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Manganese superoxide dismutase gene therapy protects against irradiation-induced cystitis

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Kanai, Anthony J., Mark L. Zeidel, John P. Lavelle, Joel S. Greenberger, Lori A. Birder, William C. De Groat, Gerard L. Apodaca, Susan A. Meyers, Richard Ramage, and Michael W. Epperly. Manganese superoxide dismutase gene therapy protects against irradiation-induced cystitis. *Am J Physiol Renal Physiol* 283: F000–F000, 2002; 10.1152/ajprenal.00228.2002.—Urinary bladder cystitis occurs in patients receiving radiation therapy for pelvic tumors. Radiation-induced formation of superoxide radicals is believed to damage the urothelium, exposing the underlying bladder smooth muscle to urine, culminating in nerve irritation and muscle dysfunction. We tested whether overexpression of MnSOD could decrease superoxide levels and protect the bladder from radiation damage. Pelvic irradiation led to sloughing of urothelial umbrella cells, with decreased transepithelial resistance, increased water and urea permeabilities, and increased expression of inducible nitric oxide synthase. Six months after irradiation, cystometrograms showed elevated intravesical pressures and prolonged voiding patterns. However, urothelia transfected with the MnSOD transgene recovered from radiation injury more rapidly, and detrusor function was much closer to that of control bladders than irradiated bladders without the transgene. We conclude that MnSOD gene therapy is protective, which could lead to its use in mitigating radiation cystitis and preventing dysfunction of the urinary bladder.

radiation cystitis; urinary bladder; copper-zinc superoxide dismutase, magnesium superoxide dismutase; radioprotective gene therapy

A LARGE NUMBER OF PATIENTS in the United States undergo irradiation for pelvic malignancies each year. However, the total allowable radiation dose is limited by the potential for developing irradiation-induced cystitis (35). Pathologically, irradiation damage to the bladder occurs in three distinct phases (36). Acute damage after a single dose consists of urothelial swelling, ulceration, and vascular endothelial cell damage. A subacute phase occurs within 4–6 days of radiotherapy, with infiltration of inflammatory cells (30). A third chronic phase is associated with collagen deposition and fibrosis, leading to a decrease in bladder compliance (30, 36). Clinically, the long-term side effects of radiation therapy include decreased bladder volume and increased frequency, urgency, and dysuria (7, 25).

A few studies have investigated the pathological changes associated with irradiation cystitis in animals and humans. In mice receiving <15 Gy (wherein Gy is the absorbed energy of 1 J/kg), 20% developed fibrotic bladders, while all the mice receiving 15–20 Gy exhibited increased frequency and decreased bladder volume (36). Rats irradiated with 20 Gy all exhibited small and contracted bladders 6 mo after treatment (19, 40). In human bladders, subepithelial fibrosis became more extensive with time after irradiation (1). The loss of bladder muscle and damage to vascular endothelial cells and bladder neurons was also evident (39). Thus irradiation may result in damage to multiple bladder cell types, including urothelium, nerve, and muscle. It is presently unclear whether nerve and muscle cell damage is related to the inflammation that accompanies disruption of the bladder permeability barrier.

Although little is known about the mechanism of radiation-induced urinary bladder damage, ionizing radiation is known to increase formation of superoxide (O_2^-) free radicals. These in turn can cause DNA strand breaks, oxidize membrane lipids, or react with nitric oxide (NO) to form peroxynitrite, which can cause nitration of proteins; each or all of these processes can lead to apoptosis. Because O_2^- radicals are thought to mediate much of the radiation damage, strategies to decrease O_2^- have been tested as approaches to mitigating radiation damage. These strategies include overexpression of the MnSOD transgene in cells to protect them from irradiation-induced damage both in vitro and in vivo (12, 14, 15). In vitro, overexpression of MnSOD in 32D cl 3 murine hematopoietic progenitor cells prevents irradiation-induced apoptosis (12, 15). This occurs at the level of the mitochondria, because increased MnSOD activity stabilizes the mitochondria, preventing mitochondrial membrane depolarization, release of cytochrome *c*, and activation of caspase 3,

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thereby preventing apoptosis (15). Overexpression of the MnSOD transgene in the mouse lung and esophagus protected normal tissue from irradiation-induced damage by preventing pulmonary fibrosis and esophagitis (14). Because the function of MnSOD is the removal of O_2^- produced in the mitochondria (16), increased MnSOD may be acting by reducing O_2^- produced by irradiation.

The O_2^- free radical is generated within the mitochondria as the toxic by-product of oxidative phosphorylation. The major O_2^- antioxidants are the SODs. There are three SOD isoforms that are all of nuclear DNA origin: cytoplasmic Cu/Zn-containing SOD (Cu/ZnSOD or SOD1), mitochondrial Mn-containing SOD (MnSOD or SOD2), and extracellular Cu/ZN-containing SOD (EC-SOD or SOD3). Although all three SOD isoforms are constitutively expressed, it is the mitochondrial isoform that has been shown to undergo a 100-fold increase in enzyme levels in response to oxidative stress without an appreciable increase in the level of Cu/ZnSOD. Therefore, MnSOD is often referred to as an inducible enzyme and Cu/ZnSOD and EC-SOD

