# Mediators and Mechanisms of Radiation Nephropathy (44483)

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*Abstract.* **Normal tissue radiation injury occurs after sufficient irradiation, thus limiting the curative potential of x-ray therapy. In the kidney, radiation injury results in fibrosis and, ultimately, renal failure. The mediators of fibrosis in radiation nephropathy have received scant attention. Therefore, we evaluated the sequential presence of alpha smooth muscle actin (αsma), fibrin, collagen, and TGFβ$_1$ in a porcine model of radiation nephropathy using 9.8 Gy single-dose local kidney irradiation. During the 24-week study, there was progressive and significant collagen accumulation in glomeruli and in interstitium. In glomeruli, this was associated with significant mesangial αsma expression by 2 weeks after irradiation, a further rise at 4 weeks, and then a gradual fall to baseline. Glomerular fibrin deposition was significant by 4 weeks after irradiation, and remained elevated thereafter. There was little or no glomerular TGFβ$_1$ expression at any time point. Tubular fibrin deposition was significant at 4 weeks after irradiation but declined thereafter. There was little or no tubulo-interstitial αsma expression at any time after irradiation. At 6 weeks after irradiation, there was a significant peak of tubular epithelial TGFβ$_1$ expression that declined thereafter. The early glomerular injury is evident as mesangial αsma expression but is not proceeded by TGFβ$_1$ expression. There is sustained glomerular fibrin deposition with deposition of fibrin in tubular lumens, suggesting that tubular fibrin derives and flows out from injured glomerular tufts. We conclude that i) αsma expression is an early marker of glomerular radiation injury, presaging scarring; ii) fibrin deposition is involved in glomerular and tubular radiation injury; and iii) TGFβ$_1$ is not an early event in radiation nephropathy, and not apparent in glomeruli in this model, but may correlate with later tubulo-interstitial fibrosis. Thus, the mediators of scarring in this model differ according to time after injury and also according to the affected tissue compartment.**

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Sufficient irradiation of the kidney causes progressive injury that results in fibrosis and organ failure. This injury is an example of normal tissue radiation injury, which is a dose-limiting problem for all therapeutic irradiation. The threshold x-ray dose and fractionation have been amply described, with radiation nephropathy occurring reliably after single doses of 10 Gray (Gy) or more to the kidneys (1, 2). Radiation nephropathy has been well characterized by light and electron microscopy, which show the features of endothelial injury, mesangiolysis, and glomerular basement membrane expansion (3). Classical radiobiology teaches that normal tissue injury is the unavoidable result of radiation–induced mitotic cell death (4). However, recent interventional studies suggest that radiation nephropathy may be prevented or treated successfully (5). This suggests that the initial radiation effect does not proceed inexorably toward cell death, fibrosis, and organ failure, but that there are intervening steps between irradiation and organ failure. Successful therapies can interrupt these intermediate steps.

Fibrosis itself may be more than just a marker of organ injury, and may hasten the loss of function in kidney disease (6, 7). The mediators of fibrosis that might be involved include transforming growth factor beta, Isoform 1 (TGFβ$_1$)

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and hemostatic factors. Cellular mechanisms of fibrosis involve activation of fibroblasts into myofibroblasts, which are activated fibroblasts that contract and synthesize extracellular matrix proteins including collagen (8, 9). Myofibroblasts contain the α isoform of actin, which is normally found in contractile vascular smooth muscle cells. In the kidney, glomerular mesangial cells acquire α smooth muscle actin staining (αsma) when there is inflammatory injury or proliferation (10). Glomerular and interstitial αsma immunostaining have been associated with progressive glomerular and interstitial fibrosis in nephrotoxic serum nephritis (11).

In a rat model of radiation nephropathy, we showed substantial interstitial myofibroblast activation and its reduction with captopril (12). The captopril treatment also prevented renal collagen deposition and scarring. We interpreted those studies as suggesting that myofibroblast activation was a precursor to fibrosis, and that activation was prevented by captopril. Those studies did not address what mediators might have caused the myofibroblast expression.

The present studies used a porcine model of radiation nephropathy to identify the chronology and intrarenal location of mechanisms that could initiate the scarring process.

