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ORIGINAL ARTICLE

Fractalkine: An important candidate for directing periglomerular leukocyte accumulation in irradiated mouse kidneys

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Abstract

Radiation-induced impairment of renal function is preceded by capillary endothelial cell damage, which initiates a cascade of inflammatory and thrombotic events. Accumulation of leukocytes in the irradiated kidney, especially in areas surrounding the glomeruli, has been clearly demonstrated. The chemokine fractalkine has recently been identified as a key mediator of leukocyte adhesion that functions without the requirement of integrins or selectin-mediated rolling. In this study we investigate the possible involvement of fractalkine in the inflammatory response of the irradiated kidney. Mouse kidneys were irradiated with single doses of 16 or 0 Gy, and protein and mRNA levels of fractalkine and PECAM-1 were examined after 10 to 40 weeks. These changes were correlated with the progressive increase and distribution of leukocytes in the irradiated kidneys. Increased fractalkine immunoreactivity was seen at glomerular sites 30 to 40 weeks after irradiation. This fractalkine expression was strongly associated with the presence of leukocytes surrounding the Bowman's capsule of the same glomeruli. No significant changes in mRNA levels of fractalkine were seen in whole kidney extracts after irradiation, but expression levels were not determined for isolated glomeruli. PECAM-1 protein levels did not change with time after irradiation, although a significant decrease in mRNA expression was seen at 10 weeks. This study is the first demonstration of increased fractalkine after irradiation and the results suggest that fractalkine may be an important mechanism of leukocyte trafficking in the development of a radiation induced inflammatory response.

The kidney is one of the critical organs in abdominal irradiation, particularly where large volumes of both kidneys are included in the radiation field. Impairment of renal function is not usually detectable until about 4 to 6 months after irradiation, although cellular changes occur much earlier. A key process in the cascade of events occurring after irradiation is endothelial cell (EC) swelling and detachment from the basement membrane, followed by progressive cell loss. Remaining ECs exhibit increased permeability leading to platelet adhesion, thereby initiating the formation of mural microthrombi. If unopposed, subintimal proliferation occurs with subsequent thickening of the basement membrane and progressive enlargement of platelet-rich thrombi, which may lead to total vessel occlusion, thereby jeopardizing organ function [1–5].

A prominent feature associated with vascular changes seen after irradiation is leukocyte attach-ment, which is likely to occur at sites of platelet activation and is a characteristic component of both acute and chronic inflammation. Accumulation of leukocytes has been demonstrated after kidney irradiation in the cortex, glomeruli and around the Bowmans capsule of glomeruli, where leukocytes form crescent-shaped clusters [1,2,6,7].

Migration of leukocytes into extravascular tissues involves a cascade of molecular events, including the production and secretion of chemotactic and cell adhesion factors. Fractalkine (CX3CL1) is a recently discovered chemokine with several unique features. In addition to its chemotactic properties as a soluble chemokine, fractalkine is also expressed on activated endothelial cells in a membrane-bound form, which acts as an adhesion molecule, capturing fractalkine receptor-positive leukocytes without requiring selectin-mediated rolling or activation of integrins. The presence of a functional fractalkine

receptor (CX3CR1) has recently been demonstrated on platelets, thereby providing evidence for a functional role of fractalkine in platelet activation [8]. We hypothesize that these unique features make fractalkine well suited for platelet activation and leukocyte recruitment in tissues with high blood flow, such as in the renal glomerulus.

Platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) is highly expressed in the vasculature with approximately one million copies on the surface of each EC. PECAM-1 is involved in the initial formation and stabilization of cell-cell contacts, and maintenance of the vascular permeability barrier [9]. It has also been implicated in neutrophil recruitment *in vivo* [10] and leukocytes adhesion and transendothelial migration *in vitro* [11,12].

Preventing the influx of inflammatory cells could help to reverse or inhibit the progression of renal injury after irradiation. The aim of the present study is to investigate the effects of ionizing radiation on the expression of key inflammatory markers involved in directing leukocyte infiltration at sites of inflammation, thereby elucidating their possible role in the pathogenesis of radiation nephropathy.

Materials and methods

Irradiation procedures

Female C3H mice (substrain HenAf-nu$^+$) were irradiated at 12–14 weeks of age (body weight 23–27 g). Food and acidified water were provided *ad libitum*. Experiments were approved by the local animal welfare committee and were in accordance with the national regulations for animal experiments.

