Development of features of glomerulopathy in tumor-bearing rats: a potential model for paraneoplastic glomerulopathy

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Abstract

Background. It has been well-recognized that cancer patients occasionally develop renal disorders independently of direct tumor invasion. However, the clinical entity of paraneoplastic glomerulopathy remains poorly understood, in part due to the lack of an animal model for basic research. In the present study, we investigated whether cancer-bearing rats develop features of glomerulopathy.

Methods. RCN-9 rat colon cancer cells (1 × 10⁷) were injected into F344 rats (n = 13) and T cell-deficient F344 rats (nude rats; n = 7) via the portal system. Urinalysis and histological examinations were performed in comparison with control rats (n = 6) that received a vehicle injection.

Results. Metastatic growth of RCN-9 cells exclusively in the liver was observed in the cancer-injected F344 rats, whereas direct invasion into the kidney was not evident even microscopically. Two of the cancer-injected F344 rats died within 2 days, but 9 of the 11 that avoided early death showed elevation of urinary protein (up to 158.0 mg/day) compared to controls (mean values: 60.8 ± 12.9 versus 17.8 ± 2.1 mg/day, P = 0.0291). Although morphological changes were not evident in light microscopy, abundant IgG in the glomerular tufts of the proteinuric rats was shown immunohistochemically. Ultrastructure analysis revealed electron-dense deposits in the glomerular basement membrane zone and effacement of the podocytic foot process. Interestingly, none of the nude rats showed proteinuria despite of cancer growth, suggesting that a specific immune response was involved.

Conclusions. The tumor-bearing rats developed features of glomerulopathy, as expected from the clinical perspective, and this animal model may provide new insights into the development of paraneoplastic glomerulopathies.

Keywords: cancer; proteinuria; rat model; renal pathology; secondary glomerulopathy

Introduction

Cancer patients occasionally develop renal disorders independently of the direct tumor burden [1–3]. These disorders are collectively referred to as paraneoplastic glomerulopathies. Membranous nephropathy (MN), a major cause of adult-onset nephrotic syndrome (NS), is the best known of these conditions [1]. Lefaucheur et al. [2] provided epidemiologic evidence of an additional cancer risk in patients with MN (10% of 240 MN patients; standardized incidence ratios of 9.8 for men and 12.3 for women) and showed that age, smoking and the presence of glomerular leukocytic infiltrates strongly increased the likelihood of malignancy in MN patients (more than eight inflammatory cells per glomerulus led to a diagnosis of cancer-associated MN with a specificity of 75% and a sensitivity of 92%). Of particular interest, a complete remission of NS occurred in 6 of 12 patients with a tumor in remission, but in none of the 12 patients whose tumor was not in remission [2]. Relationships between cancer and other glomerular...
diseases including IgA nephropathy (prevalence of cancer: 3% [4]), minimal-change disease and membranoproliferative glomerulonephritis have also been documented [1]. We have recently reported a case of rapidly progressive glomerulonephritis that showed marked improvement in renal function after rectal cancer resection [3]. Increased risk of malignancies among patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis and Henoch–Schönlein purpura, which often presents as a secondary glomerular disease, has also been shown, with relative risks of 6.02 and 5.25, respectively [5]. These clinical data indicate that paraneoplastic glomerulopathies should be viewed as a distinct clinical entity, but these disorders remain as a poorly understood aspect of nephropathy.

The pathophysiologic links between glomerulopathy and cancer have been sought for many years, with a focus on factors such as tumoral antigens [6] and specific antibodies [7] in clinical samples. However, a definite proof of the pathogenic nature of these factors has not been obtained [2]. Previous studies have been limited by the small number of available clinical samples and by the lack of an animal model for cancer-associated glomerulopathy [1]. Therefore, establishment of an experimental animal model would be of particular value for establishing paraneoplastic glomerulopathy as a disease entity and studying the underlying pathogenic mechanisms. In the present study, we show that colon cancer-bearing rats develop features of glomerulopathy, and we propose that this animal model can be used for studies of paraneoplastic glomerulopathy.