## Materials and Methods

Radiation nephropathy was established in mature, 40–43-week-old female Large White pigs using cobalt 60 γ-irradiation, 9.8 Gy single-dose, to both kidneys as described previously (13). In this model, there is glomerular and interstitial scarring and reduction of the renal function to 30%–40% of normal (14). Sequential studies in this model have shown worsening of glomerular injury over time, with development of sclerotic glomeruli. Tubulo-interstitial injury also occurs but is more variable than the glomerular. This model of bilateral renal irradiation, as opposed to single kidney irradiation, has the advantage of closer analogy to human diseases resulting in renal injury and scarring. These studies were performed under the rules and guidelines for animal care and experimentation, as mandated by the Home Office of the United Kingdom. Sixteen animals were used, of which three were unirradiated controls. Two animals were sacrificed at 2, 4, 12, 20, and 24 weeks after irradiation. Three were sacrificed at 6 weeks after irradiation. One kidney from each animal underwent perfusion fixation with a mixture of 1% acetic acid in 10% formal saline. Table I shows the animal and slide numbers for each time point and for each part of the study. For the αsma and $TGF\beta_1$ studies, slides were freshly made and stained. For the fibrin and collagen studies, archived, previously stained slides were used. For the fibrin and collagen studies, tissue was paraffin-embedded, cut to 5-μm sections, and stained with Martius Scarlet Blue (for fibrin) or Mallory trichrome (for collagen).

**Immunohistochemistry.** For the αsma studies, 5-μm thick sections were stained by immunohistochemistry. Following deparaffination, and rehydration in graded ethanols, endogenous peroxidase was blocked with 0.3% $H_2O_2$ in methanol. Sections were then exposed to monoclonal antibody to αsma (Sigma, Poole, UK) diluted in phosphate buffered saline (PBS)/2% bovine serum albumin (BSA) (Sigma) at 1:250 dilution for 45 min at room temperature, then washed with PBS-Tween and PBS. Sections were then incubated with biotinylated secondary antibody (Vector, Peterborough, UK) for 45 min, then washed with PBS-Tween and PBS. This was followed by a 45-min exposure to an avidin-biotin conjugate, a PBS wash, and exposure to 3-amino-9-ethylcarbazole for 5–7 min, a water wash, a PBS wash, and a light hematoxylin counterstain. Negative control sections were incubated with PBS in 2% BSA instead of primary antibody but were otherwise treated identically. Coverslips were attached with aquapolymount (Polysciences, Warrington, PA) and viewed by light microscopy.

For the $TGF\beta_1$ studies, a similar protocol was followed, with the following exceptions. First, just before the endogenous peroxidase block, pronase 0.1% (Sigma) was applied to the sections for 8 min, then washed off with water. Second, the primary antibody was to $TGF\beta_1$ (ProMega, Madison, WI) and was layered on at 1:80 dilution, and left overnight at 4°C in a humidified chamber. This antibody detected active $TGF\beta_1$. Third, the biotinylated secondary antibody was anti-rabbit instead of anti-mouse because the $TGF\beta_1$ antibody was raised in rabbits.

**Quantitative Histology.** For each group of slides, five adjacent eyepiece grid areas were studied, starting just below the renal capsule, and proceeding toward the medulla. A 100-point eyepiece grid was used for point counting (Zeiss, Welwyn Garden City, UK).

For αsma expression, glomeruli were scored individually, and by quadrant as being negative (0) or fractionally positive (0.25, 0.5, 0.75 or 1.0). Scores were summed and averaged for each time point. The tubulo-interstitium was scored by counting the fraction of the tubules that had peri-

**Table I.** Animals Used and Slides Evaluated, According to Time Point After Irradiation

| Study | Weeks of irradiation | 0 | 2 | 4 | 6 | 12 | 20 | 24 |
|---|---|---|---|---|---|---|---|---|
| αsma | Animal no./slide no. | 2/2 | 2/3 | 2/4 | 3/2 | 2/2 | 2/2 | 2/2 |
| Fibrin | | 1/2 | | 1/3 | | 2/8 | 2/12 | |
| TGF $\beta_1$ | | 1/1 | 2/3 | 2/3 | 3/5 | 2/4 | 2/4 | |
| Collagen | | 1/3 | | 1/3 | | 2/9 | 2/12 | |

tubular αsma positivity. Scores were calculated for each field, summed, and averaged for each time point. Glomerular scoring was done at 100x, and the tubular at 400x magnification.

