Prevention of Radiation-Induced Nephropathy and Fibrosis in a Model of Bone Marrow Transplant by an Angiotensin II Receptor Blocker

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Nephropathy, interstitial pneumopathy, and renal and lung fibrosis are major complications of bone marrow transplantation (BMT). This study evaluated the antifibrotic property of an angiotensin II (A_2) type-1 receptor blocker (L-159,809) and compared it with those of Captopril and Enalapril, two angiotensin-converting enzyme (ACE) inhibitors, in a rat model of BMT. Male WAG/Rij/MCW rats received a preparative regimen of 60 mg/kg body wt of cytoxan (i.p., Days 9 and 8) and 18.5 Gy of total body irradiation (TBI) in six twice daily fractions (Days 2, 1, and 0) followed immediately (Day 0) by BMT. Modifiers were given in drinking water from Day 10 until autopsy, 8 weeks after BMT. Rats treated with TBI plus cytoxan alone developed severe nephropathy. Trichrome staining showed marked collagen deposition in glomeruli, renal interstitium, and renal arteries and arterioles (especially in their adventitia). Collagen deposition and renal damage were markedly reduced by the three modifiers. Of the three, L-158,809-treated rats had slightly thinner vessels and slightly less collagen than nonirradiated normal controls. The study shows the effectiveness of these drugs in the protection of the renal parenchyma from the development of radiation-induced fibrosis. It also indicates a role for angiotensin II in the modulation of collagen synthesis.

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Injury to normal tissue characterized by inflammation, increased collagen synthesis eventually leading to organ fibrosis, and ultimate loss of function is one of the most important limiting factors to radiotherapy. Kidneys are particularly prone to this injury. A wide variety of drugs has been used in the attempt to prevent (or at least to reduce) such damaging consequences of radiation. These drugs include compounds such as penicillamine, cysteine, and cysteamine, all of which contain a sulphydril (-SH) group, as well as glucocorticoids, vasodilators, and angiotensin 1-converting enzyme (ACE) inhibitors (1–6).

Among these ACE inhibitors, Captopril has been widely studied and has been found effective in its ability to protect kidneys, lungs, and the heart from radiation-induced damage (6–8). This drug is widely used for the treatment of hypertension, congestive heart failure, and diabetic nephropathy with limited side effects. Captopril protects the kidney structures under irradiation, exerting its effect on the glomeruli, the tubuli, the renal vasculature, and the renal interstitial tissue (6). It also exerts *in vitro* a cytostatic action on many normal and neoplastic cells such as fibroblasts, endothelia, and a few cell lines of human breast carcinomas, Wilms' tumors, and pancreatic carcinomas of rats and hamsters (9–12). It also delays the growth of experimentally induced neoplasms, including radiation-induced squamous cells, carcinomas, and sarcomas (7). How this protective effect is exerted is still unclear. One possible hypothesis is that Captopril, like other ACE inhibitors, acts by preventing the synthesis of angiotensin II, thus limiting the mitogenic activity of this compound at cellular level. However, work in experimental animals as well as *in vitro* (fibroblasts and endothelial cell cultures) has shown that the radioprotective and cytostatic effects of ACE inhibitors lacking a thiol (SH) radical in their structure were weaker than those of the ACE inhibitors having the SH group (Captopril and C1 24817) (7, 10, 13). These observations suggest that some of the protective effects of Captopril might be the consequence of other pharmacological properties of the compound rather than being related to the inhibition of AII synthesis. Since Captopril is also a powerful radical scavenger, a protease

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inhibitor, and it forms complexes with copper that possess an antioxidant activity, some of Captopril's protective effects may be attributed to these additional properties (14–16). To better ascertain the role of angiotensin II in the pathogenesis of radiation induced fibrosis, we compared the effect of Captopril with that of the angiotensin II-type one-receptor blocker L 158,809 (recently synthesized by Siegl [17]) in a rat model of bone marrow transplant. The antifibrotic potency of Enalapril, an ACE inhibitor without the SH radical, was also evaluated in order to better define the possible role played by the thiol radical and by angiotensin II in the protective action from radiation.