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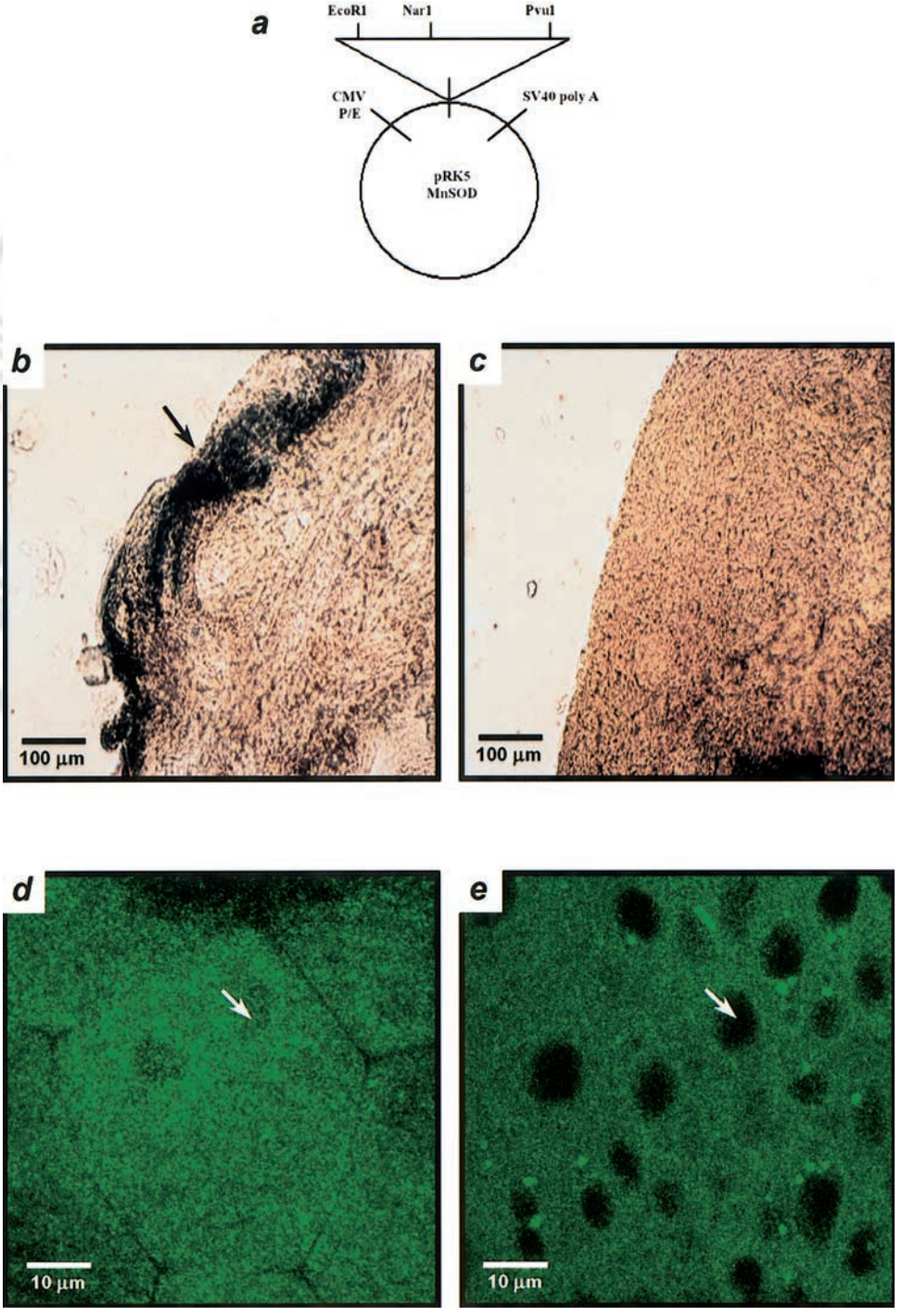

Fig. 1. *a*: plasmid/liposome complex used in these experiments contained the complete human manganese SOD (MnSOD) transgene. To visualize transfection efficiency at the tissue level, a construct containing the LacZ transgene in place of MnSOD was instilled into rat bladders. CMV P/E, . *b*: LacZ-transfected bladders show positive staining all along the urothelial surface (arrow) as well as several cells in depth. *c*: nontransfected animals do not show LacZ staining ($n = 4$). *d*: at the cellular level, in hemagglutin-MnSOD-plasmid/liposome-transfected urothelium, laser confocal immunofluorescence shows hemagglutinin epitope-tagged transgene throughout the cell. *e*: lack of immunofluorescence demonstrates the absence of the construct in control/nonirradiated bladders transfected with liposomes alone. Arrows, nuclei within the umbrella cells (*d* and *e*).

as constitutive enzymes. The SOD isoforms catalyze the dismutation of O_2^-. A dismutase converts two identical molecules (i.e., O_2^-) to two molecules of differing oxidation states (i.e., H_2O_2 and O_2)

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

We examined in detail the urothelial effects of pelvic irradiation. We hypothesized that disruption of the urothelial permeability barrier caused by irradiation would lead to inflammation of underlying musculature and that this inflammation might cause much of the reduced compliance and detrusor dysfunction seen in radiation cystitis. Given that induced MnSOD can reduce radiation injury to other epithelia, we developed a gene therapy approach to augment MnSOD expression in urothelium before irradiation (13, 31, 37, 38). Our results show that MnSOD-transfected bladders are protected from some of the damaging effects of ionizing radiation.

