tubular regeneration. Myoglobinuric acute renal failure (ARF) provides a case in point. During its initiation phase, probably all proximal tubular cells are subjected to some degree of myoglobin loading via endocytic reabsorption [6]. Although the globin and porphyrin components are rapidly catabolized within tubular cells, the released iron is retained [7]. Since iron-catalyzed oxidant stress is a critical determinant of heme protein nephrotoxicity [8–11], intracellular iron loading could alter the regenerative process. For example, iron-induced DNA, protein, or lipid oxidation could each have profound effects on cell division and repair. Alternatively, since iron is an obligate growth factor, increments in its intracellular content might accelerate the proliferative process. In support of the latter possibility are observations that $FeSO_4$ addition to cultured human proximal tubular (HK-2) cells can stimulate their outgrowth [12]. Furthermore, H_2O_2-induced oxidant stress and antioxidant agents can exert pro- and anti-proliferative effects, respectively [12, 13].

Given these considerations, the goals of this study were twofold: first, to develop a cell culture model of myoglobin-induced proximal tubular cytotoxicity that simulates some of the essential features of *in vivo* heme protein-induced tubular damage; and second, using this model, ascertain the impact of sublethal myoglobin toxicity on the ability of proximal tubular cells to mount a proliferative response.

Methods

Cell culture conditions

All experiments were performed using HK-2 cells, a proximal tubular cell line derived from a normal adult human kidney [14]. The cells were grown at 37°C in tissue culture flasks (Costar, Cambridge, MA, USA) with keratinocyte serum free medium (K-SFM; Gibco/BRL, Grand Island, NY, USA) supplemented with EGF (5 ng/ml) and bovine pituitary extract (40 μg/ml). For experimentation, the cultures were trypsinized and transferred to either 6 or 24 well cluster plates. Unless stated otherwise, they were plated at a concentration of either ~0.25×10^5 or ~1×10^5 cells per well (for the 24 well and 6 well plates, respectively). After allowing a 24-hour post-transfer recovery period, the cells were used for experimentation, as denoted below.

Model of myoglobin-induced cytotoxicity

Horse skeletal muscle myoglobin (M-0630; lot # 61H7100; Sigma Chemical, St. Louis, MO, USA) was added to HK-2 subcultures in final concentrations of 0.5, 1, 2, or 4 mg/ml. A 40

Received for publication October 25, 1995
and in revised form April 10, 1996
Accepted for publication April 11, 1996

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mg/ml stock myoglobin solution was prepared in Hank's balanced salt solution (HBSS; sonicated to complete solubilization; Gibco). Prior to application, it was passed through a 0.45 μm filter (Millex-HV; Millipore Corp. Bedford, MA, USA). After allowing a 20 to 24 hour exposure, the myoglobin-containing culture media were removed, the cells were washed twice with HBSS, and fresh K-SFM was added. The cultures were either studied immediately, or they were maintained under routine culture conditions for an additional one to four days to ascertain the rates of cell proliferation, as described below.

Characterization of myoglobin-induced cytotoxicity

Assessments of cell viability. Loss of cell viability following myoglobin treatment was assessed by the % of cells demonstrating vital dye uptake (ethidium bromide, 8 μg/ml; acridine orange, 2.3 μg/ml used as a counterstain; India ink addition to quench background fluorescence). One hundred cells in each of three fields were counted.

Lipid peroxidation. Since lipid peroxidation/malondialdehyde (MDA) generation is a correlate of both *in vivo* and *in vitro* myoglobinuric injury [8–11] it was sought in HK-2 cells following myoglobin exposure to help substantiate the relevance of this cell culture system. To this end, 2×10^6 cells were cultured in K-SFM containing 0 to 4 mg/ml myoglobin for 24 hours. After removing the culture media, the cells were washed × 3 with HBSS. They were detached from the culture plates with a rubber policeman, collected by centrifugation, and snap frozen with liquid N_2. The samples were stored at −20°C until the time of assay. MDA concentrations were determined by a thiobarbituric acid method [15]. In brief, each sample pellet was suspended in 100 μl 1.15% KCl and then 50 μl were mixed with 188 μl 0.44 M H_3PO_4 + 75 μl 1.15% KCl + 63 μl 0.6% thiobarbituric acid. The samples were boiled for one hour, they were brought to pH 6.0 with 100 μl NaOH, and then centrifuged. Supernatant MDA concentrations were analyzed by HPLC [15, 16] as follows: 100 μl samples were injected into a Hewlett Packard HPLC 1500 series equipped with an auto sampler, a multiple wave length detector, and a Supelcosil column (15 × 0.4 cm LC-18-S; Supelco, Bellefonte, PA, USA). The mobile phase was 50 mM potassium phosphate. Retention time was 2.9 minutes. Values were expressed as % increase from control values, adjusting for the number of cells present (total LDH; see below).

Iron dependence of myoglobin-induced HK-2 cytotoxicity. Previous work has demonstrated that myoglobinuric renal tubular injury is iron dependent. This is based on observations that iron chelation therapy attenuates *in vivo* myohemoglobinuric ARF [8–10] and mitigates heme protein-induced cytotoxicity in an isolated proximal tubular segment system [11]. The following experiment tested whether the present HK-2 model of myoglobin toxicity is also iron dependent. HK-2 cells were maintained under routine culture conditions either in the absence or presence of 0.5 mM DFO. After allowing a 20 hour period for cell DFO uptake, myoglobin was added (either 2 or 4 mg/ml). The DFO pre-treated cells also had 0.5 mM DFO added along with the myoglobin challenge. Cell viability was assessed 20 hours following the myoglobin addition by determining % vital dye exclusion.

Myoglobin effects on cell proliferation

The effect of myoglobin loading on HK-2 proliferation was initially assessed at the completion of 24 hours of myoglobin exposure. Cultures were exposed to either no myoglobin (controls) or myoglobin doses (0.5 or 1 mg/ml) that were not overtly cytotoxic (that is, did not cause vital dye uptake; **Results**). After completing the 24 hour incubations, total cell mass was assessed by two independent means: the MTT proliferation assay [14, 17, 18] and total intracellular LDH (each described below). The absence of tubular cell death in these experiments was confirmed by demonstrating no increase in vital dye uptake in the myoglobin-exposed versus the co-cultured normal HK-2 cells.