Materials and Methods

Syngeneic Bone Marrow Transplant (BMT). A rat syngeneic BMT model was used for the study. Seven- to 8-week-old male rats of the WAG/Rij/MCW strain that had been bred and housed in a moderate security barrier were used. The animals were free of *Mycoplasma pneumonis*, *Pseudomonas*, and common rat viruses. No antibiotics or known nephrotoxic agents were used in the animal facility. The rats were maintained in the animal care facility of the Medical College of Wisconsin, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Ten days before the BMT procedure, the animals were randomly divided into five groups. One group (control, n = 6) did not receive any treatment. The four other groups (n = 7–8), received a preparative regimen of Cytoxan (CTX), a drug that is routinely used in BMT treatment, (60 mg/kg, i.p.) at Day 9 and 8 before BMT, and 18.5 Gy of total body irradiation (TBI) in six twice daily fractions, using an orthovoltage x-ray machine. During irradiation, the unanesthetized rats were immobilized in a specially constructed plastic jig. The methods for the syngeneic BMT were identical to those previously described (18).

Antihypertensive and Antifibrotic Therapy. Beginning 10 days before the transplant, three of the four groups that were to receive TBI/CTX were started on Captopril (a thiol-containing ACE inhibitor) at the dose of 500 mg/l, on Enalapril (a non-thiol ACE inhibitor) at the dose of 100 mg/l, or on the L 158,809 A II type-1 receptor blocker at the dose of 20 mg/l in their drinking fluid. The drugs were given in the rat's drinking water until the end of the experiment, 56 days post-BMT. The selected dose for Captopril was the one that had been proved effective in previous experiments in our laboratory (6). The doses for Enalapril and L 158,809 were pharmacologically equivalent, in their antihypertensive potencies, to that of Captopril, in consideration that they reduced the systolic blood pressure in spontaneously hypertensive rats of the Okamoto Aoki strain by the same amount induced by the Captopril dose (7, 17). The experimental design is summarized in Figure 1.

Laboratory Evaluation. At the sacrifice, blood was collected by venipuncture of the abdominal aorta with the rats under pentobarbital anesthesia. Routine clinical chemistry, which included sodium, potassium, calcium phosphorus, iron, alanine aminotransferase, γ-glutamyl transferase, carbon dioxide, total protein, creatinine, uric acid, blood urea nitrogen (BUN), plasma aldosterone levels, plasma renin activity (PRA), and routine hematological tests were performed on the serum and plasma obtained from this collection. The tests for the routine clinical chemistry were done using a SMA 20 automatic analyzer. Red and white cells were counted automatically by a STKS instrument (Coulter, Hialeah, FL), and PRA was measured by an radioimmunoassay (RIA) kit supplied by Dio-Sorin (Stillwater, MN). Plasma aldosterone was also measured by RIA using a kit supplied by Coat a Count (Diagnostic Product, Los Angeles, CA).

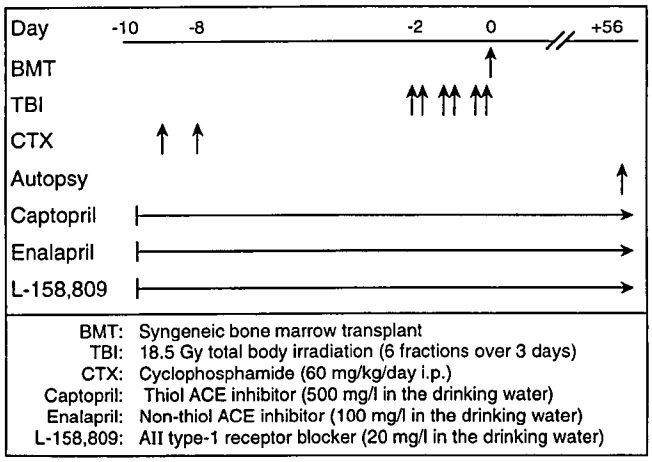

Figure 1. Graphic depiction of the experimental design.

Radiation nephropathy in the rat is characterized by proteinuria (as the urine protein to creatinine ratio) as early as 4–5 weeks after irradiation, azotemia (as BUN) by 8 weeks, and hypertension (as systolic blood pressure) by 9–10 weeks (19, 20). The injury is progressive and leads to renal failure (uremia) as early as 22 weeks (19, 20). In this mode, BUN correlates well with serum creatinin and creatinine clearance (Kendal tau $\geq$0.70), and the error of measurement of BUN is less than that for serum creatinine or creatinine clearance (19, 21). Azotemia (rather than proteinuria, hypertension, or creatinine clearance) is the best indicator that uremia will develop, and a BUN of 100 mg/dl (18–22 mg/dl is normal) gives a 100% incidence of renal failure within 15 weeks (22). Histopathological evidence of injury can be detected as soon as the BUN doubles, and as soon as a quantitative relationship between azotemia and the severity of histopathological damage has been demonstrated (6, 23).