METHODS

Animal groups. Sprague-Dawley rats were divided into five groups with the first group consisting of control/nonirradiated rats. These rats were anesthetized but were instilled with only vehicle. The second group consisted of control/irradiated rats instilled with vehicle and irradiated to 35 Gy to the bladder 24 h after instillation. A third group was MnSOD/irradiated rats that were instilled with the MnSOD-plasmid/loposome (PL) containing 500 μg of MnSOD plasmid DNA and irradiated to 35 Gy to the bladder 24 h after instillation. The plasmid/liposome complex used in these experiments contained the pRk5 MnSOD construct (Fig. 1*a*). A fourth group consisted of rats instilled with the LacZ-PL in lieu of the MnSOD-PL and were not irradiated. The fifth group were rats instilled with a hemagglutin (HA) epitope-tagged MnSOD-PL (HA-MnSOD-PL) and were also not irradiated.

Instillation of the MnSOD-PL and irradiation of the bladder. PL complexes were prepared by mixture of 56 μl of lipofectin liposomes with 600 μg of plasmid DNA in a volume of 200 μl of PBS, incubation at room temperature, and dilution to 600 μl. Female Sprague-Dawley rats were anesthetized with halothane (5% for induction and 2.5% for maintenance) in oxygen, and urethras were catheterized with a 3.5 French Tom Cat catheter. The bladder was then drained and irrigated with 0.5 ml of water to remove the urine, in which high-salt concentration can prevent the PL complex from binding to the umbrella or surface cells of the urothelium. Finally, 0.5 ml of the complex containing 500 μg of pRK5 MnSOD plasmid DNA containing the full human MnSOD transgene (Fig. 1*a*) was instilled and held in place in the bladder for 10 min to allow for transfection. Alternatively, vehicle alone was instilled for 10 min. The catheter was then removed and the rat was allowed to recover. At 24-h post-transfection, the rats were anesthetized with ketamine/xylazine and given a single dose of irradiation equivalent to 35 Gy. The irradiation was delivered by a 6 MeV Varian linear accelerator, which produces a beam of X-rays. The accelerator was configured to produce the designated output in a 1.5-cm region × 30-cm field at a depth of 100 cm to the skin. This region of the beam was set to encompass the bladder, assuring uniform irradiation. The rats were shielded so that only an area close to the bladder was exposed. Bladders were excised at times ranging from 1 h to 6 mo.

Histopathology for detection of plasmid expression. Rats were injected intravesically with LacZ-PL complexes as described above for MnSOD-PL. The rats were killed 24 h after instillation, and bladders were excised, cut longitudinally, pinned flat, and frozen in optimum cutting temperature embedding medium. Tissues were sectioned (10 μm thick) on a cryostat and stained for LacZ expression.

Confocal immunofluorescence microscopy. Rats were instilled with HA-MnSOD-PL or water only as described above for MnSOD-PL. The animals were killed 24 h later, and bladders were excised, frozen in optimum cutting temperature embedding medium, sectioned, and stained with FITC anti-HA antibodies. Studies were then performed as previously described (20, 22).

Nested RT-PCR for detection of human MnSOD transgene expression in bladder. Groups of rats were injected with MnSOD-PL complexes and killed 24, 48, or 72 h later, and bladders were excised and snap-frozen in liquid nitrogen. Bladders were homogenized in 3 ml of TRIzol (GIBCO-BRL)

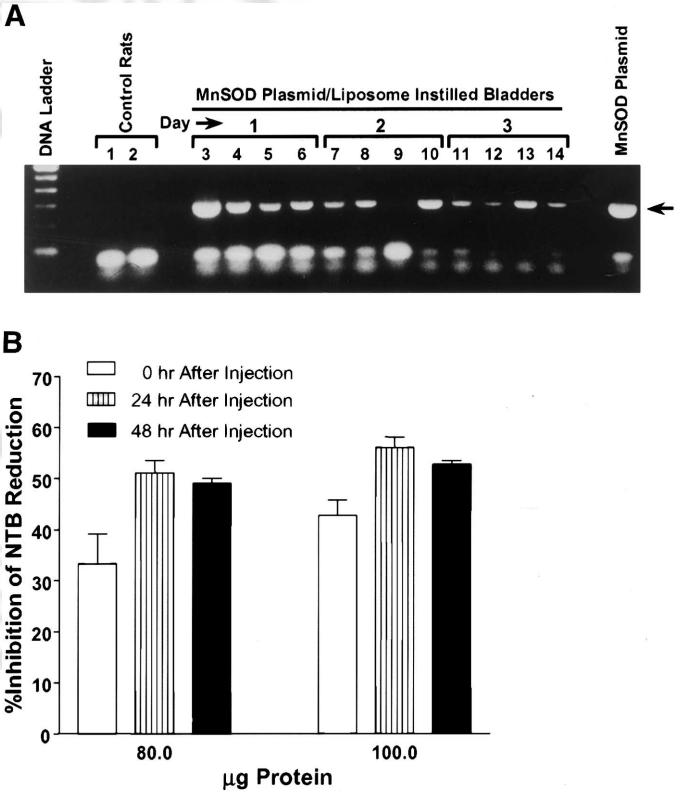

Fig. 2. *A*: nested RT-PCR detected the human MnSOD gene in rat bladder after intravesical MnSOD-PL administration. MnSOD gene expression was detected in 11 of 12 rats instilled with the construct but none of the control/nonirradiated rats. Expression was detected for at least 3 days after instillation. The *top* band at 283 bp represents the MnSOD transgene (arrow), whereas the *bottom* band represents primers used for the PCR reaction. *B*: increased MnSOD biochemical activity, measured as the function of the percentage of inhibition of the reduction of nitroblue tetrazolium (NBT) by SOD, was also detected in rats instilled with the transgene. Bladders were removed from rats at 0, 24, 48, and 72 h after instillation of MnSOD, and the biochemical activity was calculated. There was a significant increase in biochemical activity (change in absorbance measured at 550 nM) at 24 (12.8 ± 0.6 U) and 48 h (12.3 ± 0.3 U) when compared with control rats (9.7 ± 0.5 U; $n = 5$). At 72 h, MnSOD biochemical activity was back to control levels (not shown).

