# Radiation Nephropathy

## *The Link Between Functional Damage and Vascular Mediated Inflammatory and Thrombotic Changes*

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The extent of radiation-induced nephropathy, which develops progressively over periods of months to years after treatment, is strongly influenced by both total dose and dose per fraction. In this study we examined the relationship between the early expression of various thrombotic and inflammatory markers of endothelial cell (EC) damage in irradiated mouse kidneys and the subsequent development of nephropathy. Decreased levels of glomerular ADPase and increased levels of glomerular Vwf were seen from 4 or 20 weeks after irradiation, respectively. These pro-thrombotic changes were associated with increased fibrin/fibrinogen deposits, indicative of microthrombus formation, at later times. These events were, however, not sensitive to changes in total dose or dose per fraction, therefore they cannot be quantitatively linked to the development of radiation nephropathy. Increased leucocyte invasion of the renal cortex was also seen after irradiation; this was quantitatively dependent on both total dose and dose per fraction. Linear quadratic analysis of the leucocyte dose-response curves yielded an $\alpha/\beta$ ratio of 7.7 Gy, which is significantly greater than the $\alpha/\beta$ ratio or 2.7 Gy determined for nephropathy, indicating less fractionation sensitivity for the inflammatory response. We conclude that inflammatory changes contribute to, but do not entirely explain, radiation nephropathy. The role of thrombotic changes is less clear.

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The kidney is one of the dose-limiting organs in abdominal irradiation, particularly where large volumes of both kidneys are included in the field. Renal functional impairment is not usually detectable earlier than 6 months after irradiation and both the onset and rate of progression of damage are dose-related (1–3). There is a strong influence of dose per fraction, with sparing of damage for low doses per fraction and estimated $\alpha/\beta$ ratios of 2–3 Gy for radiation nephropathy (4).

The underlying processes that lead to radiation nephropathy remain to be fully elucidated, but capillary endothelial damage may play a role. Prospective clinical studies in The Netherlands Cancer Institute demonstrated progressive deterioration of both tubular and glomerular function in the left kidneys of gastric non-Hodgkin's lymphoma (NHL) patients treated with a total dose of 40 Gy. Loss of function progressed at a rate of approximately 2% per month, to 25 to 30% of pretreatment values at 6 to 9 years (2). Fifteen of these malignant lymphoma patients were further investigated, using selective angiography and captopril renography, to assess the type and extent of vascular changes. Both structural and functional vascular changes were identified (truncated and tortuous vessels, stenosis, vascular leakage), leading to renovascular hypertension in 33% of cases (5).

Serial histological examination of irradiated kidneys in mice also identified structural vessel changes, with a loss of fine capillary network and capillary distension from about 10 weeks after irradiation. These changes preceded vascular leakage and reduced renal perfusion, which were apparent from about 20 weeks after high single doses. Injection with fluorescein dextran indicated that about 30% of glomeruli were effectively 'non-perfused' by 30 weeks after 16 Gy to mouse kidneys (unpublished results Kuin et al 2001). A predominance of vascular lesions has previously been reported after kidney irradiation of several other species of experimental animals, including dogs, monkeys and pigs (6–8). This is characterized by endothe-

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lial cell (EC) swelling and detachment from the basement membrane, followed by progressive endothelial cell loss and microthombus formation which ultimately results in vascular occlusion. Radiation-induced changes in production and release of pro-thrombotic factors, e.g. von Willebrand factor (VWF) from damaged ECs may well be involved in this process.

Our previous in vitro studies demonstrated a significant increase in VWF released from irradiated human umbilical vein endothelial cells (HUVECs) at 48 to 72 h after irradiation. A causal relationship was also established between VWF release and platelet adhesion to the extra cellular matrix produced by the ECs irradiated in vitro (9). Interestingly, an even greater stimulation of VWF release was found after fractionated irradiation of HUVECs (10), which is in marked contrast with the sparing of radiation-induced cell death seen after fractionated irradiation. Separate studies have shown that the increased release of VWF from ECs irradiated in vitro is due to upregulation of VWF transcription (11).

In subsequent in vivo studies we also demonstrated significant increases in the amount of Vwf protein in glomeruli of irradiated mouse kidneys. Immunohistochemical staining for glomerular Vwf increased from 20 to 40 weeks after irradiation (10, 12). This increase preceded the onset of measurable renal functional impairment, assessed by [$^{51}$Cr]EDTA retention in separate groups of mice, but no increase in VWF was seen during the first 10 weeks after irradiation. The timing of this increase is therefore inconsistent with direct radiation-induced up-regulation of gene expression. Indeed, no significant change in expression levels of mRNA for Vwf was found after in vivo irradiation, either in mRNA isolated from whole kidney extracts or from isolated glomeruli (13). The increased glomerular Vwf protein seen after kidney irradiation is therefore presumably secondary to other events, such as increased release by damaged ECs and entrapment in the irradiated mesangial matrix.