Next, the long-term impact of myoglobin treatment on cell proliferation was assessed. HK-2 cells were cultured for 24 hours in the presence of a sublethal myoglobin challenge (1 mg/ml). Co-cultured cells maintained under normal conditions served as controls. At the end of the 24 hour challenge, all of the cultures were washed (which did not cause cell detachment) and then they were maintained for an additional 0 to 4 days under normal culture conditions. Total cell numbers in individual wells were gauged daily by MTT and total intracellular LDH in individual wells.

MTT assay. The MTT assay is based on the principle that a tetrazolium dye (MTT: 3-[4,5,-dimethylthiazol-1-y1]-2,5 diphenyltetrazolium bromide; Sigma Chemicals) is actively transported by respiring mitochondria, resulting in intramitochondrial formazin precipitation [19]. Therefore, the amount of intra-mitochondrial MTT/formazin precipitation is a reflection of total mitochondrial mass, and hence, cell number [14, 17, 18]. At the appropriate time points (from 0 to 4 days following myoglobin removal), 1 mg/ml MTT (in K-SFM) was added to the cultures and incubated at 37°C for two hours. Then, the culture medium was removed and dimethylsulfoxide (DMSO) was added to solubilize the intramitochondrial formazin precipitate. The absorbance of the latter was measured at 570 nm with an ELISA reader, using a 630 nm reference wave length [14].

LDH assay. To further gauge the extent of proliferation, additional wells of cells, grown under control or myoglobin exposure conditions, were washed and then lysed with 10% Triton X-100. The lysate was assayed for LDH by autoanalyzer technology, as previously performed by this laboratory (such as [11]). LDH, rather than total protein, was used for this purpose to avoid extraneous extracellular protein contamination (such as from the culture medium, matrix proteins, any contaminating myoglobin).

Validation of MTT and LDH assays as indices of cell numbers. To validate the MTT assay and total LDH as markers of HK-2 cell number, serial dilutions of HK-2 cells [2, 1.5, 1.0, 0.5, and 0×10^5 cells] were added to cluster plates and cultured for two days. Cell numbers were then assessed by both techniques.

The possibility was considered that despite the fact that 1 mg/ml myoglobin did not cause cell death (vital dye uptake) at the completion of the 24 hour challenge, delayed cell death (that is, after myoglobin washout) potentially could have occurred. This could have diminished cell numbers in the above proliferation experiments. To exclude this possibility, % vital dye exclusion was ascertained at 24 and 48 hours following myoglobin washout in three separate experiments.

Effect of non-iron containing proteins on HK-2 proliferation

The following experiment was undertaken to ascertain whether myoglobin's anti-proliferative effect was due to myoglobin *per se*, or just exogenous protein addition. HK-2 cells were cultured either in the absence or presence of fatty acid free bovine serum

albumin (A-6003; Sigma) or lysozyme (L-6876; Sigma), a non-iron containing protein similar in size to myoglobin. These test proteins were added in concentrations ranging from 1 to 4 mg/ml. After 20 to 24 hour incubations, the cells were washed and cultured for 0 to 4 days ($N = 3$ each). Degrees of proliferation were gauged by the MTT assay, as noted above. Cell viability at the completion of the protein challenges was determined by vital dye exclusion.

Effect of gentamicin on HK-2 cell proliferation. To test whether any well defined nephrotoxin can reproduce myoglobin's anti-proliferative effect, the impact of gentamicin on HK-2 cell outgrowth was determined. Since gentamicin is handled by proximal tubular cells like myoglobin (endocytic uptake, followed by storage within the endolysosomal system), its effects were determined. HK-2 cells were cultured under normal conditions or in the presence of 1 mg/ml gentamicin for 24 hours (preliminary data indicated that this dose, like the 1 mg/ml myglobin dose, caused no loss of cell viability, as assessed by vital dye uptake). After completing the 24-hour gentamicin exposure, these cultures and co-cultured controls were washed. They were then maintained in culture for an additional three days under routine conditions. Cell numbers were gauged at the completion of the initial 24-hour gentamicin loading period and one, two, three, and four days thereafter by MTT and LDH assay ($N = 4$ separate determinations on 4 separate cultures at each time point).

Effect of iron chelation therapy on HK-2 cell proliferation

The following experiments were undertaken to ascertain whether iron chelation therapy, introduced after HK-2 myoglobin loading, could reverse the block in cellular proliferation. HK-2 cells were exposed to 1 mg/ml myoglobin for 24 hours, followed by its washout as noted above. Fresh medium was added ± 0.5 mM DFO. Cell proliferation was determined over the next four days. Co-cultured cells not subjected to myoglobin loading, but treated in an identical fashion (± DFO), served as controls ($N = 3$ separate experiments).

Since the above experiments indicated that prolonged DFO exposure can independently inhibit cell proliferation (**Results**), the following experiment ascertained whether short-term DFO treatment might reverse myoglobin's anti-proliferative effect while circumventing it own. In brief, the above experiment was repeated in its entirety except that DFO was applied only for the first 24 hours following myoglobin exposure ($N = 3$ experiments).

To test whether DFO's anti-proliferative effect on HK-2 cells was due to iron chelation, rather than a non-specific drug effect, normal HK-2 cells were cultured in the presence or absence of a different iron chelator (50 μM 1,10-phenanthroline). Cell numbers (MTT assay; total intracellular LDH) were assessed each day for three consecutive days.

Myoglobin's effect on protein synthetic rates

One potential mechanism by which myoglobin might inhibit cell proliferation is via a blockade in protein synthesis. To test this possibility, rates of protein synthesis following 1 mg/ml myoglobin treatment were gauged by ^{35}S-methionine incorporation as previously described in detail [20]. In brief, 10 μCi/ml ^{35}S-methionine were added to the culture plates which contained methionine free K-SFM. After three hours of incubation, the cells were washed with HBSS, the cells were lysed, the labeled proteins were solubilized, precipitated with 10% trichloroacetic acid using BSA as a carrier, and then collected on filters. After washing and air drying, the amount of precipitated radioactivity was determined by addition to liquid scintillation fluid (Sigma-fluor; Sigma) and counting in a scintillation counter. Protein synthetic rates were determined just after completion of the myoglobin challenge and one to four days thereafter. Co-cultured cells treated in an identical fashion except for no myoglobin exposure served as controls.