Histological Studies. All rats were sacrificed 8 weeks after irradiation. At the time of death, kidneys from each rat were bisected and immediately placed in 10% buffered formalin. Specimens from each animal were paraffin embedded and 4-μm-thick sections were stained by hematoxylin eosin, by Masson Trichrome for collagen, and by Verhoff method for elastin. The degree of injury visible

under light microscopy was scored by two researchers who were not aware of the slide identification, according to a technique previously described (6). As in the previous publications, we assessed the renal damage evaluating the changes observed in the glomeruli (from the thickening of the basal membrane to the hyalinization of the entire glomerulus), the tubuli (from cloudy swelling to the presence of hyaline casts), the interstitial tissue (degree and severity of inflammation and scarring), and the presence or the absence of vasculitis. The percentage of occlusion of the lumen of small arteries (diameter ranging from 20–200 μm) as well as the thickness of the arterial wall measured in photographs taken from the slides were evaluated by two independent investigators. The mean arterial thickness of the arteries was determined by a computerized image analysis as previously described by one of us (24). A subjective rating of each slide ranging from ± to ++++ (minimal to severe and extensive damage) was assigned to each component of the organ, and was then quantified with a value of 5 given to every ± and a score of 10 for each +, or of any of its multiple. In the previous publications, all the components of the histopathological scoring system were strongly correlated, so that the composite gives as much information as the details. Therefore, a general overview of the renal damage was considered adequate.

Statistics. Data are shown as means ± 1 SD. Group data are compared by the Mann-Whitney test for two-group comparisons and the Kruskal Wallis test for *n* group comparison.

Results

General Conditions. The rats tolerated the treatment well, and all of them survived until the end of the experiment. A difference in body weight gains was evident among the five groups (Table I). All the irradiated rats gained less weight than their nonirradiated controls ($P < 0.01$). Among the irradiated animals, the smallest gain in weight was recorded for the group not receiving any additional treatment. The groups treated either with ACE inhibitors or the A II receptor blocker registered weight gains intermediate between those of the TBI/CTX animals and those of the nonirradiated controls. The gains in weight were similar for the three groups. No statistically significant changes were observed for the weights of adrenals.

Hematopoietic Studies. Irradiated rats had lower white blood cell counts than the nonirradiated controls, with the lowest values observed in the group of rats treated with Enalapril. This decrease was mostly related to the number of neutrophils and it was counterbalanced by a modest increase of lymphocytes, thus changing the ratio Ne/Ly. Red cell numbers and their related parameters (HgB, HCT, MCV, MCH, etc.) were not affected either by the radiation or by the drug's treatment. No statistically significant changes were likewise observed for the platelets. Data from these parameters are summarized in Table I.

General Chemistry. No statistically significant differences were observed for 13 of the 15 serum analytes when the values of the controls were compared with those of their irradiated counterparts. Treatment with Captopril, Enalapril, and L 158,809 did not affect any of those 13 analyses, which include evaluation of liver function, electrolytes, calcium, iron, and creatinine. On the other hand, significant changes were observed for the levels of BUN and uric acid (Fig. 2). Rats given TBI/CTX alone had marked increases in the concentration of BUN and of uric acid; both analytes reached values approximately 3-fold higher than those of the untreated controls. The difference, however, was statistically significant for BUN. These increases were practically corrected when Captopril, Enalapril, or L 158,809 was given to the rats in addition to the TBI/CTX treatment. Again, the difference was statistically significant only for BUN.

PRA. Rats given TBI/CTX alone had greatly reduced values of PRA compared with the controls (Fig. 3). Such a reduction was totally prevented by the addition of the two ACE inhibitors, and the animals of these two groups had PRA values higher than those of the controls ($P = 0.004$). When the A II receptor blocker was administered, PRA was

Table I. Body Weight, Adrenal Weight, Leukocytes, Neutrophil Granulocytes, Lymphocytes, and Erythrocytes of Controls, TBI/CTX-Treated Irradiated Rats, and TBI/CTX-Treated Irradiated Rats Receiving Captopril, Enalapril, or the L 158, 809 A II Blocker