by using a Polytron PT2000 homogenizer (Brinkman Instruments, Westbury, NY). The homogenized samples were incubated for 5 min at room temperature, followed by the addition of 0.6 ml of chloroform, mixed, and incubated at room temperature for 3 min. The samples were centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was removed and transferred to a new tube in which 1.5 ml of isopropyl alcohol was added and incubated at room temperature, followed by centrifugation at 12,000 rpm for 10 min at 4°C. The pellet was washed with 75% ethanol and centrifuged at 7,500 rpm for 5 min at 4°C, air dried, and resuspended in diethyl pyrocarbonate water. Two micrograms of each sample were used in the RT reaction by mixing the RNA with poly-dT, 10 μM mixture of dCTP, dATP, dTTP, and dGTP, and Superscript II RT (GIBCO-BRL). The tubes were incubated for 50 min at 42°C and 10 min at 95°C, followed by incubation at 4°C.

Nested PCR was used to amplify the human MnSOD transgene by using primers specific for the human MnSOD gene (9–11). For the nested reaction, 0.01 μl of the RT reaction was mixed with the first set of 5′ and 3′ oligonucleotide primers, 10 μM mixture of dATP, dCTP, dTTP, and dGTP, and 0.4 U *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The mixture was subjected to 20 cycles of 94, 60, and 72°C (30, 50, and 90 s, respectively) in a Perkin-Elmer (Foster City, CA) 9600 Gene Amp PCR system. A second round of PCR was performed using 1 μl of a 1:100 dilution of the first reaction mixed with 24 μl of the PCR mixture described above, except oligonucleotide primers, internal to the first set of primers and not overlapping in sequences, were used. Thermocycling was identical to the first PCR reaction, except the reaction was 35 cycles. Electrophoresis of the PCR products was carried out in a 1% agarose gel and stained with ethidium bromide. The first set of primers consisted of a 5′ primer of CGGCGGCATCAGCGGTAAGCCAGCACTA (nucleotides 61–89) and a 3′ primer of TGAGCCTTGGACACCAACAGATGCA (nucleotides 505–529). The internal primers consisted of a 5′ primer of GCTGGCTCCGGCTTTGGGGTATCTG (nucleotides 128–152) and a 3′ primer of GCTGAGCTTTGTCCAGAAAATGCTC (nucleotides 388–412). The expected size of the PCR product was 284 bp.

MnSOD biochemical activity. Bladders were instilled with MnSOD-PL as described above. Those rats were killed along with control/nonirradiated rats at 0–96 h, and bladders were removed and snap frozen in liquid nitrogen. Bladders were homogenized and sonicated, and MnSOD biochemical activity was analyzed with an assay whereby samples having higher levels of MnSOD activity exhibit a higher percentage of inhibition. Protein concentrations of 0, 75, and 100 μg were added to assay tubes containing 20 mM Tris (pH 7.6), 1 mM diethylenetriaminepentaacidic acid, 1 U of catalase, 5.6×10^{-8} M nitroblue tetarzolium (NBT), 0.1 mM xanthine, 0.05 mM bathocuproine-disulfonic acid, 10 mg/ml defatted BSA, and 5 mM sodium cyanide. The tubes were incubated for 45 min at room temperature. Xanthine oxidase was added and the change in absorbance was measured at 550 nM.

NO measurement from isolated bladder. In vitro measurement of NO production in isolated bladders was accomplished as previously described (3–5).

Diffusive permeability studies. The transepithelial resistance and [^{3}H]water and [^{14}C]urea permeabilities were measured with a specially designed Ussing chamber as previously described (22, 23, 26, 32, 42).

Scanning electron microscopy. Bladders were handled, fixed, and imaged as previously described (1, 2, 22).

Constant infusion cystometry. Rats were anesthetized with urethane (1.2 g/kg sc) (27–29), and their bladders were catheterized and drained (28, 29). Cystometry was performed as previously described (6, 8, 40), with a constant infusion of PBS to elicit repetitive voids (44, 45).

RESULTS

The efficiency of transfection of the PL complexes was demonstrated in the bladders of rats instilled with LacZ-PL vs. control/nonirradiated rats killed 24 h later. Bladders from LacZ-instilled rats showed positive LacZ staining that was restricted to the epithelial cell layer (Fig. 1*b*), whereas bladders from control/nonirradiated rats did not show LacZ staining (Fig. 1*c*). Rats instilled with the HA-MnSOD-PL also had their bladders removed 24 h after instillation. Immunohistochemical staining of the bladders of rats instilled with the HA-MnSOD-PL was demonstrated with an FITC conjugate antibody to HA and confocal microscopy. Umbrella cells in the bladders of the mice instilled with the HA-MnSOD-PL exhibited fluorescence throughout the cell, indicating the presence of MnSOD (Fig. 1*d*), whereas bladders of control rats exhibited little fluorescence (Fig. 1*e*). These results demon-