Non-anesthetized mice were sham-irradiated or irradiated with a single dose of 16 Gy via two lateral opposed tangential fields on both kidneys. 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 kidney was 2.35 Gy per minute and mice were rotated 180° halfway through each irradiation in order to ensure a homogeneous dose distribution.

Immunohistochemistry

Groups of 4–7 irradiated and age-matched control animals were sacrificed at intervals of 10 to 40 weeks after irradiation. Immediately before killing, mice were anesthetized and kidneys were perfused with 5 ml 0.9% NaCl. Kidneys were excised, frozen in liquid nitrogen and stored at −80°C. Cryosections (4 μm) were cut for immunohistochemistry, fixed for 10 min in cold acetone and incubated with specific primary antibodies (Table I) in 1% bovine serum

Table I. Specific primary antibodies against proteins of interest.

Protein of interest	Primary antibody	Catalog #
Fractalkine (CX3CL1)	Goat-anti rat anti Fractalkine (R&D)	AF537
Leukocytes (CD45)	Biotinylated rat anti-mouse anti-CD45 (PharMingen)	Old#: 01112D
		New#: 553078
PECAM-1 (CD31)	Purified rat anti-mouse anti CD31 (PharMingen)	Old#: 01951D
		New#: 553370

albumine (BSA)/PBS (w/v) at room temperature for 60 min. After washing in PBS, sections were incubated with specific biotinylated secondary antibodies for 30 min, followed by peroxidase labeled avidin-biotin complex (Vector Laboratories, Burlingame, USA). The reactions were visualized using 3,3′ diaminobenzidine (Vector Laboratories, Burlingame, USA) as the chromogen (brown reaction product). Sections were lightly counterstained with hematoxylin to discriminate the glomeruli from the surrounding tissue. The immunostaining conditions were optimized for each antibody reagent. Omission of primary antibodies served as negative controls. Tissue sections were analyzed without knowledge of the treatment.

Semi-quantitative scoring

The number of fractalkine positive glomeruli was determined as a percentage of the total number of glomeruli per section using a semi-quantitative scoring system based on the following criteria: ring formation or relative surface coverage of <30% or >30% (Figure 1). Data are expressed as mean ± S.D (n = 6–7 mice per group).

mRNA expression

Total RNA (free of genomic DNA) from irradiated and age matched control kidneys (n = 4/group) was isolated with RNAsol B according to the manufacturer's instructions (Bioteck Laboratories, Houston, USA). RNA concentrations were measured spectrophotometrically at 260 nm and purity was confirmed with 260/280 OD spectrophotometer readings.

Pooled RNA from four individual animals (total 5 μg) was reverse transcribed with Superscript II Reverse transcriptase (Invitrogen) and random hexamers in a total volume of 20 μl, according to the manufacturer's protocol. After cDNA synthesis, the volume was adjusted to 100 μl with water.

Real time PCR (RT-PCR) assays were performed to quantify levels of RNA transcripts of fractalkine, and PECAM-1. Specific primers for the genes of interest were designed using Primer Express 2.0
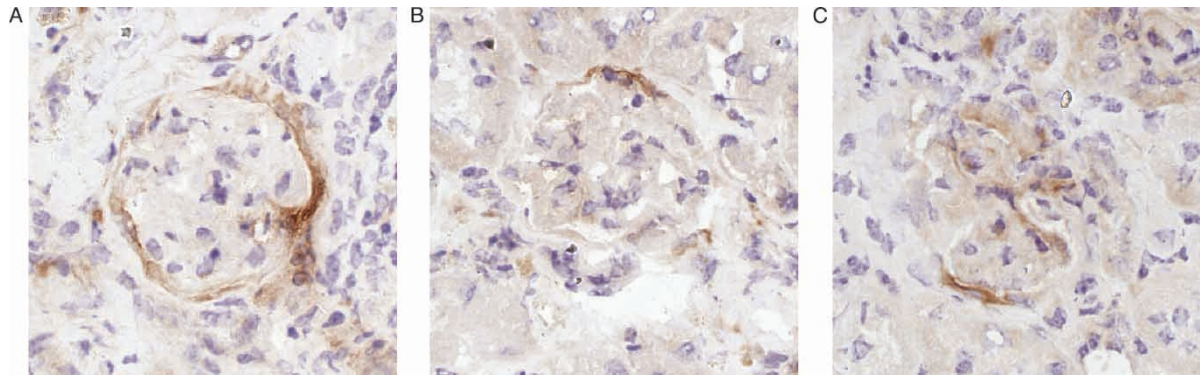
Figure 1. Immunohistochemical staining patterns of fractalkine. (A) ring formation, (B) surface coverage $<30\%$ and (C) surface coverage $>30\%$.