Materials and methods

Cell preparation, animals and procedures for cancer injection

Rat colon cancer-RCN-9 cells, which were originally established in inbred F344 rats [8], were obtained from Riken cell bank (Tsukuba, Japan) and maintained in RPMI 1640 medium (Sigma–Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Invitrogen) at 37°C. F344 rats [8], were obtained from Riken cell bank (Tsukuba, Japan) and Rat colon cancer RCN-9 cells, which were originally established in inbred F344 rats [8], were obtained from Riken cell bank (Tsukuba, Japan) and were maintained in RPMI 1640 medium (Sigma–Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Invitrogen) at 37°C. Inbred male F344 rats (F344/N Slc) and T cell-deficient F344 rats (nude rats; F344/Ncl-run) of 6–8 weeks of age were purchased from Charles River Japan (Yokohama, Japan) and CLEA Japan (Tokyo, Japan). After laparotomy under anesthesia by inhalation of isoflurane, cells suspended in warm PBS and tissue samples were fixed in 10% neutral formalin (pH 7.4). For electron microscopy, a small part of the kidney cortex was minced and fixed with 2.5% glutaraldehyde solution containing 2% paraformaldehyde. Paraffin embedding, sectioning and subsequent silver and Masson stains were performed by Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Hematoxylin–eosin and Periodic acid-Schiff stains were performed in our laboratory.

For immunohistochemistry (IHC) against rat IgG, paraffin sections dewaxed with xylene and rehydrated with descending grades of alcohol were blocked with 3% H2O2 for 15 min each, 10% horse serum albumin for 20 min and an avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA) for 10 min each. Subsequently, the sections were incubated with a goat-raised primary antibody (Catalog no. 55740; dilution 1:10 000; MP Biomedicals, Solon, OH) for 90 min, followed by a biotinylated horse anti-goat IgG (dilution 1:500; Vector Laboratories) and horse-radish peroxidase streptavidin (dilution 1:250; Vector Laboratories). Immune complexes were visualized with 3,3’-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan). The sections were then counterstained with hematoxylin solution, washed in running water, dehydrated with ascending grades of alcohol and mounted with Permount (Fisher Scientific, Pittsburgh, PA). The specificity of the primary antibodies was verified using control sections in which the primary antibody was replaced with normal goat IgG (R&D Systems, Minneapolis, MN). All staining procedures were performed at room temperature. The slides were rinsed thrice with PBS between steps. Electron microscopy was used to analyze the representative electron photomicrographs, prepared by Mitsubishi Chemical Medience Corporation (sampling method).

Statistical analysis

The data are expressed as mean values ± SEM. Intergroup comparisons of the maximum urinary protein levels were made by unpaired t-test and those for changes in the rate of BW gain from baseline [7] were evaluated using the Mann–Whitney U-test and those with P < 0.05 were considered to be significant.

Results

Tumor growth in the liver of rats causes proteinuria

As a first step, various numbers of RCN-9 cells (5 × 10^6 to 5 × 10^6) were injected into the ilieocolic vein of F344 rats. Consistent with our previous reports using the RCN-H4 subline of RCN-9 cells [9, 10], a successful tumor engraftment in the liver was achieved (Figure 1A and B). The cancer cell-injected rats presented with abdominal distension due to bloody ascites but were otherwise thin. The extent of tumor growth in the liver depended on the injected cell number. In contrast, direct tumor invasion into the kidney was not evident, even microscopically, in any of the cancer-bearing rats (Figure 1C and D). Marked
proteinuria (up to 173.3 mg/day) emerged 3 weeks after the injection only in rats that received the highest dose (5 × 10^7) of RCN-9 cells (Figure 1E). These results suggest that extrarenal tumor growth causes proteinuria, to some extent in a dose-dependent manner.

Reproducible cancer-induced proteinuria depends on the transplanted cell number

Based on the above results, we injected 5 × 10^7 RCN-9 cells into 10 F344 rats to verify the reproducibility of proteinuria. However, the high mortality was problematic, since six of the rats died within 2 days after cancer injection and the others died before development of proteinuria (one rat was sacrificed at Day 28 because of its moribund condition). This indicates that events such as an acute immune response and tumor thrombolism might occur upon injection of an excessive number of cancer cells. Consequently, we reduced the number of transplanted cells to 1 × 10^7 (20% of the initial level) and extended the observation period to avoid early death before onset of proteinuria. Nevertheless, two rats died on Day 2, even with this modified dose. However, as expected, 9 of the 11 surviving rats that received 1 × 10^7 RCN-9 cells showed elevation of urinary protein levels up to 158.0 mg/day from the fourth week post-injection, whereas there was little change in the urinary protein level in the six control rats that received an injection of vehicle (PBS) (Figure 2A). The maximum urinary protein levels in cancer-bearing rats after Week 4 were significantly higher than those in control rats (60.8 ± 12.9 versus 17.8 ± 2.1 mg/day, P = 0.0291). We also subcutaneously injected 1 × 10^5 RCN-9 cells (1% of the proteinuric-inducible level) into another five F344 rats before the ileocolic injection with the goal of achieving a booster effect mimicking other kinds of experimental nephritis, but augmentation of the urinary protein level was not observed (data not shown). These findings demonstrated that reproducible proteinuria with RCN-9 colon cancer is dependent on the cell number injected into rats.