For fibrin deposition, glomeruli were scored as positive when any focus of orange-staining fibrin was found, and were scored as negative otherwise. The fraction of fibrin-positive glomeruli was calculated as an average for each time point. Tubules were scored as positive when any focus of fibrin was found within the lumen or adjacent to the tubular basement membrane and were scored as negative otherwise. The fraction of tubules that were positive per total number in a grid area was calculated as an average for each time point. Glomerular scoring was done at 100x magnification, and the tubular at 400x magnification.

For glomerular $TGF\beta_1$ expression, 100 glomeruli were studied per slide. A glomerulus was scored positive if two or more cells showed distinct granular reactivity. The fraction of positive glomeruli was summed and averaged for each time point. For tubular $TGF\beta_1$ expression, tubules were scored positive if there was distinct tubular epithelial staining. The fraction of positive tubule sections per grid area was summed and averaged for each time point. Glomerular scoring was done at 200x and the tubular at 200x magnification.

Collagen deposition was assessed as blue-staining areas on the 100-point eyepiece grid. The fraction of points positive per hundred was calculated per grid area, and the scores were summed and averaged for each time point. This quantitation was done at 200x magnification.

**Statistical Analysis.** The data are shown as a mean + SD. Comparisons between time points were made by first testing for group differences by the Kruskal-Wallis test, then by the Mann-Whitney test between the data of two time points. Correction for multiple comparisons was made according to the recent guidelines (15). Statistical significance was achieved at corrected $P \leq 0.05$.

## Results

There was no glomerular αsma staining in unirradiated animals. Both irradiated and unirradiated animals had the expected αsma staining of arterial and arteriolar vessel walls. This provided a consistent internal positive control for every slide. In irradiated animals, glomerular αsma staining was distinct, and was in a mesangial pattern (Fig. 1). It reached a peak at 4 weeks after irradiation, which was significantly different from baseline values or 12-, 20-, and 24-week values (Fig. 2). Overall, the 2- and 4-week peaks in αsma staining were significantly elevated above baseline, and the 20- and 24-week points declined to become equal to baseline (Table II).

There was no interstitial αsma staining in unirradiated animals. Interstitial αsma staining of irradiated animals' kidneys was visually unimpressive and quantitatively 10–100-fold less intense than the glomerular. The fractional interstitial scores are shown by time point in Table III; there were no statistical differences between any time points.

There was no tubular or glomerular fibrin in unirradiated animals. Tubular fibrin deposition was significantly present starting at 4 weeks after irradiation (Fig. 3). It declined thereafter although remained above baseline even at the 20-week time point. Qualitatively, intraluminal fibrin appeared to predominate in proximal as opposed to distal tubules. When compared to baseline, unirradiated animals, there was enhanced glomerular fibrin deposition at 4, 12, and 20 weeks (Fig. 4) but because of the six multiple comparisons, this only achieved statistical significance for the baseline versus 20-week time point comparison.

There was no $TGF\beta_1$ expression in unirradiated animals' kidneys. In irradiated kidneys, $TGF\beta_1$ expression was not easily identified in glomeruli, where it appeared to be confined to endothelial and mesangial cells. There was a trend for more $TGF\beta_1$-positive glomeruli with time, when compared to baseline, but the intensity of staining was 10-