software (Applied Biosystems) and sequences are presented in Table II. Samples were subjected to 45 cycles of amplification of 15 s at 95°C followed by 1 min at 60°C, using an ABI PRISM 7700 detection system and SYBR Green buffer according to the manufacturer's instruction with minor modification (Applied Biosystems). Water and RNA that had not been subjected to reverse transcription reactions were included to ensure specificity. Integrity was assessed for each sample through analysis of amplification plots and dissociation curves. In addition, the size of the PCR products was checked by agarose gel electrophoresis. The relative quantification was performed using the standard curve method with sham-irradiated kidney as a calibrator according to User Bulletin #2 (Applied Biosystems). To establish the reproducibility of the PCR in different runs, all amplifications were carried out in duplicate. Expression levels were normalized for the expression of GAPDH, a housekeeping gene.

Statistical analysis of mRNA expression

Values are presented as relative mRNA levels compared to sham-irradiated controls, after normalization for a housekeeping gene, GAPDH. The Student's t-test was used to compare mean values ($\pm$S.D.) of sham-irradiated control and irradiated groups. A p-value <0.05 was considered significant.

Table II. Primersequences.

Gene of interest	Primersequence
Fractalkine	Forward 5′-GGGTGGCCATGTTTGCTTAC-3′
	Reverse 5′-CAGGCAAGCAGCTCACACTG-3′
PECAM-1	Forward 5′-GAGCCCAATCACGTTTCAGTTT-3′
	Reverse 5′-TCCTTCCTGCTTCTTGCTAGCT-3′

Results

Immunohistochemistry

Figure 2 shows kidney sections stained for the presence of leukocytes, fractalkine and PECAM-1 at 40 weeks after irradiation or sham treatment. Previous studies using quantitative immunohistochemistry demonstrated a progressive increase in leukocyte invasion in the mouse kidney from 10–40 weeks after irradiation [7]. This was confirmed in the current study. At early time intervals (10–20 weeks) after irradiation, leukocytes were distributed randomly throughout the interstitium (data not shown), whereas at later times (peri)glomerular accumulation of leukocytes was found (Figure 2A&B).

There was no detectable fractalkine immunoreactivity in normal sham-irradiated kidneys (Figure 2C) or at 10–20 weeks after irradiation (data not shown). Increased expression of fractalkine was specifically found in and/or around the glomeruli at 30 and 40 weeks after irradiation (Figure 2D). The immunohistochemical staining patterns for fractalkine ranged from ring formation to surface coverage of <30 or $>30\%$ (Figure 1). Semi-quantitative scoring demonstrated a significant increase in the number of fractalkine immunopositive glomeruli as a percentage of the total number of glomeruli at 30 and 40 weeks after irradiation (Figure 3). Staining of serial sections demonstrated that periglomerular fractalkine expression at late times after irradiation correlated with leukocytes surrounding the Bowmans capsule of those glomeruli (Figure 2B,D). In contrast, leukocyte invasion of the renal cortex, which has been demonstrated with time after radiation was not associated with fractalkine expression (data not shown).