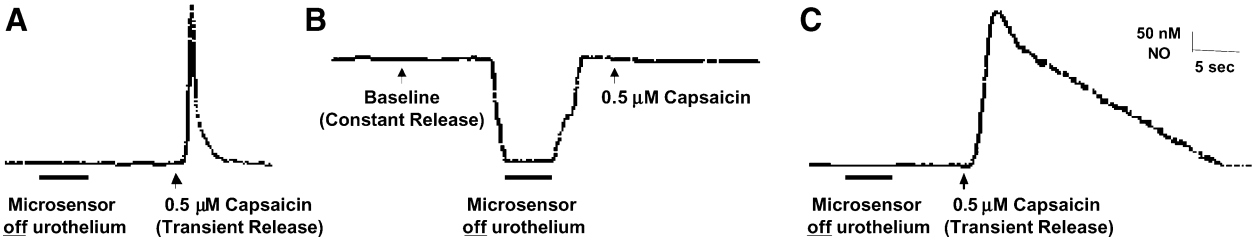

Fig. 3. The effect of irradiation and MnSOD transfection on nitric oxide (NO) production in the urothelium. In control/nonirradiated bladders, placing a prophyrinic microsensor on the urothelium, then raising it (>100 μm, bar), did not shift the baseline demonstrating the absence of basal NO production. Elevating the microsensor allows basally produced NO to degrade before it reaches the microsensor, causing the signal to decay. *A*: however, the vanilloid receptor agonist capsaicin (0.5 μM) evoked transient (~4 s) NO production (367 ± 28 nM; $n = 4$). *B*: in control/irradiated bladders (24-h postirradiation), there was basal NO production (232 ± 48 nM; $n = 4$) due to inducible NO synthase (NOS) expression. Unlike the constitutive NOS isoforms, the inducible isoform does not require Ca^{2+} and thus produces NO constantly. These cells did not respond to capsaicin. *C*: in MnSOD/irradiated bladders (24-h postirradiation), similar to control/nonirradiated bladders, the cells did not express inducible NOS but transiently produced NO (332 ± 31 nM; $n = 4$) in response to capsaicin.

strated that exposing the urothelium to the vector results in the expression of proteins encoded within it.

To determine whether the MnSOD construct leads to production of MnSOD mRNA, we used nested RT-PCR to detect mRNA with primers specific for the human MnSOD transgene. Human transgene expression was detected in the bladders of 11 of 12 rats treated with the MnSOD-PL but none of the control/nonirradiated rats (Fig. 2*A*). Human MnSOD transgene expression was detected for 3 days after instillation. To determine whether exposure to the MnSOD construct increased MnSOD enzymatic activity, bladders were removed from rats at 0, 24, 48, and 72 h after transfection, and the increase in MnSOD biochemical activity was measured as the percentage of inhibition of the reduction of NBT by SOD (the 72-h time point was omitted because MnSOD was back to control levels) (Fig. 2*B*). In this inhibition assay, increased SOD activity dismutates O_2^-, preventing the reduction of NBT leading to decreased absorbance at 550 nm. MnSOD activity was significantly increased for up to 48 h posttransfection, suggesting that the MnSOD mRNA detected at 3 days did not generate sufficient protein to maintain elevated levels of MnSOD activity. These data clearly demonstrate that the plasmid is taken up by the urothelium and induces overexpression of a functional MnSOD protein in the urothelium that remains active for at least 48 h.

To determine the effects of irradiation and MnSOD transfection on NO production in the bladder, we measured the free radical with a prophyrinic microsensor positioned directly on the urothelial surface. In control/nonirradiated bladders, NO was not basally produced, as demonstrated by the lack of a baseline shift when the microsensor was raised off the bladder surface (Fig. 3*A*). Previous studies have demonstrated that when the microsensor is elevated >100 μm off the bladder surface, the NO degrades before it can reach the sensor (17). However, these bladders did transiently produce NO in response to the vanilloid receptor agonist capsaicin (Fig. 3*A*). We have previously demonstrated that capsaicin evokes NO production in both intact urothelium and cultured urothelial cells (3, 5). Alternatively, in 24-h control/irradiated bladders, there was basal NO production due to inducible nitric oxide synthase (NOS) expression (Fig. 3*B*). Unlike the constitutive NOS isoforms, the inducible isoform does not require Ca^{2+} and thus produces NO constantly. These cells did not respond to capsaicin. In 24-h MnSOD/irradiated bladders, similar to the control/nonirradiated bladders, the cells did not express inducible NOS but transiently produced NO in response to capsaicin (Fig. 3*C*).

Transepithelial resistance is a measure of the ability of the epithelium to prevent ionic movement across the tissue and represents a critical component of urothelial barrier function. Permeabilities to water and urea also relate to the integrity of the apical membrane and tight junctions. To investigate changes in permeability, rats were killed at 1, 48, and 96 h and 7 and 24 days after 35 Gy (Fig. 4,*A–C*). MnSOD treatment did not prevent the acute changes induced by irradiation at 1–96 h but did allow transepithelial resistance and water and urea permeabilities (Fig. 4, *A–C*, respectively) to recover to near normal levels within 4 wk. These barrier functions did not recover in control/irradiated animals.