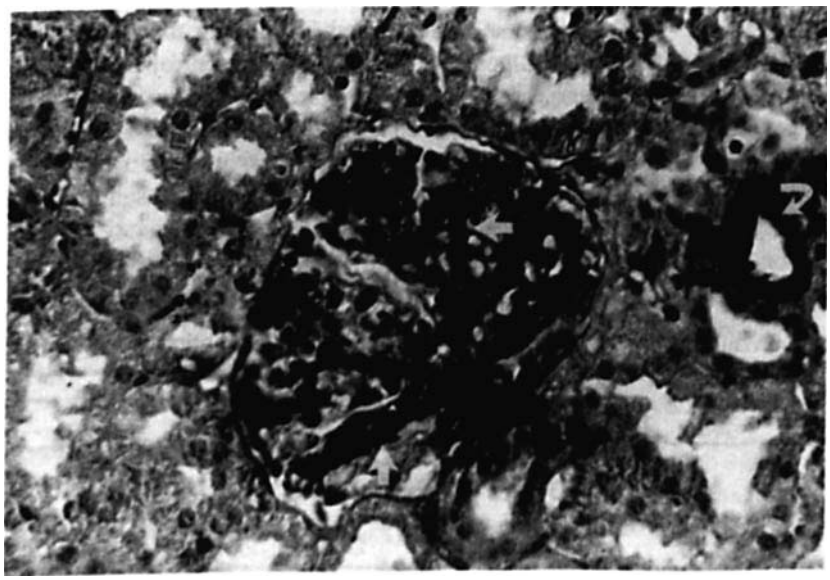

**Figure 1.** Glomerular alpha smooth muscle actin (αsma) staining in 9.8 Gy irradiated pig kidney, at 4 weeks after irradiation. There is distinct positivity in a mesangial pattern (white arrows). There is an arteriolar wall positivity (curved white arrow).

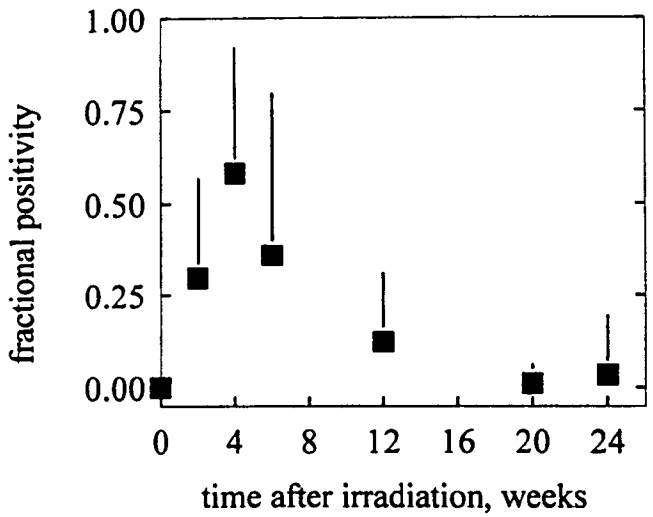


**Figure 2.** Evolution of glomerular αsma staining versus time after irradiation. The fraction of glomerular positivity is shown as a mean + SD. The 2- and 4-week time points are significantly elevated above baseline.

fold less than that of the tubular epithelial cells. In the tubules, the positive cells of irradiated animals contained numerous large granules of reaction, whereas positive cells within the glomerulus contained one or only a few granules (Fig. 5). Statistical analysis was therefore not done for $TGF\beta_1$ glomerular staining. By 2 weeks after irradiation, there was some tubular positivity, but this did not reach a peak until 6 weeks (Fig. 6). The 2- and 4-week time points were not statistically different from baseline, when correcting for multiple comparisons. By 20 weeks, $TGF\beta_1$ expression had fallen to levels significantly below those of 6 weeks, but was still higher than the baseline and 2- or 4-week time points.

Collagen deposition had a distinct and significant upward trend with time (Fig. 7). This was significant for all time points compared to baseline but not between 4-, 12-, or 20-week time points.

## Discussion

These studies clearly indicate that radiation nephropathy does not depend on a single mediator for its evolution to collagen deposition and scarring. The earliest marker of future fibrosis occurred by 2 weeks, shown by distinct mesangial αsma expression. Fibrin deposition was evident by 4 weeks, especially in tubules. $TGF\beta_1$ expression was not significantly increased until 6 weeks after irradiation.