Various inflammatory cytokines and their receptors on ECs and leucocytes are also induced by irradiation. Leucocyte extravasation from the circulation and accumulation in tissue is mediated by these selectins and integrins. This occurs at sites of platelet activation and is a characteristic component of both acute and chronic inflammation. A prominent feature associated with the vascular changes seen in irradiated pig kidneys is leucocyte attachment to glomerular ECs (6). We have also found a progressive increase in leucocytes in the renal cortex of irradiated mice from 10 to 40 weeks after irradiation (10, 12).

These observations prompted us to investigate in more detail the relationship between renal functional impairment and Vwf expression and leucocyte adhesion, as markers of pro-thrombotic and inflammatory changes, after a range of single and fractionated irradiation doses to mouse kidneys. The dose dependence and fractionation sensitivities for these two markers of EC damage were compared with the extent of late functional damage at 40 weeks after irradiation.

## MATERIAL AND METHODS

### *In vivo irradiations*

Female C3H/HenAf-nu$^{+}$ mice were irradiated at 12–14 weeks of age (body weight 23–27 g). Food and acidified tap water were provided ad libitum. Experiments were performed in accordance with the national regulations for animal experimentation and experimental protocols were approved by the local animal welfare committee before the start of the studies.

Non anaesthetized mice were irradiated or sham irradiated via two lateral opposed tangential fields on both kidneys, as previously described (1, 4). Irradiation was performed with 250 kV x-rays, operating at 15 mA and filtered with 0.5 mm Cu. The dose rate at the position of the kidneys was 2.35 Gy per min and the mice were rotated 180° halfway through each irradiation in order to ensure a homogeneous dose distribution. Fractionated irradiation schedules employed daily irradiation, 5 days per week.

### *Immunohistochemistry*

Earlier studies had demonstrated elevated glomerular Vwf levels from 20 to 40 weeks after irradiation and a progressive increase in the number of leucocytes in the renal cortex from 10 to 40 weeks (10, 12). However, significant impairment in kidney function was not expected much earlier than 40 weeks after the fractionated doses used in this study. For a direct comparison between functional damage and markers of EC damage, an evaluation time of 40 weeks was therefore chosen. Groups of 5–6 mice per dose group were anaesthetized with ether and the kidneys were perfused with 5 ml 0.9% NaCl prior to sacrifice. Kidneys were excised and frozen in liquid nitrogen, stored at −70°C, and cryosections (4 μm) were cut for immunohistochemistry. Cryosections were fixed for 10 min in cold acetone, and stained with polyclonal rabbit anti-human Vwf antibody (Dako, Glostrup, Denmark) or biotinylated monoclonal rat anti-mouse CD45 antibody (PharMingen, San Diego, CA) in 1% bovine serum albumin (BSA)/PBS (w/v). Sections were incubated at 37°C for 90 min (Vwf) or at room temperature for 60 min (CD45), prior to washing and incubation with secondary antibodies for 30 min at room temperature (swine anti-rabbit peroxidase labelled, Dako (Vwf), or biotinylated rabbit anti-rat, Dako, followed by avidin-peroxidase complex, Vector Laboratories, Burlingame, CA (CD45)). The reactions were visualized using 3,3′ diaminobenzidine (Vwf) or 3,3′ diaminobenzidine plus nickel (CD45) (Vector Laboratories). Omission of primary antibodies served as negative controls.

*Image analysis*

For digital image analysis, all kidney sections were stained and captured in one run to minimize variation in signal intensity due to technical manipulations. Tissue sections were viewed through a microscope (Carl Zeiss, Jena, Germany) fitted to a cooled CCD black and white camera (model KAF 1400, Photometrics, Munich, Germany). The CCD camera was coupled to a Macintosh Quadra 650 computer with frame grabber (Photometrics) and IPlab software (Signal Analytics Corporation, Vienna, VA). Analysis of Vwf was restricted to staining in the glomeruli and analysis of leucocytes was restricted to the renal cortex. For each immunohistochemical stain five different fields were randomly selected per tissue section. The immunopositive areas were quantified according to a threshold as determined by the observer and expressed as a percentage of the total area in the region of interest.