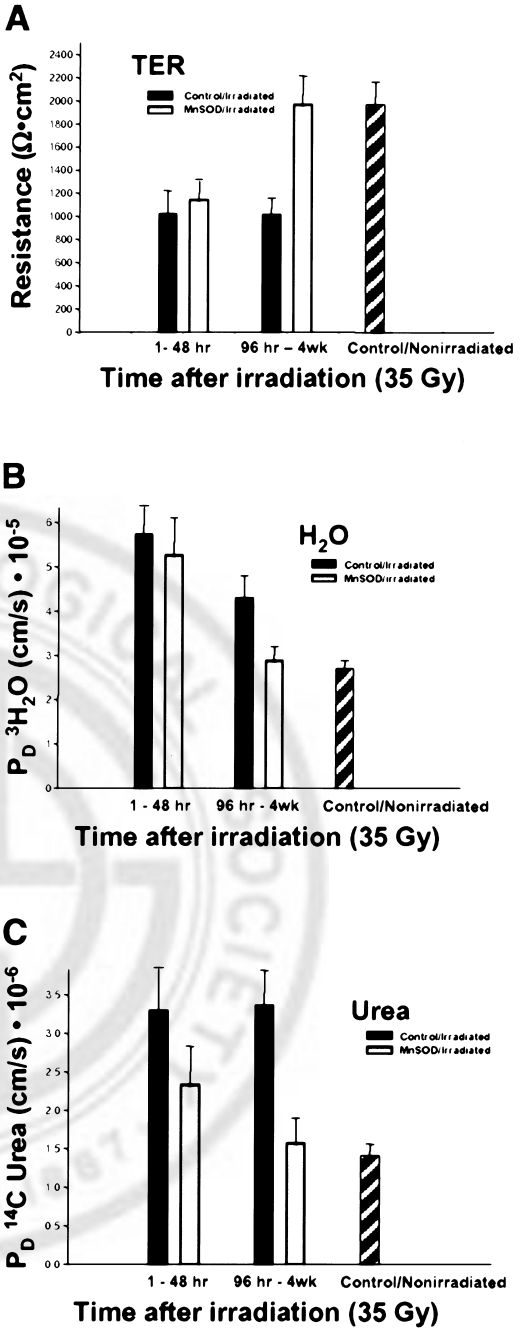

Fig. 4. The transepithelial resistance (TER) and water and urea permeabilities (*A–C*, respectively) for bladders were determined at the indicated times after irradiation [35 Gy (wherein Gy is the absorbed energy of 1 J/g)]. At 1–48 h, there was a decrease in the TER and an increase in water permeability for both control/irradiated (filled columns; $n = 16$) and MnSOD/irradiated (gray columns; $n = 11$) bladders. However, MnSOD treatment did help limit the increase in urea permeability seen in control/irradiated bladders. At 96 h to 4 wk, there was a remarkable improvement in MnSOD/irradiated bladders, with TER and water and urea permeabilities almost back to pretreatment values ($n = 13$). On the other hand, the values for control/irradiated bladders did not improve significantly ($n = 20$). P_D, .

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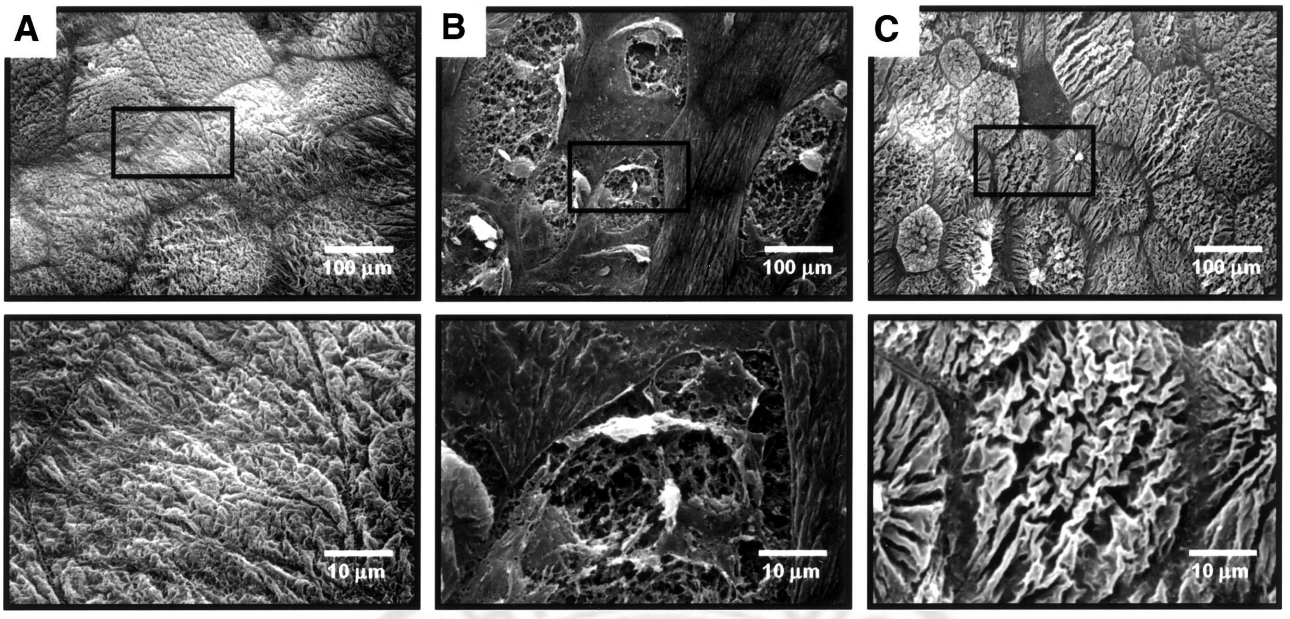

Fig. 5. Scanning electron micrographs depict the changes that occurred in the urothelium after irradiation (35 Gy). *Bottom*: areas enclosed by the rectangles in the *top* micrographs are shown magnified. *A*: control/nonirradiated bladder shows normal intact urothelium ($n = 7$). *B*: however, 48 h after irradiation the control/irradiated bladder shows areas of superficial ulceration of the umbrella cells ($n = 6$). *C*: alternatively, in MnSOD/irradiated bladders transfected with the human MnSOD transgene before irradiation, the urothelium shows only minimal ulceration ($n = 5$).