The earliest evidence of injury in these studies was the glomerular αsma expression, which peaked at 4 weeks. It is possible that even earlier evidence might have been found had tissue been obtained from time points before 2 weeks. However, previous ultrastructural studies using this model have not shown evidence of injury until 3 weeks after irradiation (3). The pattern of staining for αsma was a centerspoke, which is strongly suggestive of mesangial staining (Fig. 1). Generally, αsma expression correlates with mesangial injury or proliferation (10). The role of $TGF\beta_1$ in causing mesangial αsma expression is possible but chronologically inconsistent with the present study. It is possible that an initial peak of $TGF\beta_1$ expression occurred before the 2-week time point. For instance, very early expression of $TGF\beta_1$ has been found in irradiated mouse mammary glands (16). Yet the pattern of $TGF\beta_1$ expression that we found, which was slightly but not significantly elevated at 2 and 4 weeks, does not suggest another peak, at a time before the 2-week time point. Nonetheless, it remains possible that specimens from an earlier time point, such as 1 week, could have shown enhanced presence of tissue $TGF\beta_1$.

These studies do not permit conclusions as to the cause of the glomerular mesangial activation "peak" (Fig. 2). It is worth noting that this peak is very similar to one found in the same radiation nephropathy model using bromodeoxyuridine (BrDu) as a proliferation marker (17). It is possible then that the earliest effect of renal irradiation is to set in motion a proliferative response in the glomerulus.

Tubular and glomerular fibrin staining could not be assessed at the 2-week time point because of lack of suitably stained tissue specimens. But at 4 weeks, there was a trend toward increased glomerular fibrin positivity and a significant tubular fraction positivity compared to baseline. The notion of radiation-induced fibrin deposition is not new. It has been documented in irradiated heart and kidney (18, 19). The studies of Oikawa *et al.* (20) showed that plasminogen activator inhibitor 1 (Pai-1) mRNA is enhanced over control at 12 weeks after local kidney irradiation. If increased Pai-1 expression is involved, its cause remains unclear. Angiotensin II ($A_{II}$) could be involved (21), but there is no evidence of systemic angiotensin excess in radiation nephropathy (Cohen *et al.*, unpublished data). It is possible that renal irradiation could cause local, intrarenal $A_{II}$ excess

**Table II.** *P* Value Table for Glomerular αsma Staining

| Weeks | 2 | 4 | 6 | 12 | 20 | 24 |
|---|---|---|---|---|---|---|
| 0 | 0.0002[a] | 0.0001[a] | 0.0045 | 0.0196 | 0.4700 | 0.4600 |
| 2 | | 0.0032 | 0.8124 | 0.0194 | 0.0001 | 0.0001[a] |
| 4 | | | 0.0196 | 0.0001[a] | 0.0001[a] | 0.0001[a] |
| 6 | | | | 0.1039 | 0.0004[a] | 0.0009[a] |
| 12 | | | | | 0.0072 | 0.0149 |
| 20 | | | | | | 0.9493 |

*Note.* The values shown are uncorrected. Values with superscript are significant when taking multiple comparison into account, for $p = 0.05$.

**Table III.** Fractional αsma Interstitial Positivity, by Week

| Week | Mean | SD |
|---|---|---|
| 0 | 0 | 0 |
| 2 | 0.005 | 0.007 |
| 4 | 0.005 | 0.012 |
| 6 | 0.003 | 0.005 |
| 12 | 0.006 | 0.007 |
| 20 | 0.002 | 0.004 |
| 24 | 0.004 | 0.007 |

or enhanced response to normal amounts of $A_{II}$. Whatever the means of its appearance, excess glomerular Pai-1 could blunt fibrinolysis and thereby lead to fibrin accumulation (22). Since Pai-1 is of endothelial origin, this mechanism would be relevant for the glomerular fibrin accumulation.

Fibrin and its degradation products have long been associated with glomerular injury (23). Glomerular injury is a constant feature of radiation nephropathy, and there is abnormal glomerular permeability (14). The significant tubular fibrin that we found probably is derived from the injured glomerular tufts, *via* "downstream" egress. The fibrin that we saw in the tubules was sometimes right up against a denuded tubular basement membrane. This would be facilitated by the tubular epithelial desquamation that has been documented in this model between 4 and 12 weeks after irradiation (24). The fibrin and its smaller catabolite fibrinopeptides (M.W. ≈ 1500 Da) could then contribute to tubulo-interstitial fibrosis by crossing the tubular basement membrane of the denuded segments at those times. This could then lead to a stimulation of interstitial fibroblasts (25).