Bromodeoxyuridine incorporation

The following experiment utilized nuclear bromodeoxyuridine (BrDU) incorporation [21, 22] to gauge DNA synthesis following myoglobin treatment. Cells were challenged with either 0, 0.5, or 1 mg/ml myoglobin for 24 hours. They were washed twice with HBSS, fresh K-SFM was added, and they were maintained under routine culture conditions for an additional two days. At that time, BrDU (50 μM; Sigma) was added to the cultures and the cells were incubated for six hours. Then, they were fixed with 10% formalin in phosphate buffered saline (PBS) for 20 minutes, washed three times with PBS, and permeabilized with 0.2% Triton X-100 in PBS for five minutes. The DNA was nicked by incubating with DNAase I (Worthington Biochemical Co., Freehold, NJ, USA) for 30 minutes in a buffer containing 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 10 mM $CaCl_2$. The plates were washed four times with PBS, and 5% BSA was added for 15 minutes as a protein blocking agent. The plates were incubated for 18 hours with anti-BrDU antibody (Sigma; 1:400 dilution in the blocking solution). After washing × 3 with PBS, biotinylated anti-mouse IgG (Vector; Burlingame, CA, USA) was added in a 1:250 dilution (in PBS). Incubations and washings were carried out as noted above. The biotinylated antibody was detected using Vectastain ABC-AP and alkaline phosphatase substrate kit III (Vector) according to the manufacturer's instructions. The % of control cells and post-myoglobin treated cells showing BrDU nuclear incorporation was determined by counting > 500 cells per well.

Fluorometric DNA unwinding assay

To assess whether myoglobin-induced oxidant stress might have culminated in DNA damage, DNA strand breaks were sought by the DNA unwinding assay [23], as previously utilized by Ueda and Shah [24]. In brief, 2.5×10^6 cells were cultured with either 0 or 1 mg/ml myoglobin × 24 hours, and they were washed × 3 with HBSS, and scraped off with a rubber policeman. They were recovered by centrifugation and suspended in 3 ml HBSS (Ca^{2+}/Mg^{2+} free). The suspension was mixed with 9 ml of a hypotonic lysis buffer (0.87% NH_4Cl; 10 mM Tris-HCl, pH 7.2) and held at 4°C × 20 minutes. Following centrifugation (1800 g × 20 min; 0°C), the pellet was suspended in 3 ml of the above buffer, centrifuged again, and suspended in 1 ml of a buffer containing 0.25 M meso-inositol, 10 mM sodium phosphate, 1 mM $MgCl_2$; pH 7.2. The samples ($N = 3$ for control and myoglobin treated cells) were then used for the DNA unwinding assay, as originally described by Birnboim and Jevcak [23].

Calculations and statistics

All values are given as means ± SEM. The number of replicate experiments which were performed are given in the Figure legends. Unless stated otherwise, statistical comparisons were

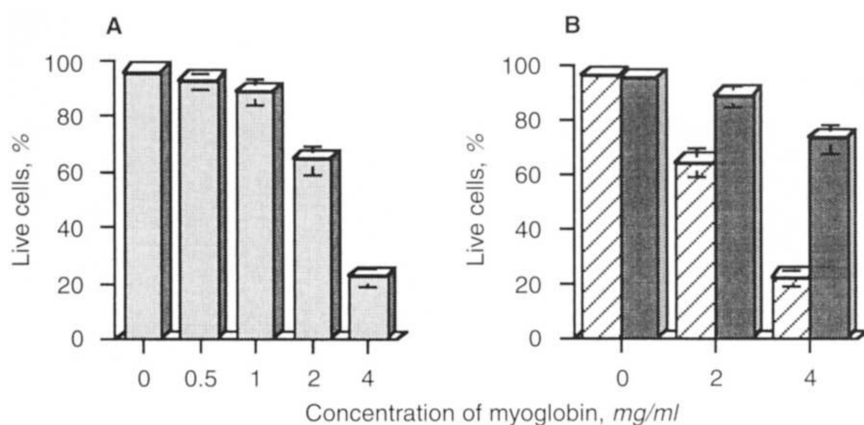

Fig. 1. A. *When cells were incubated with 0, 0.5, or 1 mg/ml of myoglobin for 20 to 24 hours, no loss of cell viability resulted, as determined by vital dye exclusion (NS vs. cells incubated under normal culture conditions, that is, 0 myoglobin).* Conversely, 2 and 4 mg/ml myoglobin exposure caused ~ 30% and 80% vital dye uptake (each $P < 0.002$, compared to controls). $N = \geq 3$ separate experiments for each. **B.** Treating the cells with 0.5 mM DFO for 24 hours prior to and during a 2 or 4 mg/ml myoglobin challenge conferred significant protection against myoglobin-induced vital dye uptake ($P < 0.001$; vs. no DFO treatment). DFO had no effect on cell viability in the absence of the myoglobin challenge (0 myoglobin). Symbols are: (▨) 0 to 4 mg/ml myoglobin without DFO; (■) 0 to 4 mg/ml myoglobin with DFO. $N = \geq 3$ separate experiments for each. Percent live cells was determined by vital dye exclusion.

performed by unpaired Student's *t*-test (in all proliferation experiments, the results for the experimental groups were contrasted to those obtained in co-cultured, independent control groups). If one set of control values was compared to more than one experimental group (such as in the BrDU experiments), unpaired Student's *t*-test with the Bonferroni correction was applied.

Results and Discussion

The first goal of this study was to establish an *in vitro* model of myoglobin toxicity which simulates the essential features of *in vivo* heme protein-induced tubular damage. This laboratory has previously used freshly isolated proximal tubular segments harvested from myoglobinuric rats or mice to study cell-specific determinants of acute heme protein cytotoxicity [11]. However, the short lifespan of freshly isolated tubules in suspension precludes their use for direct investigation of regenerative events. Hence, in this study, myoglobin was added to HK-2 proximal tubular cells to ascertain whether a relevant model of heme protein cytotoxicity would result. The available data indicate that this is the case. First, as shown in Figure 1A, myoglobin addition to HK-2 cells caused dose dependent cytotoxicity: whereas neither 0.5 or 1.0 mg/ml caused cell death, ~30% and 80% of cells manifested vital dye uptake following 2 and 4 mg/ml myoglobin exposure, respectively. Neither BSA nor lysozyme addition reproduced this effect (~90% vital dye exclusion; data not shown), indicating that myoglobin toxicity was not merely due to "protein loading." Second, DFO pre-treatment markedly decreased myoglobin cytotoxicity (Fig. 1B). Since DFO has consistently protected against *in vivo*- and isolated tubule segment-models of myohemoglobinuric renal injury [8–11], this finding further supports the relevance of the HK-2 cell line for studying heme protein toxicity. Third, myoglobin's cytotoxic effect correlated with increasing MDA generation in the residual cell population (Fig. 2). Since lipid peroxidation is a hallmark of *in vivo* myohemoglobinuric injury [8, 9], this finding further speaks to the utility of the employed cell culture system. Finally, it is noteworthy that myoglobin-initiated lipid peroxidation and cell killing were produced with clinically relevant myoglobin concentrations (≤ 4 mg/ml). In this regard, during the initiation phase of experimental myohemoglobinuric ARF, urine myoglobin concentrations of ~35 mg/ml are typically observed [25, 26].