*Measurement of renal function*

Separate groups of mice (6 to 10 per dose) were irradiated with the same single-dose or fractionated schedules as described above and were repeatedly tested for renal function. Kidney function was measured at approximately 4-week intervals, by plasma clearance of [$^{51}$Cr]EDTA, as previously described (1, 4). Briefly, intraperitoneal injections of [$^{51}$Cr]EDTA were given at a dose of 0.37 MBq in 0.1 ml per mouse. Single blood samples (75 µl), from the retro-orbital plexus, were collected in heparinized capillary tubes 30 min after injection and centrifuged. A 20 µl plasma sample was taken and counted in an autogamma counter. Results are expressed as residual activity per millilitre plasma (percentage of injected dose).

## RESULTS

*Renal function*

The mean residual [$^{51}$Cr]EDTA activity for young control mice (in plasma samples taken at 30 min) was 1.3% of the injected dose. There was no significant change in renal function of unirradiated mice with increasing age ($p = 0.47$). After single-dose or fractionated irradiation there was a progressive and dose-dependent deterioration in renal function, as assessed by [$^{51}$Cr]EDTA clearance. The time course for expression of renal damage after 20 fractions of irradiation is presented in Fig. 1. The rate of progression of damage increased with increasing dose; the mean latencies ($\pm$ SEM) before expression of moderate to severe damage, i.e. residual plasma [$^{51}$Cr]EDTA > 4%, were $74.3 \pm 3.2$ weeks, $53.7 \pm 2.2$ weeks and $42.0 \pm 3.3$ weeks for $20 \times 1.2$ Gy, $20 \times 1.6$, and $20 \times 2.0$ Gy, respectively, or < 30 weeks for higher total doses.

Dose-response curves for functional damage at 40 weeks after irradiation with 1, 4, 10 or 20 fractions are shown in Fig. 2. There was a steep dose-response relationship for

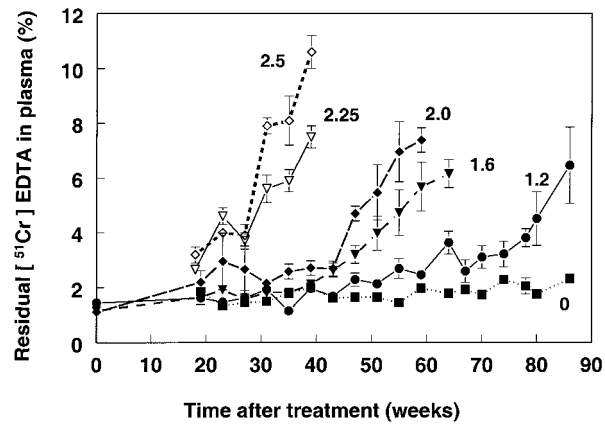


*Fig. 1.* Residual plasma [$^{51}$Cr]EDTA (functional kidney damage) versus time after the start of treatment with 20 fractions of bilateral irradiation to mouse kidneys. Total doses of 24–50 Gy were given, the dose per fraction used in each schedule is written beside each curve in Gy. Closed symbols indicate data (means $\pm$ SEM) from the current series; open symbols denote previously published data from reference (14).

renal damage within each fractionation schedule and a marked sparing of damage with increasing fractionation. A linear quadratic analysis of these data yielded an $\alpha/\beta$ ratio of 2.7 Gy (95% CI 1.8–3.6 Gy), which is consistent with previous, more extensive analyses of functional damage in mouse kidneys, using this assay (4, 14, 15).

*Number of leucocytes*

The mean area of the renal cortex covered by leucocytes in age-matched control animals was $1.4 \pm 0.2$ (SD)%. At 40 weeks after irradiation there was an increase in leucocyte coverage with increasing dose within each fractionation schedule (Fig. 3). There was also a marked sparing effect

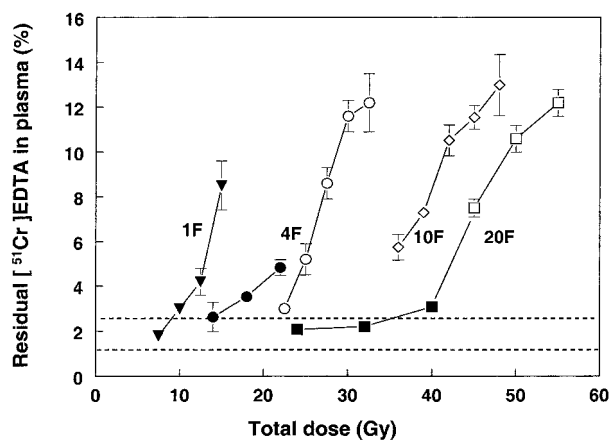


*Fig. 2.* Dose-response curves for functional kidney damage (mean values $\pm$ SEM) at 39–43 weeks after irradiation with 1 to 20 fractions. Open symbols denote previously published data (from references 4, 14, 15). The dashed lines indicate the SEM of values for age-matched control mice.