Nonetheless, and in contrast to work done in a rat radiation nephropathy model (12), interstitial αsma expression was minimal in these specimens. Adequacy of staining was ensured by the consistent internal positive control of well-stained arteries and arterioles. Thus, the tubulo-interstitial fibrosis that occurs in this model may not occur *via* αsma-positive fibroblasts.

As noted, the $TGF\beta_1$ expression found in these studies was predominantly in tubular epithelial cytoplasm. At 6, 12, and 20 weeks after irradiation, it was significantly elevated over all other time points. Moreover, qualitatively, its expression appeared first in tubules that were morphologically normal by light microscopy without adjacent interstitial expansion. Thereafter, there was interstitial expansion along with the appearance of interstitial cells expressing $TGF\beta_1$. This sequence is consistent with a role for $TGF\beta_1$ in the collagen deposition that occurs in the later phases of radiation nephropathy, but probably not as an early mediator.

$TGF\beta_1$ expression has been implicated in normal tissue radiation injury of lung, breast, skin, and gut (16, 26–29). Its known pro-fibrotic capacities provide a logical rationale for its importance in the fibrosis that is a critical side effect of therapeutic irradiation. Yet our present studies suggest that it is not the only mediator involved, and it probably is not the earliest mediator of the scarring process in radiation nephropathy.

Multiple studies have implicated $TGF\beta_1$ in renal scarring. Border *et al.* (29) showed its association with the scarring that occurred in an immune model of glomerulonephritis. A causal role for its importance is further suggested by the work of Oikawa *et al.* (20) in a radiation nephropathy model that showed enhanced expression of $TGF\beta_1$ that was significantly reduced by treatment with an angiotensin II antagonist. Datta *et al.* (30) have also shown that the rise in glomerular $TGF\beta_1$ that occurs after renal irradiation is prevented by angiotensin II antagonist. Similar reductions of $TGF\beta_1$ expression by angiotensin blockade have been found in the remnant kidney model, cyclosporine nephrop-

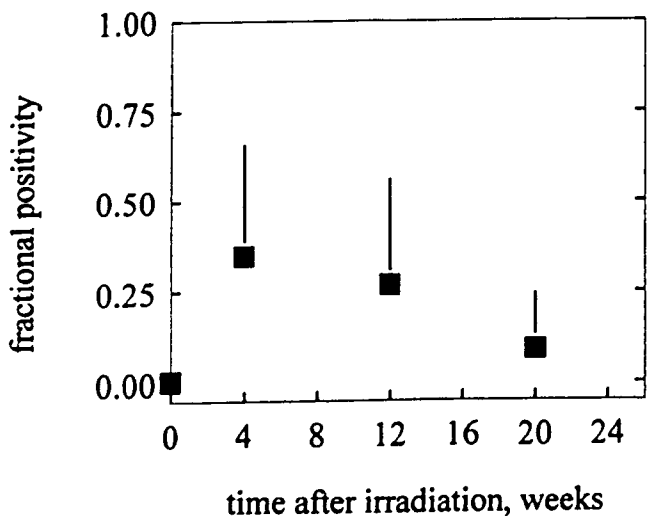


**Figure 3.** Evolution of tubular fibrin staining versus time after irradiation. The fraction of tubules with positive staining is shown as a mean + SD. The 4-week time point is significantly elevated above baseline.

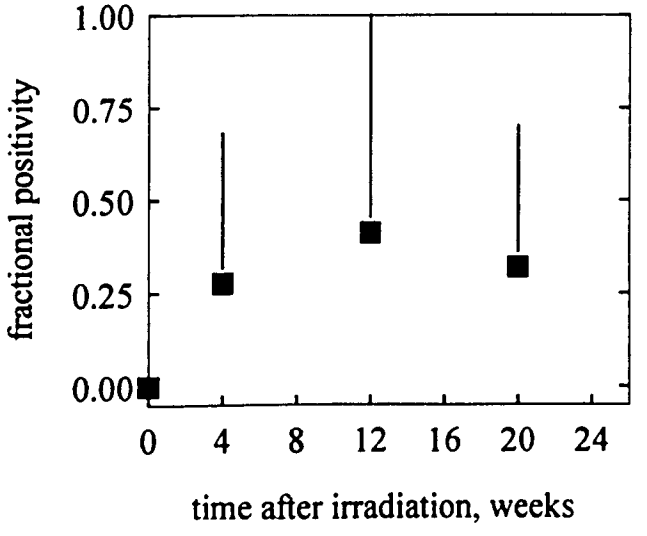


**Figure 4.** Evolution of glomerular fibrin staining versus time after irradiation. The fraction of glomeruli staining for fibrin is shown as a mean + SD. The 20-week time point is significantly elevated above baseline.