The lowest epithelial resistances were recorded 1–96 h after irradiation in both MnSOD/irradiated ($0.96 \pm 0.5\ k\Omega \cdot cm^2$) and control/irradiated ($1.03 \pm 0.7\ k\Omega \cdot cm^2$) animals (Fig. 4*A*). High resistance averaged $1.97 \pm 0.9\ k\Omega \cdot cm^2$ in control animals, with similar values ($1.97 \pm 0.4\ k\Omega \cdot cm^2$) being recorded in treated (MnSOD) animals 1–4 wk after irradiation.

Scanning electron microscopy was used to examine the ultrastructure of urothelial cells and to evaluate the changes that occur after ionizing radiation. In control/nonirradiated bladders, the urothelium was intact (Fig. 5*A*). However, 48 h after irradiation (35 Gy), bladders showed areas of superficial ulceration of the umbrella cells (Fig. 5*B*). The intermediate cells can be clearly seen to have gaps in the junctions between cells. However, the application of the human MnSOD transgene before irradiation protected the urothelium, because it only showed minimal ulceration (Fig. 5*C*). It is notable that irradiated urothelium in which MnSOD had been induced revealed small surface cells, indicating regeneration of the urothelium after injury. We have observed a similar pattern of regeneration of rat urothelium after selective injury of umbrella cells with protamine sulfate (21).

To determine the effects of irradiation on detrusor function, slow infusion cystometrograms were performed under urethane anesthesia, which preserves the voiding function. The cystometrograms for control/nonirradiated rats showed low baseline intravesical pressures and even voiding patterns (Fig. 6*A*). The cystometrograms from rats at 6 mo postirradiation (35 Gy) showed elevated baseline pressures, instability, and prolonged voiding patterns (Fig. 6*B*). However, the cystometrograms of rats that were transfected with the MnSOD transgene showed similar baseline pressures but more stable voiding patterns (Fig. 6*C*).

DISCUSSION

The bladder stores, for extended periods, urine that differs markedly in composition from the plasma. Because urine contains many substances that are noxious to normal cells (including high levels of potassium and ammonium, low pH, osmolalities that vary in humans from 50 to 1,200 mosmol/kgH_2O, and other toxins), the integrity of the urothelial permeability barrier protects underlying bladder structures from damage and inflammation that would result from exposure to urine.

In the early period after irradiation, urothelial barrier function was compromised equally in bladders treated with MnSOD or with vehicle. However, at later time points, urothelia with enhanced MnSOD activity at the time of irradiation recovered barrier function, whereas those treated with vehicle did not. This difference was reflected as well in the scanning electron micrographs, which reveal much more integrity of the umbrella cell layer in urothelia pretreated with MnSOD transgene. Because MnSOD overexpression lasted only 48 h and the protective effect was noted many days later, it is possible to speculate that MnSOD protected intermediate and basal cells of the urothelium so that they were better able to regenerate umbrella cells in the days after irradiation.

Consistent with prior reports, we found that at 6 mo after irradiation, cystometrograms of irradiated blad-

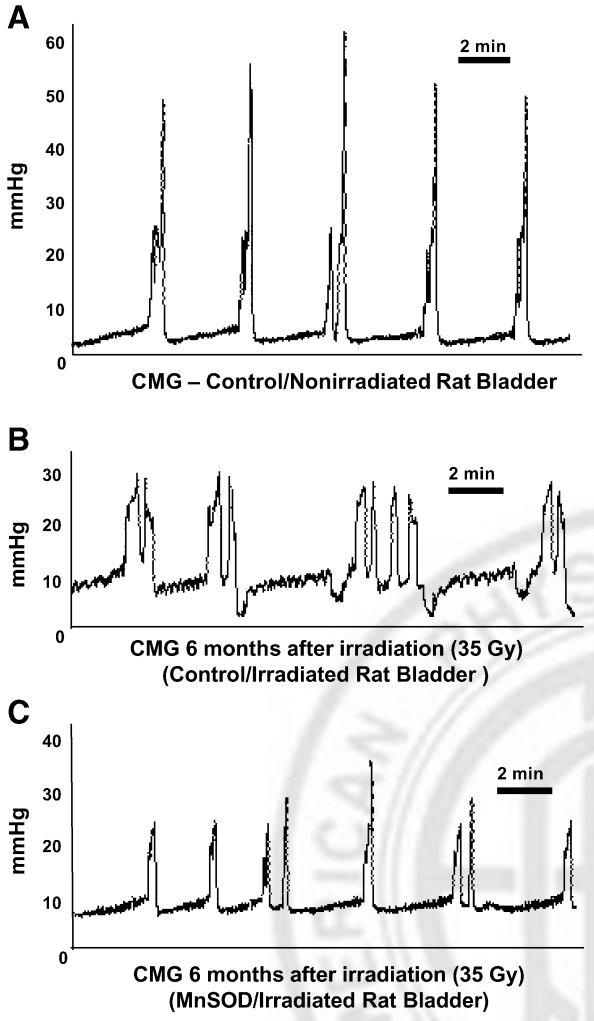

Fig. 6. Cystometrograms (CMGs) were performed under urethane anesthesia. *A*: in control/nonirradiated rats there were regular, even voiding patterns with low intravesical baseline pressures ($n = 6$). *B*: cystometrograms from control/irradiated (35 Gy, 6 mo earlier) rats show voiding patterns that are prolonged and unstable, consisting of multiple peaks in intravesical pressure as well as elevated baseline pressures ($n = 4$). *C*: in contrast, the cystometrograms from MnSOD/irradiated (35 Gy, 6 mo earlier) rats showed more even voiding patterns but comparable baseline pressures ($n = 4$).