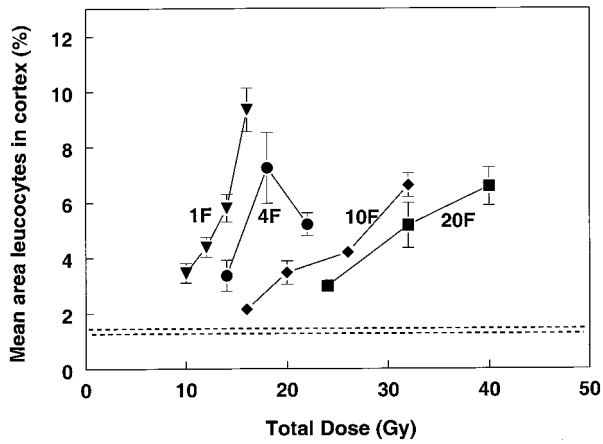


*Fig. 3.* Dose-response curves for mean leucocyte coverage ($\pm$SEM) in the renal cortex (calculated as a percentage of total area) at 40 ($\pm$4) weeks after single-dose or fractionated irradiation. The dashed lines indicate the SEM of values for age-matched control mice.

of fractionation. The total dose required to give a three-fold increase in leucocyte coverage increased from approximately 12 Gy in a single dose to 30 Gy in 20 fractions. A linear quadratic analysis of these data yielded an $\alpha/\beta$ ratio of 7.7 Gy (95% CI 4.7–10.6 Gy) for leucocyte presence in the kidney at 40 weeks after irradiation. This is significantly higher than the $\alpha/\beta$ ratio obtained from the analysis of functional response data.

*Glomerular Vwf expression in vivo*

The mean glomerular area expressing Vwf protein (as detected by immunohistochemistry) in age-matched control kidneys was $18.2 \pm 5.1\%$ (SD). At 40 weeks after irradiation there was a $>1.5$-fold increase in glomerular Vwf in most dose groups tested (Fig. 4). The values for the

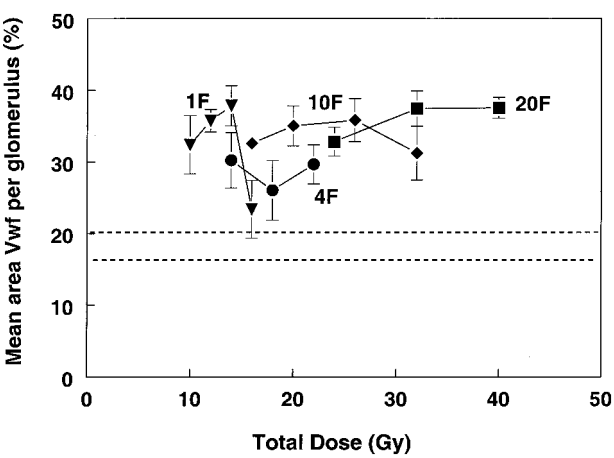


*Fig. 4.* Dose-response curves for mean glomerular Vwf expression, $\pm$SEM, (calculated as a percentage of glomerular area) at 40 ($\pm$4) weeks after single-dose or fractionated irradiation. The dashed lines indicate the SEM of values for age-matched control mice.

16 Gy single-dose group were not significantly different from those for controls in this particular staining experiment. Sections from kidneys of these same mice which were stained in previous runs did have a significant, two-fold increase in glomerular Vwf at 40 weeks after 16 Gy (10, 12). When all the irradiated groups were compared with the age-matched controls (all sections stained in a single run), there was a significant increase in the level of glomerular Vwf staining in the irradiated groups (mean values of 32 $7 \pm 7.6\%$ (SD) and $18.2 \pm 5.1\%$ ($p < 0.0001$). There was, however, no significant influence of increasing radiation dose within each fractionation schedule, and no separation of the curves according to the number of fractions given. These data therefore cannot be analysed according to the linear quadratic formulation.