	Control	BMT/TBI cytoxan	BMT/TBI cytoxan + L 158,809	BMT/TBI cytoxan + captopril	BMT/TBI cytoxan + enalopril
Number of rats	6	8	8	7	7
Body weight (g)	295 ± 13.0	175.0 ± 5.0[a]	198.0 ± 5.0[a]	187.0 ± 5.0[a]	192.0 ± 4.0[a]
Adrenal weight (mg/100 g body weight)	24.5 ± 1.5	23.1 ± 1.3	20.3 ± 1.2	22.1 ± 3.5	21.7 ± 1.2
Leukocytes	4.8 ± 0.6	4.1 ± 0.7	3.0 ± 0.3	3.1 ± 0.5	2.3 ± 0.2[b]
Neutrophils	21.7 ± 4.1	24.0 ± 7.8	22.4 ± 3.5	19.1 ± 1.6	17.6 ± 2.3
Lymphocytes	77.8 ± 4.0	74.4 ± 7.7	76.0 ± 3.6	79.4 ± 1.7	81.4 ± 2.5
Erythrocytes	8.13 ± 0.11	6.47 ± 0.19	6.99 ± 0.10	7.29 ± 0.19	7.00 ± 0.30

[a] $P < 0.01$ vs controls.
[b] $P < 0.05$ vs controls.
Values given are ±1 SE.

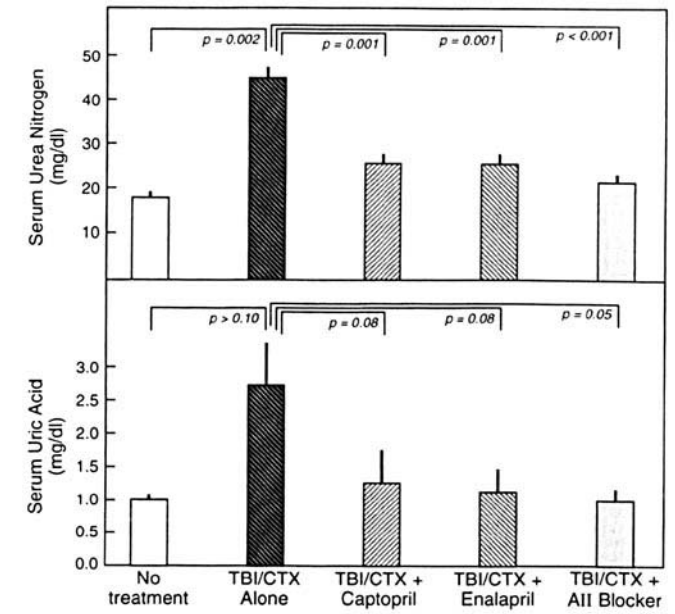

Figure 2. Serum urea nitrogen and serum uric acid concentration in the five experimental groups of rats.

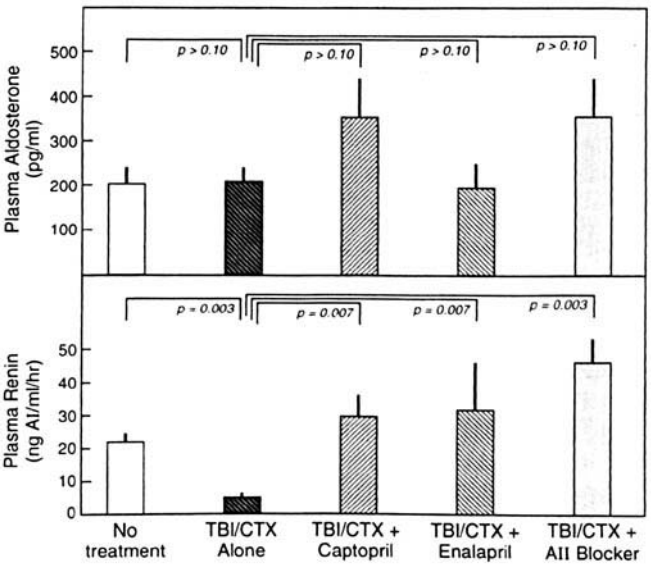

Figure 3. Plasma aldosterone and plasma renin activity levels in the five experimental groups of rats.

even more elevated when compared with that of TBI/CTX-treated rats ($P = 0.003$), but the difference was not statistically significant when compared with the values of animals treated with the ACE inhibitors.

Plasma Aldosterone. No significant difference ($P < 0.10$) in plasma aldosterone concentrations were observed among the five groups of rats (Fig. 3).