ders revealed elevated basal tone and irritability. Importantly, pretreatment of urothelium with MnSOD resulted in a more normal voiding at 6 mo than occurred when urothelium was not pretreated with MnSOD. However, baseline pressures remained elevated in MnSOD-treated bladders. Because the nerves innervating the bladder mediate voiding patterns and the compliance of the bladder smooth muscle is involved in baseline pressures, this suggests that exogenous MnSOD was more protective of the bladder nerves. We hypothesize that this is due to the closer proximity of the bladder nerves to the MnSOD-transfected urothelium. The nerves lay directly beneath the urothelium and above the detrusor.

These results provide direct evidence that reducing leakage through the urothelial permeability barrier exerts a long-term protective effect on the underlying bladder wall layers. Because the irradiation was delivered by a linear accelerator set to produce a 35-Gy beam of X-rays in a 1.5-cm-diameter × 30-cm-long field, we can discount the possibility that any portion of the bladder was not irradiated uniformly.

What is the mechanism of this apparent protective effect on urothelium of enhanced MnSOD expression? Ionizing radiation has been shown to produce O_2^- free radicals (18), a process that appears to be mitochondrial dependent (24). The importance of the mitochondria to irradiation damage is demonstrated by studies that show that mitochondrial MnSOD is effective in preventing radiation-induced cell death in SK-N-SH cells, whereas MnSOD targeted to the cytosol is not effective (41). Conversely, cytosolic CuZnSOD in not effective in preventing radiation-induced cell death in these same cells, whereas CuZnSOD targeted to the mitochondria is effective (41). Recent studies have also demonstrated that NO and ONO_2^- exacerbate the damaging effects of ionizing radiation on mitochondrial respiratory complexes I and III (33). The importance of mitochondrial DNA to apoptosis was demonstrated by studies that showed that human fibroblast cells devoid of the mitochondria genome are resistant to oxidative stress-induced cell death, whereas cells with the mitochondrial genome are not resistant (43).

On the basis of these results, it appears that MnSOD protects urothelial cells from apoptotic cell death by reducing damage caused by radiation-induced O_2^- free radicals. Although some of the urothelial umbrella cells may be damaged or lost in MnSOD/irradiated bladders during the first 48 h, enough intermediate and basal cells remain to regenerate the urothelial cell barrier within 96 h to 4 wk. On the other hand, without induction of MnSOD, it appears that underlying intermediate and basal cells may be severely damaged so that the urothelium fails to regenerate an effective barrier.

The effectiveness of the MnSOD therapy in reducing the O_2^- free radical concentration in cells is suggested by the prolonged half-time of capsaicin-evoked NO production in MnSOD/irradiated cells (Fig. 3*C*) compared with control/nonirradiated cells (Fig. 3*A*). Although it appears that a greater amount of NO is being produced by MnSOD-treated cells in response to capsaicin, these cells exhibited the same rate of rise and peak amplitude of NO production as control cells and therefore produced similar amounts of NO. Thus the increased half-time of NO is most likely due to low levels of O_2^-, which could otherwise react with NO to form ONO_2^- (34).

The present data provide potential for translation of MnSOD-PL-based bladder radiation protection to clinical radiotherapy of cervix and vulvar cancer, particularly in patients with bulky disease who may require protracted courses of external beam radiotherapy and/or volumetric brachytherapy implants. Instillation of MnSOD-PLs to the bladder can be carried out 2 or 3 times/wk during a 7-wk course of fractionated radiotherapy. MnSOD-PL administration to the bladder could also be carried out 24 h before brachytherapy

source placement, because most interstitial implants for these tumors require single or several fractions of high dose rate brachytherapy or a 2- to 3-day course of low dose rate brachytherapy implants usually lasting 50 h. In either situation, significant protection from acute cystitis and chronic radiation fibrosis might be expected.

With respect to basic radiation biology, the present data suggest that prevention of acute radiation cystitis by MnSOD-PL gene therapy, through mechanisms that block radiation apoptosis and decrease vascular and epithelial cell swelling, in this setting may translate to protection from late radiation fibrosis. The molecular and cellular mechanisms of linkage between the acute inflammatory reaction of cystitis and the late fibrotic reaction are not known. Interruption of the acute inflammatory response by this technique of gene therapy resulted in decreased fibrosis and suggests that the two are linked in ways that could be studied by using this animal model.

In summary, we have shown that radiation directly damages urothelium, leading to loss of its critical barrier function. By using a gene therapy approach, we successfully induced MnSOD selectively in urothelial cells. Induction of MnSOD did not prevent disruption of barrier function by irradiation but led to rapid regeneration of the urothelium and recovery of barrier function. This recovery was associated with reduced spasticity of detrusor function at 6 mo after the injury. We conclude that this gene therapy approach can protect urothelium from radiation damage. These results also provide strong evidence that other forms of chronic bladder dysfunction that may result from urothelial injury (e.g., chemical and chronic infectious cystitis) should respond to approaches that enhance the recovery of the urothelial permeability barrier.

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