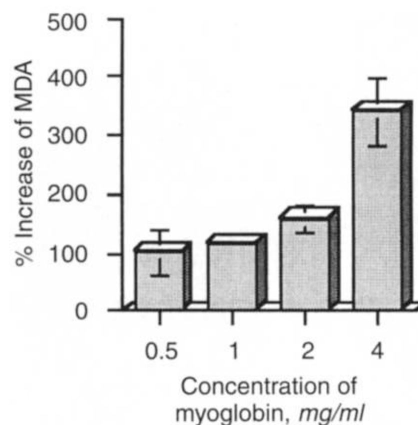

Fig. 2. *Culturing cells with myoglobin for 24 hours caused a dose dependent increase in MDA concentrations, significant at a concentration of > 1 mg/ml ($P < 0.01$; $N = 3$ determinations at each concentration).* The values are expressed as a % increase over that observed in co-cultured controls.

Since recovery from ARF requires regeneration of the damaged tubular epithelium, the principal goal of this study was to determine myoglobin's impact on HK-2 cell proliferation following sublethal damage. To gain an initial insight into this issue, cells were incubated with sublethal myoglobin doses (0.5 and 1 mg/ml), and after 24 hours cell numbers were assessed by the MTT assay and total intracellular LDH. The validity of these markers of HK-2 proliferation was first demonstrated by essentially perfect correlations between them and cell numbers in serial dilution/outgrowth experiments (Fig. 3). As shown in Figure 4, after 24 hours of culture in the presence of either 0.5 or 1 mg/ml of myoglobin, significant reductions in cell number/mass were apparent. Given that the normal HK-2 cell doubling time is ~36 hours, and since neither of these myoglobin doses caused cell death (as assessed by vital dye exclusion), these findings suggested that myoglobin had induced HK-2 growth arrest.

To further pursue this possibility, cells were pretreated with 1 mg/ml of myoglobin for 24 hours, the myoglobin was removed from the media, and then the cultures were maintained for an additional four days. As shown in Figure 5, the myoglobin loaded

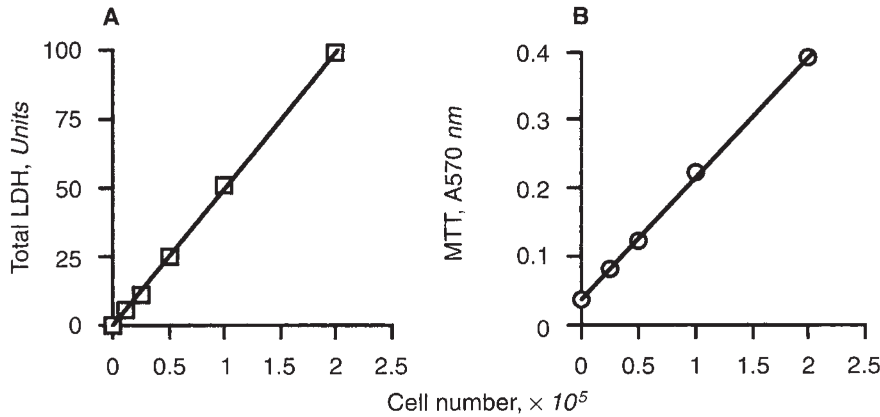

Fig. 3. *Serial dilutions of HK-2 cells were plated and cultured for two days, followed by total intracellular LDH (**A**) and MTT assay (**B**).* An essentially perfect correlation ($r > 0.99$) was noted between the number of plated cells and total LDH/MTT uptake, validating these two tests as markers of HK-2 cell numbers. ($N = 2$ separate experiments for each data point). The SEM was < the depicted symbols.

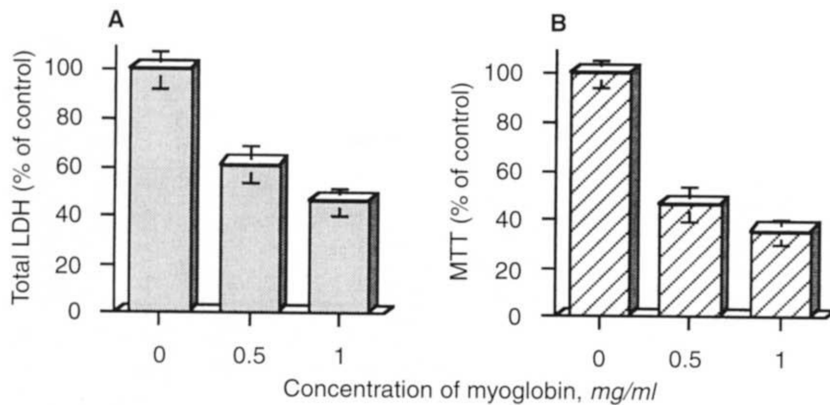

Fig. 4. *Cell numbers were assessed 24 hours following culture in the presence of 0, 0.5, and 1 mg/ml myoglobin.* Stepwise reductions in both total (**A**) LDH and (**B**) MTT values were apparent (% of the values observed with the 0 myoglobin controls). Since neither the 0.5 nor the 1.0 mg/ml myoglobin dose caused a loss of cell viability (assessed by vital dye exclusion), and since the myoglobin treatment did not cause HK-2 cell detachment (a potential artifact which could cause reductions in cell mass), these results suggested that myoglobin had induced growth arrest (0 myoglobin, $N = 16$; 0.5 or 1 mg/ml myoglobin, $N = 8$ each).