PECAM-1 immunoreactivity was prominent in ECs of both the renal cortex and glomeruli and there was no apparent difference between expression levels
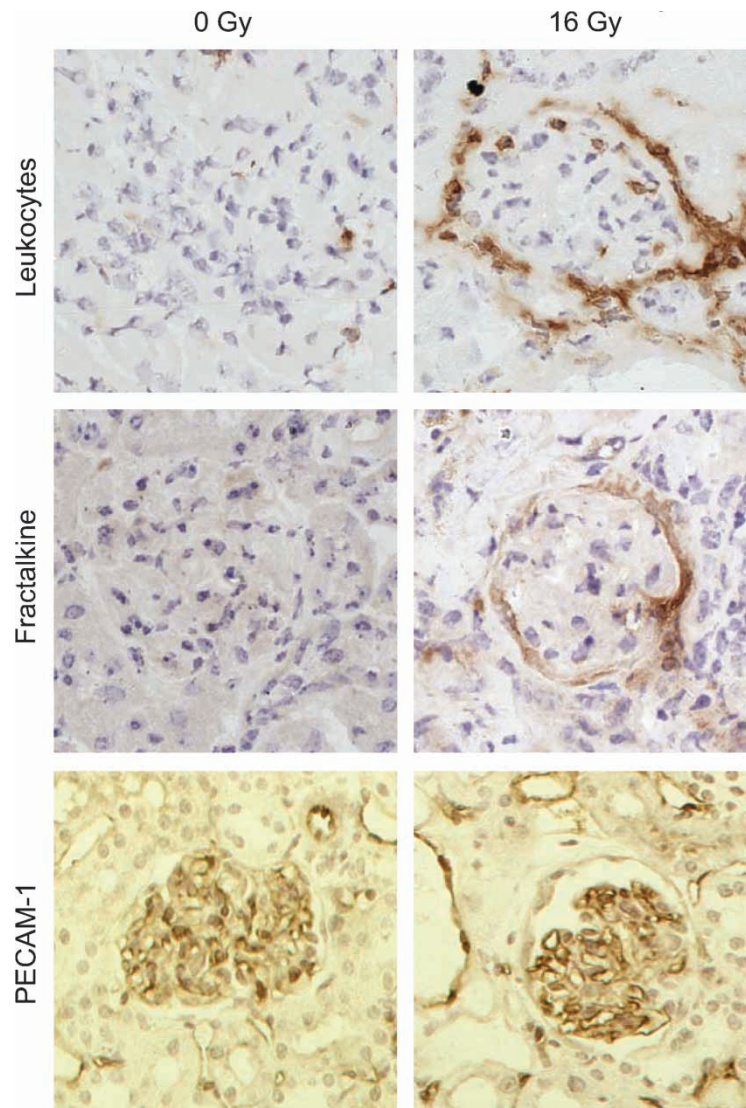
Figure 2. Photographs of mouse kidney sections stained immunohistochemically for leukocytes (A–B), fractalkine (C–D) and PECAM-1 (E–F) at 40 weeks after irradiation with 0 Gy or 16 Gy. All photomicrographs were taken at an original magnification of 400 ×. Sections were counterstained with hematoxylin and eosin.

in irradiated and unirradiated sections (Figure 2E,F). Previous studies using quantitative image analysis [13] had shown no significant change in the amount of glomerular PECAM-1 protein with time after irradiation.

mRNA expression

Messenger RNA levels of genes of interest were quantified using real time PCR at different time intervals after irradiation. Single PCR products of the expected length were amplified (confirmed by agarose gel electrophoresis) from cDNA samples of both irradiated and control kidneys; no amplification was observed with the use of water or RT negative controls. For all genes of interest, correlation coefficients of standard curves from sham-irradiated kidneys were consistently > 0.95 (mean $R^2 \pm$ S.D. $= 0.98 \pm 0.04$).

Transcripts of fractalkine were constitutively expressed during the observation period. There were no significant differences in mRNA expression between irradiated kidneys and sham-irradiated controls (Figure 4A). In contrast, mRNA expression for PECAM-1 was significantly decreased at 10 weeks after irradiation, with partial recovery towards control levels at later times (Figure 4B).

Discussion

Our previous studies showed that radiation-induced impairment of renal function in mice was preceded by a progressive increase in leukocytes, mainly in the cortex [13]. Here, we provide evidence for the
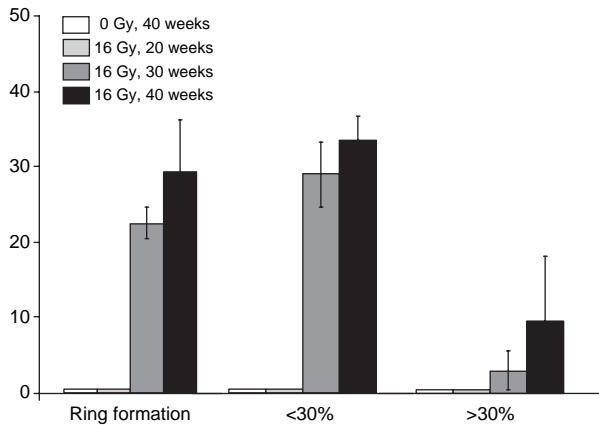
Figure 3. Semi-quantitative analysis of the number of fractalkine positive glomeruli as a percentage of the total number of glomeruli at different time intervals after irradiation or sham-treatment. Glomeruli were evaluated separately with surface coverage ranging from ring formation to < or >30%. Data are expressed as mean ± S.D.

molecular mechanism that may mediate these inflammatory changes. Our data demonstrate that fractalkine present in the glomeruli of irradiated kidneys was strongly associated with leukocytes surrounding these glomeruli. This is the first demonstration of increased renal fractalkine after irradiation, although increased expression has also been reported in glomerular endothelium upon induction of anti-glomerular basement membrane glomerulonephritis [14].