## DISCUSSION

The working hypothesis for these studies was that kidney irradiation causes early changes in EC function, resulting in changes in expression of various mediators of inflammation and platelet adhesion that shift the balance towards a pro-thrombotic microenvironment. These changes combined with a second stimulus, such as EC loss, could result in glomerular thrombosis and organ ischaemia, leading to functional impairment. Previous studies had shown time-related changes in the expression of glomerular Vwf and ADPase, as well as increased leucocytes in the cortex of irradiated kidneys (10, 12, 16). The aim of the present study was to determine whether there is a relationship between these markers of thrombosis and inflammation, and renal functional damage after a range of single-dose and fractionated irradiation schedules.

In capillary circulation, where high shear forces exist, the initial adhesion of activated platelets to the subendothelium is almost exclusively mediated by VWF. A causal link between VWF released from irradiated ECs and platelet adhesion to the extra cellular matrix has been demonstrated in vitro (9), but not yet in vivo. Another potent inhibitor of platelet adhesion is ADPase, and high concentrations of this enzyme are found in the outer membrane of glomerular ECs and in the glomerular basal membrane. Previous studies have demonstrated a significant decrease in ADPase activity in glomeruli isolated from rat kidneys at 1 day after in vivo irradiation (17). The functional importance of glomerular ADPase activity as an inhibitor of platelet activation was also implied from experiments combining renal irradiation with a nephrotoxic stimulus 1 day later. Under these pro-aggregatory conditions, the thrombotic tendency was enhanced in irradiated glomeruli, coinciding with the observed, early decrease in ADPase activity; later time points were not examined (17).

In the course of our studies, we have demonstrated significant increases in glomerular Vwf from 20 to 40

weeks after single-dose irradiation of mouse kidneys, with no early change at 1 day to 10 weeks (10, 12, and results described here). Significant decreases in glomerular ADPase levels, assessed from quantitative enzyme histochemistry of kidney sections, were also found at 4 to 40 weeks after irradiation, with no change at 1 day (16). These chronic changes in Vwf and ADPase were associated with a fourfold increase in the number of glomeruli with deposition of fibrinogen/fibrin, which is an indirect marker of platelet aggregation. It seems plausible that these glomerular changes might contribute to the chronic and progressive deterioration in renal function seen after irradiation, although no causal link has yet been definitively established.

It has been known for many years that radiation-induced nephropathy is dose related and strongly influenced by fractionation (e.g. 1, 4, 14, 15). If radiation-induced changes in expression of molecules involved in glomerular thrombus formation are the primary event responsible for the subsequent development of nephropathy, then comparable dose response and sensitivity to dose per fraction might be expected to govern these parameters. Clearly, the data for glomerular Vwf after in vivo irradiation which are described here do not show any dependency on total dose or a fractionation sparing effect. A similar lack of dose response or sensitivity to dose per fraction was found for previously published glomerular ADPase levels at 40 weeks after irradiation (16). This seems to argue against a direct causal relationship between radiation-induced changes in these thrombotic markers and the development of radiation nephropathy, although an indirect or secondary influence cannot be ruled out.

Irradiation of ECs in vitro and various tissues in vivo has been shown to cause rapid transcription of genes regulating inflammation, e.g. TNFα and various interleukins. These cytokines then stimulate production and release of adhesion molecules from ECs, e.g. E selectin and ICAM-1, which initiate leucocyte adhesion, extravasation and invasion in tissue (18, 19). In several studies it has been demonstrated that expression of these inflammatory markers is radiation dose dependent. The adhesion molecule ICAM-1 seems to play a critical role in leucocyte recruitment, since antibodies blocking its expression have been shown to abrogate leucocyte adhesion in vivo.

Our data for irradiated kidney clearly show a dose-dependent increase in leucocytes in the renal cortex. Increased numbers of leucocytes within the glomeruli were not seen, although clusters of leucocytes surrounding the glomeruli were common. The dose-response relationship for leucocyte presence in the irradiated kidney suggests a closer link between this inflammatory process and radiation-induced nephropathy than for the glomerular thrombotic events studied. However, the fractionation sensitivity for leucocyte presence was less marked than that for functional damage; with an $\alpha/\beta$ ratio of 7.7 Gy versus 2.7 Gy for functional damage. It therefore seems probable that the radiation-induced inflammatory response contributes to, but is not entirely responsible for, the progressive nephropathy.

In summary, our studies on the radiation response of the mouse kidney have identified both structural and functional vascular changes. Increased levels of glomerular Vwf and decreased levels of ADPase were associated with increased fibrin/fibrinogen deposition, but these changes were not obviously radiation dose related. Their significance in the development of radiation nephropathy is therefore unclear. Increased leucocytes in the renal cortex were dependent on total radiation dose and were sensitive to changes in dose per fraction, although to a lesser extent than functional damage.

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