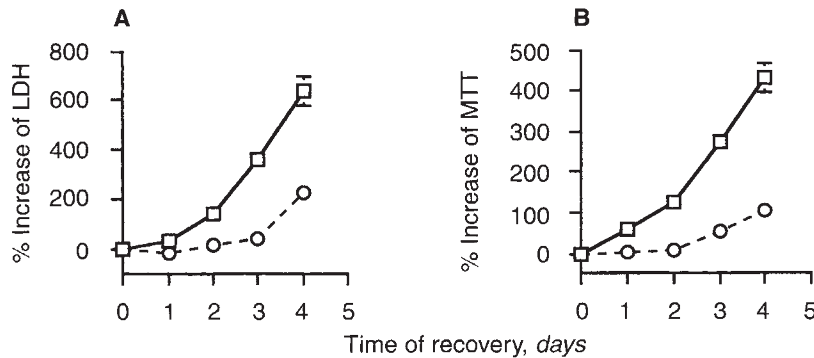

Fig. 5. *Cells were treated with 1 mg/ml myoglobin for 24 hours, the myoglobin was removed, and then cell outgrowth was assessed over 4 additional days by (**A**) LDH or (**B**) MTT assay.* Co-cultured, nonmyoglobin exposed cells treated in an identical manner served as controls (□). Whereas the normal cells manifested curvilinear increases in LDH and MTT values, the myoglobin treated cells (○) demonstrated virtually no increments for ~three days following myoglobin washout. After three days, recovery of cell proliferation was noted. Values are given as a % increase from time 0 values (the time of myoglobin washout). SEM if not depicted = less than the depicted symbols. $N > 4$ determinations at each time point ($P < 0.00005$ at days 1, 2, 3, and 4, compared to the control cells).

cells continued to manifest a marked reduction in outgrowth for ~3 additional days. By the fourth day post myoglobin washout, some improvement in cell proliferation was apparent (Fig. 5). Of note is that neither BSA nor lysozyme treatment had any impact on identically conducted cell outgrowth experiments (data not shown), indicating that myoglobin's negative impact was not simply a "protein loading" effect. Finally, when the cells were examined for vital dye uptake one to two days into these outgrowth experiments, no loss of cell viability was observed. This indicates that delayed cell death did not account for the diminished cell numbers. In sum, these experiments demonstrate that: (1) myoglobin can induce HK-2 growth arrest; (2) this effect persists beyond the period of acute myoglobin exposure (a point with obvious potential clinical relevance), and (3) this antiproliferative effect represents a transient, rather than an irreversible, defect.

Since myoglobin toxicity is iron dependent (such as Fig. 1B data), the next issue to be addressed was whether DFO treatment could reverse myoglobin's growth suppressive effect. To test this possibility, cells were continuously exposed to DFO after they had

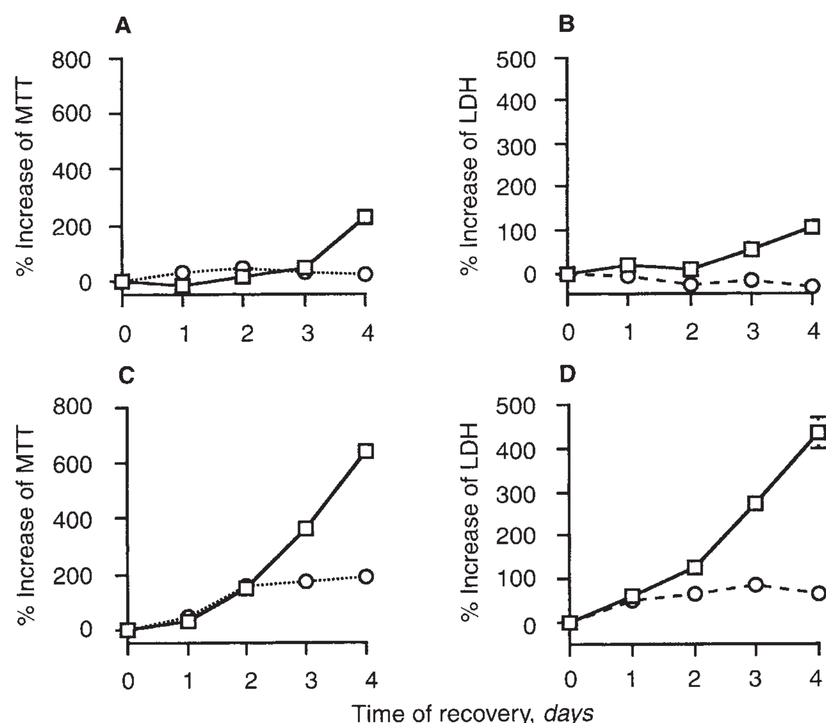

Fig. 6. A, B. *Effect of DFO treatment on HK-2 cell outgrowth following 1 mg/ml myoglobin loading.* All of the cultures demonstrated an absence of proliferation until ~ day 3. DFO treatment during this time only served to supppress cell outgrowth, as evidenced by the fact that the DFO-treated cells did not manifest the normal proliferative upsurge at ~ day 3. Thus, DFO worsened, rather than improved, cell proliferation rates. **C, D**. DFO's negative effect on HK-2 outgrowth was not confined to myoglobin loaded cells since it also blocked normal HK-2 cell proliferation. In data not shown, 50 μM 1,10 phenanthroline reproduced DFO's anti-proliferative effect. Symbols are: (□) −DFO; (○) +DFO. (All values are given as a % increase from time zero. N ≥ 3 determinations at each time point. The SEM was < the size of the symbol if no SEM bars are given).

completed 20 to 24 hours of myoglobin loading. As shown in Figure 6 A and B, DFO did not improve HK-2 proliferation from days 1 to 3 post myoglobin washout, whether assessed by MTT or LDH assay. Of particular note, DFO treatment blocked the normal proliferative "upsurge" that normally occurs three to four days post-myoglobin loading. This negative impact was not confined to the myoglobin-loaded cells, because DFO also retarded normal HK-2 cell outgrowth (Fig. 6 C, D). That 1,10-phenanthroline reproduced this latter growth suppressive effect (data not shown) indicates that DFO's negative impact was likely due to iron chelation, not DFO toxicity, *per se*. In subsequent experiments, we tested whether short-term DFO therapy (for 24 hr immediately post-myoglobin treatment) might reverse myoglobin's growth suppressive effect while, at the same time, averting its own. However, this protocol also failed to exert a beneficial influence (data not shown). Thus, these findings suggest a therapeutic paradox: whereas iron chelation therapy mitigates the *initial expression* of heme protein nephrotoxicity, it may also retard *recovery from it*. This result is by no means surprising since it has previously been demonstrated that DFO, as well as other iron chelators, can inhibit cell proliferation by blocking DNA synthesis [27, 28]. This may occur via inhibition of ribonucleotide reductase, the enzyme which mediates the free-radical reduction of ribo- to deoxyribonucleotides [27]. Since this is a rate limiting step in DNA synthesis, cell proliferation is attenuated. Alternatively, iron chelation may also inhibit the expression of cyclin A-dependent $p33^{cdk2}$ kinase [28].