Histological Studies. Using light microscopy observation, we could ascertain that the irradiated animals receiving only TBI/CTX had severe renal damage involving the glomeruli, the tubuli, the interstitial tissue, and the blood vessels. (Figs. 4A and 5A). Glomeruli showed endothelial damage and subendothelial expansion with occlusion of many capillaries. There was diffuse thickening of the glomerular basal membrane, segmented focal or total glomerular hyalinization, and significant incidence of capsular adhesions and of crescents formation. Tubular injury included diffuse cloudy swelling, diffuse balloon-like cellular degeneration, necrosis, and widespread presence of casts that was extended to the medullary portion. The interstitial spaces showed patchy areas of lymphocytic infiltration, and diffuse and, in many areas, severe fibrotic expansion. The interlobular arteries showed intimal proliferation and luminal reduction that was, in some vessels, so severe as to occlude the lumen. The media was thickened with the presence of myofibroblasts hyperproliferation. The adventitia frequently showed severe periadventitial edema with infiltration of chronic inflammatory cells and of collagen fibers. Many afferent arterioles of the glomeruli were partially or totally occluded, and the juxtaglomerular apparatus was hyperplastic.

The rats treated with ACE inhibitors or A II blockers had remarkably less severe renal damage (Figs. 4, B–D and 5, B–D). Most glomeruli were normal in appearance and only few of them had mild mesangial expansion. Those mildly injured glomeruli were more often seen in rats treated with Captopril and Enalapril (Figs. 4 and 5, C and D) than with the L 158,809 compound (Figs. 4 and 5Bs). Tubuli were mostly normal in appearance. The interstitial tissue did not show presence of abnormal amounts of inflammatory cells. Interlobular or smaller arteries did not show evidence significant vascular changes. In TBI/CTX rats receiving the A II blockers, and to a lesser extent in TBI/CTX rats receiving ACE inhibitors, most of the arterial walls of the interlobular arteries and of the arterioles were thinner than the walls of the same type vessels of the nonirradiated controls. The rats treated with Captopril, Enalapril, and L 158,809 had less collagen expression than their irradiated counterparts and also less collagen expression than the normal nonirradiated controls. In fact, the three drugs, as it can be observed from Figures 4 and 5, were inhibitors of collagen synthesis so that collagen fibers were barely seen in the kidneys of the rats treated with these drugs. The only exception was the pericapsular collagen, which remained well evident in the staining of the kidneys from all rats, regardless of treatment.

No significant differences were observed in elastin renal content and expression.

Quantitative Evaluation of the Histological Renal Damage. Figure 6 summarizes the histological semiquantitative scoring of the renal damage in the five groups of rats. As mentioned before, minimal damage was present in some of the nonirradiated control rats, whereas the damage was very severe in the animals given TBI/CTX alone. The two ACE inhibitors and L 158,809 greatly reduced the severity of this damage.