Relationships between the renal function and the overt proteinuria in the cancer-bearing rats

The cancer-bearing rats became debilitated over time, with %BW becoming significantly lower after Week 4 compared with control rats (Figure 2B; Week 2: 22.4 ± 3.4% versus 23.6 ± 6.0%, not significant (n.s.); Week 4: 27.7 ± 3.0% versus 49.1 ± 9.6%, P = 0.0177; Week 6: 31.6 ± 2.5% versus 72.3 ± 14.8%, P = 0.0085). Although there was little difference in the serum Cr levels (starting: 0.21 ± 0.01 mg/dL, n.s.; Week 2: 0.25 ± 0.01 versus 0.25 ± 0.01 mg/dL, n.s.; Week 4: 0.27 ± 0.22 versus 0.24 ± 0.01 mg/dL, n.s.; Week 6: 0.27 ± 0.02 versus 0.26 ± 0.01 mg/dL, n.s.), these rats also showed a significant decline in CCr compared to controls (Figure 2C; starting: 1.78 ± 0.14 mL/min, P = 0.0177; Week 2: 1.83 ± 0.18 versus 2.12 ± 0.15 mL/min, n.s.; Week 4: 1.78 ± 0.20 versus 2.46 ± 0.14 mL/min, P = 0.0343 and Week 6: 1.24 ± 0.22 versus 2.36 ± 0.16 mL/min, P = 0.0022). However, we noted that the decline of CCr did not precede overt proteinuria in all rats, as shown in Figure 2D.

Inflammatory responses accompanied by anemia in the cancer-bearing rats

The cancer-bearing rats showed an elevation in the white blood cell (WBC) count (n = 6; 22 033 ± 3175 versus 8160 ± 748, P = 0.0037), suggesting that the tumor growth simultaneously causes systemic inflammation. These rats also developed anemia (n = 6; hemoglobin levels: 9.3 ± 1.2 versus 15.4 ± 0.4 g/dL, P = 0.0018).

Histological alterations of glomeruli in the cancer-induced proteinuric rats

We then addressed light microscopic changes in glomeruli of the cancer-induced proteinuric rats (Figure 3A–D). Light microscopy with Periodic acid-Schiff (Figure 3A and B),
Masson (Figure 3C) and Silver (Figure 3D) stains did not reveal significant morphological changes in the cancer-induced proteinuric rats from which kidneys could be harvested (n = 6) compared with the controls (n = 6). Cellular proliferation, deposits, thickening of the glomerular basement membrane (GBM) and mesangial expansion were not evident in the kidneys of proteinuric rats. Thus, we concluded that morphological changes were less observed in glomeruli of the cancer-induced proteinuric rats at the light microscopic level.

We next examined whether immunological substances might be deposited in glomeruli of the cancer-induced proteinuric rats from which kidney specimens were available (n = 6). Diffuse and global staining was strikingly found in glomerular tufts by IHC against rat IgG in cancer-bearing rats (Figure 3E and F). In contrast, only scattered and weak staining was observed in controls (n = 6) (Figure 3G and H). Negative control specimens treated with normal goat IgG in place of the primary antibody showed no staining (data not shown). We further examined ultrastructure of glomeruli of the cancer-induced proteinuric rats (Figure 4). Along with immunohistochemical findings, scattered subendothelial electron-dense deposits (EDDs) were observed in the GBM zone of proteinuric rats (Figure 4). Thickening of the GBM was not evident. Also, effacement of the podocytic foot process was occasionally seen. Taken together, immunological substances might be involved in the development of the cancer-induced glomerulopathy.

Development of cancer-induced proteinuria needs T cell-mediated immune responses

To verify the involvement of a potential immune response in development of cancer-induced proteinuria, we injected $1 \times 10^7$ RCN-9 cells into seven nude rats via the ileocolic vein. The cancer-bearing nude rats showed wasting and the follow-up times were a little shorter than those of the immune-
competent F344 rats (one rat, up to the sixth week; four rats, up to the fifth week; two rats, up to the fourth week). There was little change in urinary protein levels of the nude rats compared to the peak level in immune-competent cancer-bearing F344 rats (mean values: 5.9/6.12 versus 60.8/6.12 mg/day, $P = 0.0041$; Figure 5A), and there were no morphological abnormalities (Figure 5B) or IgG deposition (Figure 5C) in the kidneys of the nude rats.