A number of nephrotoxins can suppress protein synthesis [29–31]. Were myoglobin to exert this influence, it could help to explain its antiproliferative effect. Hence, ^{35}S-methionine incorporation was determined in myoglobin loaded and control HK-2 cells. As depicted in Figure 7A, following 24 hours of a 1 mg/ml myoglobin challenge, an approximate 85% suppression of ^{35}S-methionine uptake was observed. This finding cannot simply be explained by diminished cell numbers, since factoring the ^{35}S-methionine data by the total cell mass (LDH) still revealed an approximate 65% reduction in protein synthesis (Fig. 7B). Whether reduced protein synthesis was a cause of the diminished cell proliferation or a marker of it remains unknown. As shown in Figure 7C, once the myoglobin was removed from the cultures, the control and the myoglobin loaded cells mounted essentially parallel increments in ^{35}S-methionine incorporation. This conceivably could have allowed the cells to escape from the myoglobin-induced growth arrest.

To further define the characteristics and possible basis for myoglobin's anti-proliferative effect, DNA synthetic rates were gauged by BrDU incorporation. Specifically, the % cells manifesting BrDU uptake two days following completion of a 0.5 or 1 mg/ml myoglobin challenge was assessed. Co-cultured normal HK-2 cells served as controls. Whereas 44 ± 4% of normal cells demonstrated BrDU positivity (Fig. 8), the cells previously treated with 0.5 or 1 mg/ml myoglobin manifested 25 ± 9% and 4 ± 4% BrDU positivity, respectively. Thus, these data provide independent confirmation of the principal conclusion from the LDH and MTT data: that sublethal myoglobin exposure induces a profound antiproliferative effect. The BrDU data also raise the possibility that a direct block in DNA replication might exist, possibly causing or contributing to this result.

A likely explanation for the myoglobin-induced block in BrDU incorporation could be iron-mediated oxidant DNA damage. In this regard, it is noteworthy that DNA strand breaks are readily induced by iron dependent oxidant stress [32]. To explore this possibility, we used the highly sensitive DNA unwinding assay to determine whether a sublethal myoglobin exposure (1 mg/ml) had

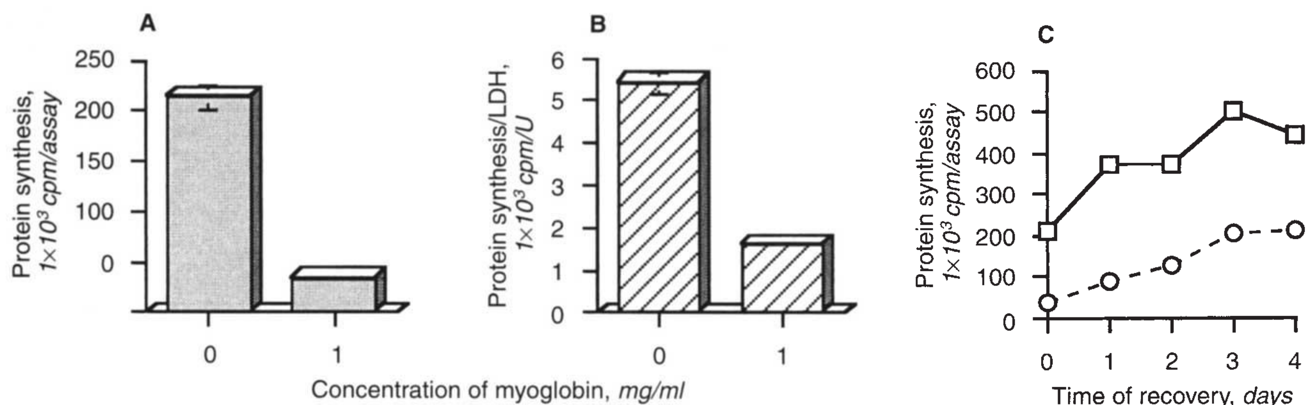

Fig. 7. **A.** *Absolute cpms ^{35}S-methionine incorporated into cells after 24 hours in culture in the absence or presence of 1 mg/ml myoglobin.* An approximate 85% suppression was noted in the myoglobin exposed cells ($P < 0.00005$; $N = 3$). **B**. Factoring the ^{35}S-methionine incorporation by the prevailing cell mass (as assessed by intracellular LDH) indicated that the above difference in protein synthesis could not be solely due to differences in cell number (0 vs. 1 mg/ml myoglobin, $P < 0.001$). **C**. Following myoglobin washout from the culture medium, the cells (○) regained what appeared to be a normal protein synthetic rate (that is, the same approximate slope as observed in the nonmyoglobin exposed cells, □).

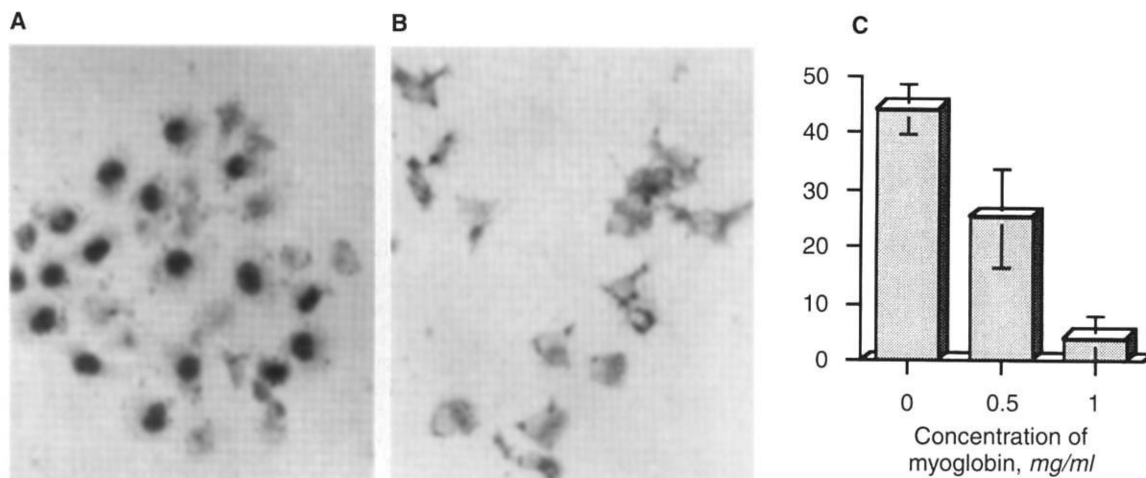

Fig. 8. *Effect of myoglobin treatment on BrDU incorporation by HK-2 cells.* **A**. Normal HK-2 cells showing a high frequency of nuclear BrDU positivity. Conversely, cells treated with 1 mg/ml myoglobin for 24 hours and then allowed a 48-hour recovery period showed a low frequency of BrDU positivity (**B**). In addition, the cells after, but not before, fixation, had a more angular appearance. The % BrDU positivity for these experiments are depicted in panel **C**. $P < 0.0001$ for each myoglobin group vs. the controls (unpaired t-test/Bonferroni correction; controls, $N \geq 9$ determinations for each).