The most convincing evidence of a causal role for fractalkine in leukocyte recruitment was obtained from experiments using neutralizing antibodies for
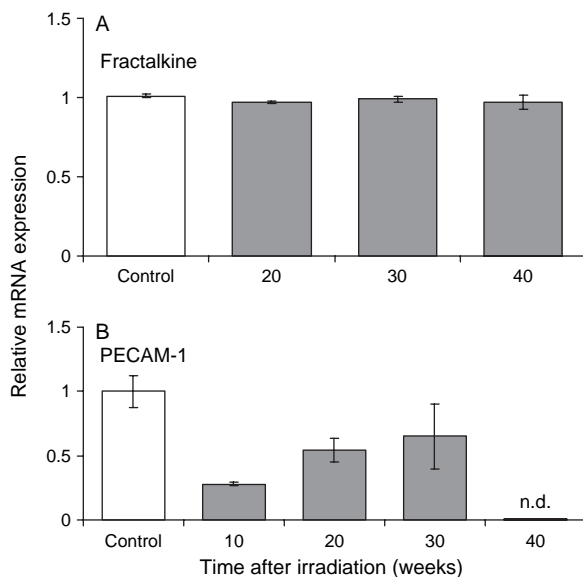

Figure 4. Relative mRNA expressions of genes encoding fractalkine (A) and PECAM-1 (B). Messenger RNAs were analyzed by real-time PCR assays and normalized for GAPDH, a housekeeping gene.

fractalkine or its receptor (CX3CR1) during the generation of inflammatory responses in experimental animals. In a model system of crescent glomerulonephritis, viral chemokine with antagonistic activity for CC-, CXC- and CX3C-chemokine receptors significantly reduced the infiltration of leukocytes and attenuated proteinuria [14]. Other studies demonstrated that daily injections of anti-CX3CR1 antibody blocked the infiltration of leukocytes into the glomeruli, prevented crescent formation and improved renal function, suggesting a role for fractalkine in the pathogenesis of the glomerulonephritis [15].

In kidneys of mice with bovine serum albumin overload proteinuria, fractalkine mRNA levels were approximately two fold higher than those in controls [16]. Fractalkine expression was also induced in tubular epithelial cells in this model. In contrast, we were not able to detect any increased expression of fractalkine mRNA in extracts from whole irradiated kidneys, suggesting post-transcriptional regulation of the protein synthesis. The increased protein levels seen after *in vivo* irradiation may be due to increased release from damaged ECs, rather than a direct increase in transcription such as occurs after *in vitro* stimulation with cytokines (such as TNF alpha). Alternatively, a local increase in fractalkine mRNA in the glomeruli could remain undetected when analysing expression levels in whole kidney extract.

The discovery of fractalkine has provided new insights into the mechanisms of platelet activation, leukocyte recruitment and organ damage at sites of inflammation. While soluble chemokines activate platelets rather unspecifically, membrane-anchored fractalkine may represent a more specific mechanism by focussing activated platelet to the original site of EC damage [8]. Changes in fractalkine protein levels might also attract mononuclear cells directly into the tissue and enhance their adhesion property, which in turn would favour inflammation and disease progression.

Taken together, these reports suggest that radiation-induced fractalkine might be an important mechanism for platelet adhesion and leukocyte trafficking at the endothelium in the high-flow glomerular circuit and, in turn, implicates its receptor as a prime drug target for therapeutic intervention of endothelium-related inflammatory renal disease after irradiation.

Due to the association between cell-cell adhesion and cell growth and migration, we also studied the expression and localization of EC adhesion molecule PECAM-1. We did observe a decrease in PECAM-1 mRNA, which was significant at 10 weeks. This might be associated with a reduction of the adhesion interaction between adjacent ECs, thereby enhancing

their permeability [9]. However, there was no apparent change in the immunoreactivity of PECAM-1 in irradiated kidneys, either in the present study or in a previous study [13]. The role of PECAM-1 in the inflammatory processes in the irradiated kidney therefore remains uncertain.

In conclusion, irradiation has been shown to increase fractalkine expression, which may regulate periglomerular leukocyte invasion in irradiated mouse kidney. These changes may be part of the tissues' attempt to protect itself or may contribute to tissue damaging processes. It is important to understand the potential limitations of extrapolating data from mice to humans. Therefore, the challenge remains how to convert this knowledge into potential therapies of radiation nephropathy.

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