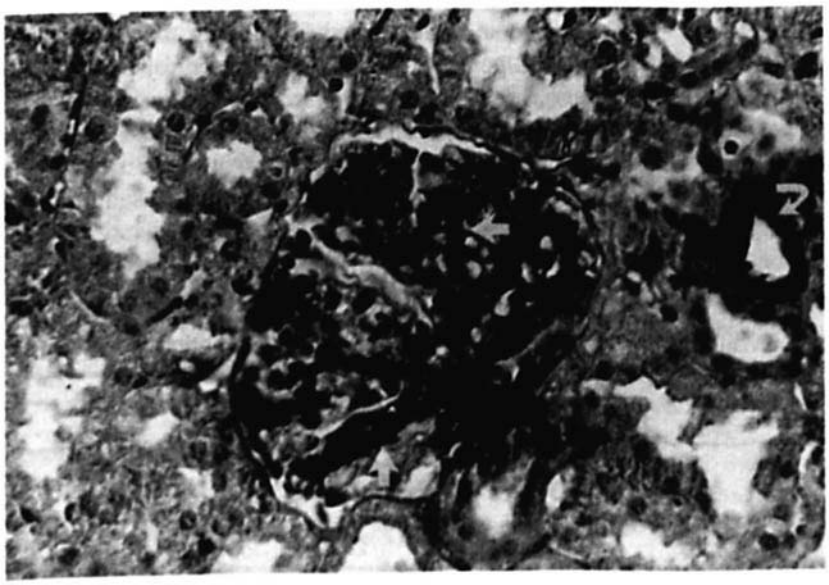

**Figure 5.** Staining for TGFβ$_1$ in irradiated pig kidney, at 6 weeks after irradiation. There is prominent tubular epithelial staining (arrow heads) and little or no glomerular staining.

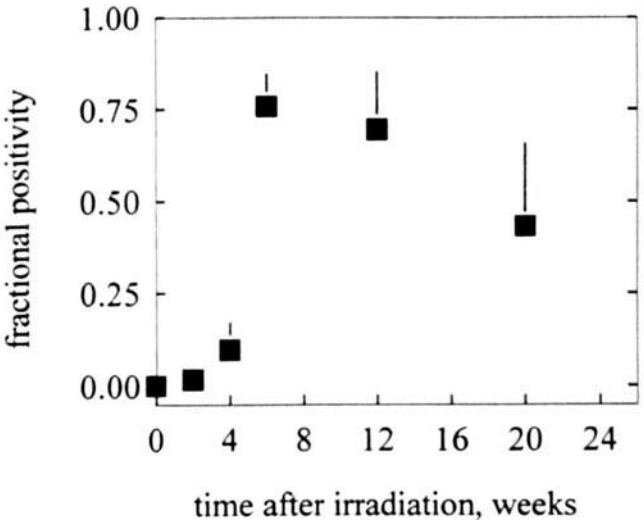


**Figure 6.** Evolution of tubular staining for TGFβ$_1$ versus time after irradiation. The fraction of tubules staining positively for TGFβ$_1$ is shown as a mean + SD. The 6-week time point is significantly elevated above baseline.