damaged HK-2 DNA. The unwinding assay is based on the principal that the rate of DNA unwinding in alkaline solutions is dependent on the length of the DNA. Hence, strand breaks (even 1 per chromosome) accelerates this process [23]. As shown in Figure 9, significantly greater DNA unwinding under alkaline conditions was observed in the myoglobin treated cells. Clearly then, DNA damage had occurred. Whether these strand breaks, and/or oxidant damage to DNA bases, were directly responsible for the decreased proliferation remains an untested, but appealing hypothesis.

A remaining question is whether the myoglobin-induced block in cell proliferation was a completely non-specific response, that is, could it be reproduced by any toxic agent? This seemed most unlikely given observations that H_2O_2 [13] and $FeSO_4$ [12] can increase tubular cell proliferation, possibly by enhancing ribonucleotide reductase activity [27]. On the basis of these results, one might anticipate that myoglobin would exert a growth stimulatory, rather than an inhibitory, effect. The explanation for this seeming paradox remains unknown, but it may stem from marked differences in rates and degrees of iron/free radical generation. For example, an H_2O_2 challenge is rapidly dissipated due to intracellular catalase activity. Conversely, $FeSO_4$ has very limited intracellular access. However, myoglobin is actively transported into tubular cells and once there, it represents a persisting iron burden. These differences in cellular kinetics could conceivably underlie these seemingly descrepant observations.

To further make the point that a persisting block in cell proliferation is not a necessary consequence of nephrotoxin exposure, the impact of a borderline toxic gentamicin concentration (1 mg/ml) on HK-2 proliferation was assessed. After a 24 hour exposure, gentamicin caused an approximate 20% decrease in cell numbers, as assessed by MTT and LDH assay ($P < 0.01$). Since this gentamicin dose caused no increased vital dye uptake (> 90% for the control and gentamicin groups), it appears that

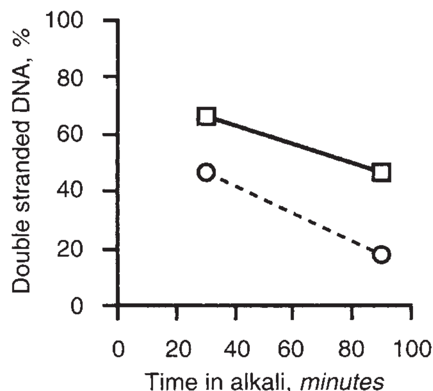

Fig. 9. *Effect of 1 mg/ml myoglobin treatment × 24 hours on DNA unwinding.* Under alkaline conditions, the myoglobin treated cells (○) manifested significantly increased unwinding, consistent with DNA strand breaks compared to controls (□). The values are statistically different after both 30 minutes and 90 minutes under alkaline conditions ($P < 0.005$). If one extrapolates the line back to time zero, 61 ± 4% and 77 ± 2% intact DNA values were obtained for myoglobin and control cells, respectively ($P = 0.02$; $N = 3$; the SEM was less than the symbol sizes).

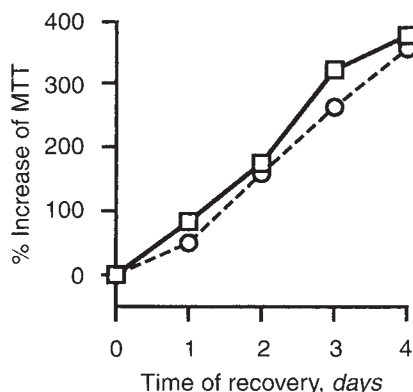

Fig. 10. *Effect of 1 mg/ml gentamicin (○) exposure for 24 hr on HK-2 proliferation.* At the completion of 24 hr of gentamicin exposure, cell numbers were reduced by ~20% (assessed by MTT and LDH assay) ($P < 0.01$, compared to co-cultured controls, □). This is consistent with gentamicin's ability to inhibit protein synthesis [30]. However, following gentamicin removal from the cultures, a normal proliferative response was observed (similar upward slope to that seen in the normal cells). This result was in marked contrast to that observed in the myoglobin washout experiments.

gentamicin had retarded cell proliferation by its ability to inhibit protein synthesis [30]. However, once the gentamicin was removed from the culture medium, a normal proliferative response was observed (Fig. 10). This further makes the point that myoglobin's ability to induce persisting growth arrest should not simply be viewed as a non-specific consequence of "nephrotoxicity".

In conclusion, the present study demonstrates that following a 20 to 24 hour period of myoglobin exposure, a marked suppression of proximal tubular cell proliferation may result. Correlates of this growth suppression, and potential causes of it, include a striking decrease in DNA synthesis (BrDU incorporation), direct DNA damage (strand breaks), and a marked suppression of protein synthesis. Whether these results, obtained with an immortalized proximal tubular cell line, are directly relevant to *in vivo* myoglobinuric renal injury remains unknown. Nevertheless, they do offer potential new insights into the nature and spectrum of heme-protein induced cytotoxicity. Most notably, the data suggest that two phases of myoglobin cytotoxicity exist: (1) the acute phase in which myoglobin may cause lethal cell damage; and (2) a delayed phase in which sublethally damaged cells have a blunted proliferative response. Since the latter might retard restoration of tubular integrity, delayed recovery from ARF could result. Lastly, it should be noted that DFO can affect both of these injury phases: it can mitigate myoglobin-induced cell killing, but it can also induce its own growth suppressive effect. The latter action, which is not uniquely expressed in HK-2 cells, needs to be considered if this agent is to be used in clinical trials of myohemoglobinuric ARF.

Acknowledgments

The authors thank Mr. Dennis Gmur for performing the HPLC analyses during the course of these experiments. This work was supported by a grant from the National Institutes of Health (DK 38432).