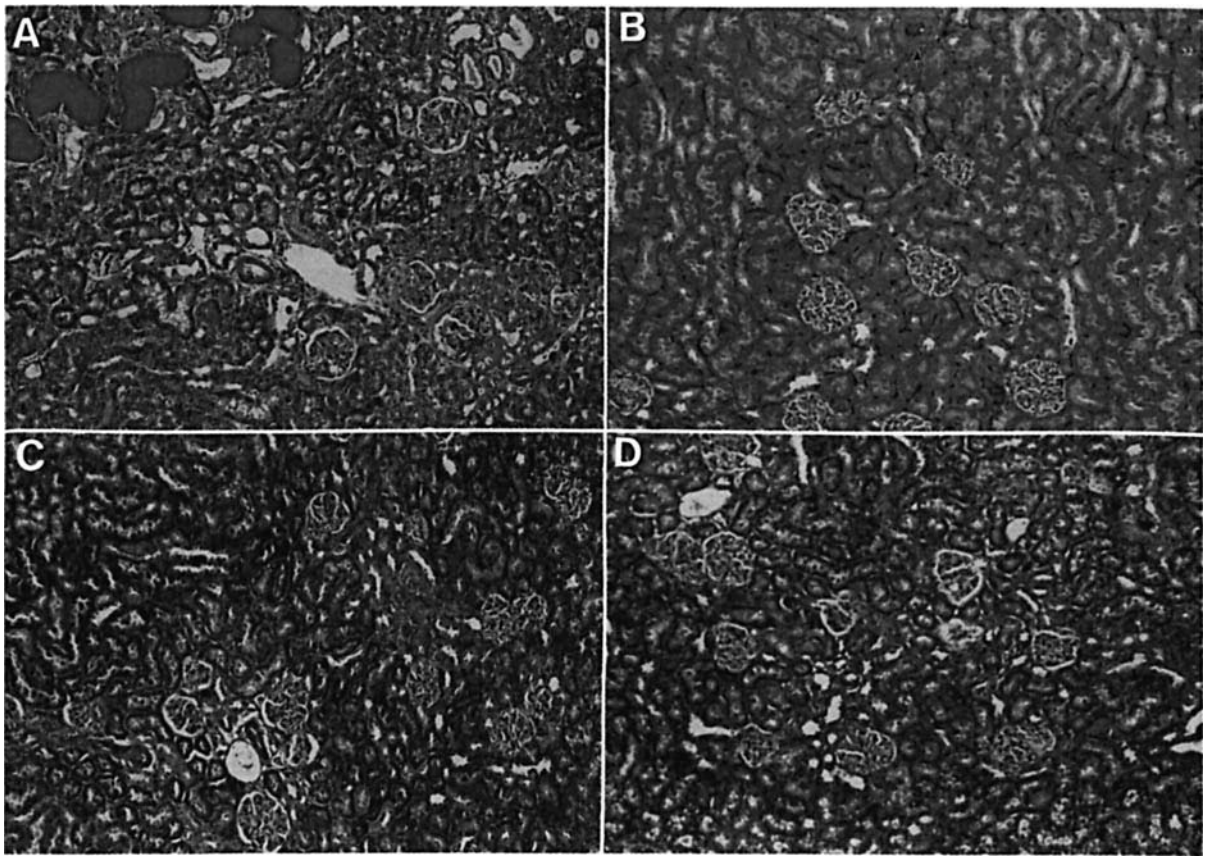

Figure 4. Representative histological sections of the kidney of a rat irradiated (TBI) and treated only with CTX (A), a TBI/CTX-treated rat also receiving L 158,809 (B), a TBI/CTX-treated rat receiving Captopril (C), and a TBI/CTX-treated rat receiving Enalapril (D). The extensive glomerular and tubular damage, the presence of tubular casts, and the interstitial inflammatory process observed in rats receiving only the TBI/CTX treatment (A) were not observed when Captopril, Enalapril and, in particular, L 158,809 were added to the treatment (B, C, and D). The arrow in B shows a moderately thickened small artery. The kidneys of these rats had a substantially normal appearance. Staining: Trichrome, 100×.

Discussion

These studies confirm and extend previous research on radiation nephropathy. The functional protection of Captopril and Enalapril are equivalent to those found in previous studies both by other investigators and by our research groups (5, 6, 25, 26) despite the fact that the radiation treatment was much more aggressive than in the previous studies by our group. They show that no single cell type is spared from radiation injury and that both ACE inhibitors exert their protective action on the entirety of the renal structure, including glomeruli, vasculature, tubular epithelia, and interstitium. In addition, the study shows similar protection was obtained when an A II receptor blocker was used, and this drug also exerted its protective effect on the entire kidney structure (27). The effect of the latter drug was so efficient that the glomeruli of the irradiated rats treated with L 158,809 were, at the histological examination, very similar to ones of the normal controls: the walls of their vessels were thinner, and the expression of collagen was even less evident than that seen in the untreated control animals. It is of interest to note that the interstitial collagen of the renal parenchyma was reduced by the three drugs, but no effect was seen on the capsular collagen.

The protective effect of Captopril, Enalapril, and L 158,809 is not limited to the kidney. We also observed severe postirradiation damage to the lungs mainly characterized by chronic pneumopathy and fibrosis (28). The three drugs affected the organ vasculature, the inflammatory process, the septal thickening, and the collagen deposition in the vascular adventitia and in the septa, and the best results were observed by administration of L 158,809 (28). It is worth mentioning that all the three drugs were again effective against collagen expression in the lung parenchyma, but they did not affect the collagen of the pleura or of the large bronchi, thus showing a "selective" control on different types of collagen. Radiation-induced inflammation and fibrosis of other organs (heart) is also prevented by Captopril treatment (8), whereas Losartan, a selective type 1 A II receptor antagonist, and SAR-T-AT-II, a type 2 receptor antagonist, are effective in preventing collagen overexpression in lungs of rats receiving bleomycin (29).

All three drugs were well tolerated. Even if the animal's growth was slower than that of nonirradiated controls,