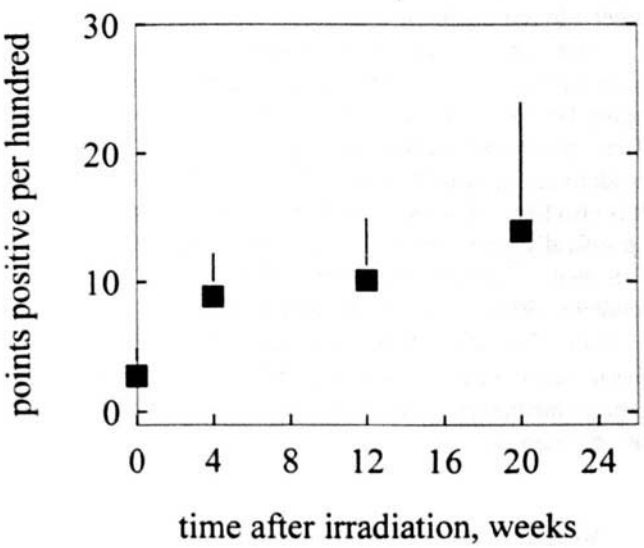


**Figure 7.** Evolution of kidney collagen deposition versus time after irradiation. The fraction of grid points overlapping tissue collagen is shown as a mean + SD. The 4-, 12-, and 20-week time points are all significantly elevated above baseline.

athy, and Heymann nephritis (31). Nonetheless, causality remains unproven. As shown by Oikawa *et al.* (20) TGFβ$_1$ mRNA expression in radiation nephropathy is reduced by enalapril and an angiotensin II antagonist, but not by captopril, yet all three agents reduced proteinuria and scarring in their studies. Thus the reduction in TGFβ$_1$ may not be essential for reduction in proteinuria and scarring.

We did attempt but were not able to obtain good tissue staining of the latency-associated peptide (LAP). Moreover, despite apparent low-level glomerular TGFβ$_1$ expression, it is possible that radiation caused activation of TGFβ$_1$, perhaps by an oxidative effect (32), thus setting in motion the glomerular scarring. This possibility remains speculative at present. Use of an antibody that recognized active as opposed to total TGFβ$_1$, appears to be most relevant to the investigation of the scarring process.

There is glomerular and tubulo-interstitial scarring in radiation nephropathy. It is generally accepted that the glomerular injury precedes the tubular in this form of renal disease (1, 14). Regarding glomerular injury and scarring, recent *in vitro* studies showed that single doses of 5–20 Gy irradiation of mesangial cells caused increased TGFβ$_1$ mRNA synthesis within 24 hr (33). This response was accompanied by a dose-related increase in fibronectin mRNA, a response that was consistent with the known effects of TGFβ$_1$ on fibronectin synthesis (34). The lack of strong TGFβ$_1$ glomerular staining in the present *in vivo* studies is thus unexpected. It is possible that glomerular mesangial TGFβ$_1$ mRNA is enhanced *in vivo* as *in vitro*, albeit with a lack of TGFβ$_1$ protein production. This would not easily explain later glomerular scarring. Furthermore, the early mesangial cell activation, expressed here as αsma staining, preceded rather than followed the TGFβ$_1$ peak. Specimens from an earlier time point might have shown enhanced

$TGF\beta_1$ expression, which would then have supported the notion that $TGF\beta_1$ is important in mesangial cell activation in this model. But specimens were not available before the 2-week time point.

In summary, using a porcine model of radiation nephropathy, we found that the development of fibrosis and scarring was not uniformly dependent on one cell type or mediator throughout the kidney. Moreover, there was variability in mediator expression depending on the time after injury in this model. The early glomerular mesangial activation was probably the starting point for later glomerular scarring, assisted by glomerular fibrin deposition. $TGF\beta_1$ did not appear to be critical to the glomerular scarring mainly because it was of low intensity and also because it occurred later than either glomerular fibrin deposition or αsma expression. On the other hand, tubular epithelial $TGF\beta_1$ expression appeared to be well associated with tubulo-interstitial fibrosis. It is likely that tubular fibrin or other filtered mediator deposition adds to this effect.

One can envisage, in this model, different or overlapping mediators of scarring depending not only on time of injury but also depending on the kidney tissue compartment. Thus, therapies that may attempt to reduce fibrosis in this model may need to be directed at more than one mediator. It is also likely that they will be important at particular times specifically after irradiation and in particular tissue compartments. Speculatively, these observations may be extended to other normal tissue radiation injuries such as lung or brain. The satisfactory prevention or treatment of normal tissue radiation injury will depend on identification of the critical mediators, their localization, and also on their time of appearance.

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