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References

1. HUMES KD: Cellular and molecular basis of renal repair in acute renal failure. *J Lab Clin Med* 124:749–754, 1994
2. LAKE EW, HUMES HD: Acute renal failure: directed therapy to enhance renal tubular regeneration. *Semin Nephrol* 14:83–97, 1994
3. HAMMERMAN MR, MILLER SB: Therapeutic use of growth factors in renal failure. *J Am Soc Nephrol* 5:1–11, 1994
4. HUMES HD, CIESLINSKI DA, COIMBRA TM, MESSANA JM, GALVAO C: Epidermal growth factor enhances renal tubule cell regeneration and repair and accelerates the recovery of renal function in postischemic acute renal failure. *J Clin Invest* 84:1757–1761, 1989
5. MILLER SB, MARTIN DR, KISSANE J, HAMMERMANN MR: Insulin-like growth factor I accelerates recovery from ischemic acute tubular necrosis in the rat. *Proc Natl Acad Sci USA* 89:11876–11880, 1992
6. ZAGER RA: Intracellular myoglobin loading worsens H_2O_2-induced, but not hypoxia/reoxygenation-induced, in vitro proximal tubular injury. *Circ Res* 73:926–934, 1993
7. BUNN HF, JANDL JK: The renal handling of hemoglobin. II. Catabolism. *J Exp Med* 129:925–934, 1969
8. PALLER MS: Hemoglobin- and myoglobin-induced acute renal failure in rats: Role of iron in nephrotoxicity. *Am J Physiol* 255:F539–F544, 1988
9. SHAH SV, WALKER PD: Evidence suggesting a role for hydroxyl radical in glycerol-induced acute renal failure. *Am J Physiol* 255:F438–F443, 1988
10. ZAGER RA: Combined mannitol and deferoxamine therapy for myohemoglobinuric renal injury and oxidant stress. Mechanistic and therapeutic implications. *J Clin Invest* 90:711–719, 1992
11. ZAGER RA, BURKHART K, CONRAD S, GMUR DJ: Iron, heme oxygenase, and glutathione: Effects on myoglobinuric proximal tubular injury. *Kidney Int* 48:1624–1634, 1995
12. ZAGER RA, FUERSTENBERG SM, BAEHR P, MYERSON D, TOROK-STORB B: An evaluation of antioxidant effects on recovery from postischemic acute renal failure. *J Am Soc Nephrol* 4:1588–1597, 1994
13. GOLIGORSKY MS, MORGAN MA, SUH H, SAFIRSTEIN R, JOHNSON R: Mild oxidative stress: Cellular mode of mitogenic effect. *Renal Failure* 14:385–389, 1992
14. RYAN MJ, JOHNSON G, KIRK J, FUERSTENBERG SM, ZAGER RA, TOROK-STORB B: HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int* 45:48–57, 1994

15. GRUNE T, SOMMERBURG O, PETRAS T, SIEMS WG: Postanoxic formation of aldehydic lipid peroxidation products in human renal tubular cells. *Free Rad Biol Med* 18:21–27, 1995
16. BIRD RP, SILAS S, HUNG O, HADLEY M, DRAPER HH: Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. *Anal Biochem* 18:21–27, 1995
17. MOSSMAN T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Meth* 65:55–63, 1983
18. PRICE P, MCMILLAN TJ: Use of the tetrazolium assay in measuring the response of human tumor cells in ionizing radiation. *Cancer Res* 50:1392–1396, 1990
19. SLATER TF, SAWYER B, STRAULI UD: Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. *Biochim Biophys Acta* 77:383–393, 1963
20. IWATA M, HERRINGTON J, ZAGER RA: Protein synthesis inhibition induces cytoresistance in cultured human proximal tubular (HK-2) cells. *Am J Physiol* 268:F1154–F1163, 1995
21. MOIR RD, LOWY-MONTAG M, GOLDMAN RD: Dynamic properties of nuclear lamins: Lamin B is associated with sites of DNA replication. *J Cell Biol* 125:1201–1212, 1994
22. KRIEGLSTEIN K, SUTER-CRAZZOLARA C, FISCHER WH, UNSICKER K: TGF-β superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP^+ toxicity. *EMBO J* 14:736–742, 1995
23. BIRNBOIM HC, JEVCAK JJ: Fluorometric method for rapid detection of DNA breaks in human white blood cells produced by low doses of radiation. *Cancer Res* 41:1889–1892, 1981
24. UEDA N, SHAH SV: Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. *J Clin Invest* 90:2593–2597, 1992
25. ZAGER RA: Studies of mechanisms and protective maneuvers in myoglobinuric acute renal injury. *Lab Invest* 60:619–629, 1989
26. ZAGER RA, FOERDER C, BREDL C: The influence of mannitol on myoglobinuric acute renal failure: Functional, biochemical, and morphological assessments. *J Am Soc Nephrol* 2:848–855, 1991
27. HOYES KP, HIDER RC, PORTER JB: Cell cycle synchronization and growth inhibition by 3-hydroxypyridin-4-one iron chelators in leukemia cell lines. *Cancer Res* 52:4591–4599, 1992
28. LUCAS JJ, SZEPESI A, DOMENICO J, TAKASE K, TORDAI A, TERADA N, GELFAND EW: Effects of iron-depletion on cell cycle progression in normal human T lymphocytes: Selective inhibition of the appearance of cyclin A-associated component of the $p33^{dk2}$ kinase. *Blood* 86:2268–2280, 1995
29. GOERING PL, FISHER BR, CHAUDHARY PP, DICK CA: Relationship between stress protein induction in rat kidney by mercuric chloride and nephrotoxicity. *Toxicol Appl Pharmacol* 113:184–191, 1992
30. BENNETT WM, MELA-RIKA LM, HOUGHTON DC, GILBERT DN, BUSS WC: Microsomal protein synthesis inhibition: An early manifestation of gentamicin nephrotoxicity. *Am J Physiol* 255:F265–F269, 1988
31. TAY LK, BREGMAN CL, MASTERS BA, WILLIAMS PD: Effects of cis-diaminedichloroplatinum (II) on rabbit kidney in vivo and on rabbit renal proximal tubules in culture. *Cancer Res* 48:2538–2543, 1988
32. GOLCONDA MS, UEDA N, SHAH SV: Evidence suggesting that iron and calcium are interrelated in oxidant-induced DNA damage. *Kidney Int* 44:1228–1234, 1993