**Discussion**

In this study, rats injected with cancer cells showed features of glomerulopathy including marked proteinuria, IgG deposition and EDDs in the glomerular tufts, while lacking evidence of direct tumor invasion into the kidney. A similar injection in T cell-deficient rats did not produce these characteristics. In the initial experiments, only the highest dose of RCN-9 cells ($5 \times 10^7$) caused significant proteinuria in a single rat at Week 3 after the injection, indicating a dose dependency between cancer growth and development of proteinuria. This rat had a urinary protein level of 173.3 mg/day, which is comparable to that in other established nephritic models [11, 12] and strongly suggests the feasibility of establishment of an animal model for paraneoplastic research. However, in subsequent experiments, this dose was found to be unsatisfactory due to high mortality (a conflict between dose dependency and mortality). Therefore, we reduced the cell number and extended the observation time, achieving reproducible proteinuria in the cancer-injected rats.

The results raise the question of the mechanism of development of proteinuria without direct tumor invasion into the kidneys in the cancer-bearing rats. It has been reported that experimental animals with cancer develop cachexia, a complex wasting syndrome [13–16]. The cancer-bearing rats showed significant declines in %BW and CCr over time (Figure 2B and C), but some proteinuric rats maintained renal function (Figure 2D), indicating that the proteinuria was not simply a secondary result of renal impairment associated with decreased blood flow. Also, various proinflammatory cytokines including tumor necrosis factor (TNF) [14] and interleukin-1 (IL-1) [16], and especially those derived from tumors [15], are important in the induction and promotion development of experimental cancer cachexia. These factors are also widely accepted as potent modulators in models of experimental autoimmune glomerulonephritis (EAG) [17–19]. For example, the pretreatment with recombinant TNF and IL-1 exacerbates the glomerular injury [17], whereas the administration of an IL-1 receptor antagonist attenuated the severity of this injury via inhibition of intercellular adhesion molecules such as CD54, CD11a and CD18 [18]. Thus, the elevation of WBC counts in the cancer cell-injected rats indicates that tumor-derived inflammatory activators may be capable of participating in renal injury via a nonspecific immune response. On the other hand, most experimental models of EAG require specific immune responses, including antigen–antibody reactions [20–22], antigen...
presentation in association with a co-stimulatory pathway [23, 24] and T cells positive for CD4 [25]. Neither IL-1 nor TNF produce clinical, morphologic or biochemical evidence of renal toxicity when given alone, except for a transient increase in the number of glomerular neutrophils observed in one study of EAG [17]. In our study, abundant IgG deposition was shown by IHC in the glomerular tufts of proteinuric rats (Figure 3E and F), despite little morphological change. Ultrastructure analysis revealed EDDs in the GBM zone (Figure 4), as also observed in an EAG model produced by immunological substances [26]; strictly, we noted that there is a difference in localization since our model is sub-endothelial, while the EAG model was sub-epithelial. Taken together, these results suggest that cancer-induced proteinuria is caused by a specific immune response. In support of this hypothesis, T cell-deficient rats that received \(1 \times 10^7\) RCN-9 cells showed no increase in urinary protein levels in Week 5, when most immune-competent F344 rats had developed proteinuria (Figure 5A), despite marked cancer growth in the T cell-deficient rats. A complete lack of IgG staining in the kidneys (Figure 5C) also suggested that deletion of the cellular and humoral immune system prevented the development of nephropathy.

A number of our findings are difficult to interpret, including the unusual localization of EDDs and the difference in the degrees of EDDs and IgG deposition observed by IHC. Interestingly, our histological findings closely resemble those observed in cancer patients by Pascal et al. [27]. These observations included electron-dense sub-endothelial deposits and positive immunofluorescent reactions for immunoglobulin (IgG dominant) in kidneys of 29 patients without clinical renal disease, indicating that cancer patients latently develop such unusual features in the kidneys. No sub-endothelial deposits were seen by electron microscopy and there was no correlation between electron microscopic and immunofluorescence findings [27]. In addition, we also observed distinctive features in our clinical experience with paraneoplastic glomerulopathy in a patient completely lacking circulating ANCA [3], whereas as many as 80–90% of patients with pauci-immune crescentic glomerulonephritis were positive [28]. These differences from primary renal diseases might be clues to the pathogenesis of paraneoplastic glomerulopathies.

In conclusion, we demonstrated that tumor-bearing rats developed features of glomerulopathies and that this animal model has the potential to provide new insights into paraneoplastic nephropathy.

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