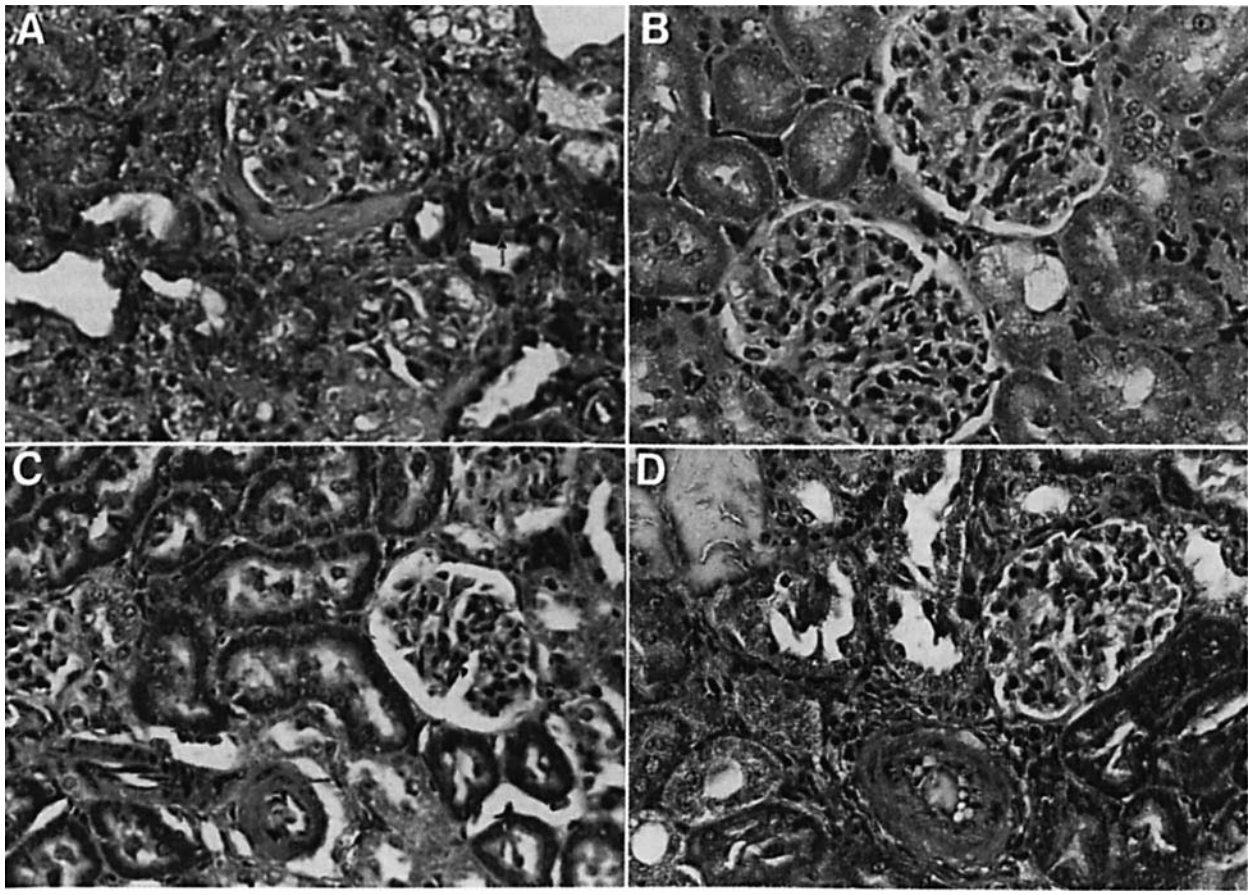

Figure 5. Higher magnifications and "close-ups" of the kidneys in Figure 4: Note that in section A, the extensive damage of the glomerulus and the thickened wall of an arteriole Arrows identify arterioles with very thick walls and reduced lumen. Some periadventitial edema is also present. By contrast, the sections of the kidneys of rats receiving the two ACE inhibitors and especially the rat receiving L 158,809 appear to be normal (B, C, and D). Staining: Trichrome, 400×.

the final weight of the irradiated rats receiving any of the three modifiers was markedly greater than that of the counterparts receiving TBI/CTX alone. None of the three modifiers significantly affected the rat's hematopoiesis, their liver function, their electrolyte balance, or their protein content. Drug treatment had a beneficial effect on the renal function and on the uric acid concentrations, and it returned these analytes to values similar to those of the control animals, despite the heavy radiation dose.

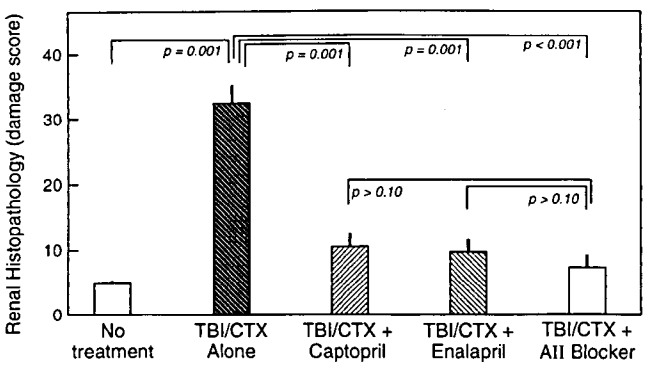

Figure 6. Summary of the semiquantitative evaluation of the histological renal damage in the five experimental groups of rats.

All three drugs had an influence on the animals renin-angiotensin-aldosterone (RAS) system. A marked decline in PRA was observed in rats receiving TBI/CTX alone, likely to be related to increased synthesis of A II. However, the treatment with the two ACE inhibitors and L 158,809 restored PRA activity to values above those of the controls. The changes of the PRA levels are probably a consequence of the interrupted feed back mechanisms of the RAS system related to a diminished presence of A II at the cellular level when the drugs were used. Notwithstanding such an increase in PRA, the juxtaglomerular apparatus of the treated rats did not appear hyperplastic, nor did the afferent arteries have reduced lumen or thicker walls. The changes in PRA and the possible changes in A II levels were not reflected in significant variation of plasma aldosterone concentration nor in significant variations of the adrenal weight, thus confirming earlier studies where Captopril was administered for several weeks to rats (30). These experimental findings are also confirmed by clinical studies related to the measurement of serum aldosterone after prolonged treatment with ACE inhibitors in hypertensive patients. Although PRA activity is very elevated as a consequence of the pharmacological intervention of this drug and the interruption of the

feedback control mechanisms of the renin-angiotensin-aldosterone system, the aldosterone concentration is not affected (31).

The role of angiotensin II in the pathogenesis of radiation-induced fibrosis is still unclear. High concentrations of angiotensin II have been observed in another model of experimentally induced vascular hypertension and fibrosis: exposure to chronic hypoxia (32–34). Extremely low levels of lung ACE and plasma A II were observed in llamas (Llama Huanaco and Llama Glama), a species of animals acclimatized at high altitude (consequently enduring chronic hypoxia) who do not develop pulmonary hypertension and fibrosis (35). Moreover, ACE inhibitors are beneficial in the prevention of pulmonary fibrosis experimentally induced by the administration of monocrotaline or exposure to radiation (24, 36–38).

However, experiments by Ward and colleagues (7, 24, 36, 37, 39) have also shown that both in radiation-induced and monocrotaline-induced pneumopathies, there was a dissociation of pharmacological activity when ACE inhibitors containing or not containing a sulphydril group were used. All ACE inhibitors were effective in the arrest of the vascular inflammatory process, but those with the thiol group (Captopril and CL 24817) were more efficient in the regulation of the inflammatory process and the pulmonary fibrosis than ones (Enalapril, Cilazapril, CGS 13945, or CGS 15385) without the thiol group, even if this second series of drug was *in vitro* more potent in its ACE inhibitory activity. An antifibrotic effect was also observed in some of the experimental models when other drugs that were poor ACE inhibitors but contained the same thiol group (Penicillamine) were used (2, 24). *In vitro* studies where ACE inhibitors were used for modulation of the growth of human fibroblasts, neovascular formation, or neoplastic cell growth confirmed our *in vivo* observations (10, 11, 13). Captopril has, in addition to the inhibition of ACE, other pharmacological activities as previously mentioned, and we suggested in previous publications that some of the protective effects of the drug were more related to those additional pharmacological properties that to its effect on A II synthesis (7). On the other hand, our experience with the A II receptor blocker, L 158,809, indicates that this compound is more effective than ACE inhibitors in preventing in the lungs (28) and it is at least equal in its effect in protecting the kidneys from the development of radiation-induced fibrosis. Moreover, in both experimental models, the protective effect from irradiation-induced damage exerted by the ACE inhibitor drugs was very similar whether these drugs contained a thiol radical or not. Even if other factors beside the presence of angiotensin II may be influential in the pathogenesis of radiation-induced fibrosis, it is clear that the role of angiotensin II must be very relevant and its blockage very important. The mitogenic role of angiotensin II on fibroblast growth has also been recently shown in the studies by Tallant *et al.* (40) when the trophic action of angiotensin II was inhibited by addition of angiotensin 1–7, a separate product of the renin-angiotensin-aldosterone system that inhibits angiotensin II synthesis.

Whatever the role of angiotensin II is in the pathogenesis of the radiation-induced fibrotic process, its suppression by ACE inhibitors and by L 158,809 proves highly beneficial during radiation therapy. Therefore, the use of the ACE inhibitors and now of the more recently available variety of angiotensin II type I and type 2 receptor blockers opens new possibilities for radiotherapeutic treatment, allowing the use of higher radiation doses and/or the control of the radiation-related side effects. The relative lack of toxicity associated with the use of these drugs is also an important contributing factor to their deployment.

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