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A role for extrarenal cells in the regeneration following acute renal failure

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A role for extrarenal cells in the regeneration following acute renal failure.

Background. Recovery of renal function following acute tubular necrosis (ATN) is dependent on the replacement of necrotic tubular cells with functional tubular epithelium. The source of these new tubular cells is thought to be resident renal tubular cells. The discovery of pluripotent bone marrow-derived stem cells has led to a reexamination of the cellular source and processes involved in the recovery from organ injury.

Methods. To test the hypothesis in humans that extrarenal cells participate in the recovery following ATN, we examined the origin of tubular cells in male patients with resolving ATN who had received a kidney transplant from a female donor. Immunohistochmistry of kidney biopsies was performed to identify renal tubular epithelial cells (cytokeratin positive) and leukocytes (CD45 positive). Fluorescent in-situ hybridization was used to detect Y chromosome containing cells with DAPI serving as a nuclear stain. All staining was performed on the same section.

Results. The Y chromosome was detected in approximately 40% of tubular cell nuclei in male kidneys (positive control) and in no nuclei of female kidneys (negative control). In male recipients of female kidneys who developed ATN, 1% of tubules contained Y chromosome cells defined by their morphology, positive staining for cytokeratin, and negative staining for CD45. When present, multiple cells in a positive tubule stained for the Y chromosome. No Y chromosome containing tubular cells were seen in similar sex mismatched transplants in male recipients who did not develop ATN, suggesting that recipient derived cells do not routinely repopulate the transplanted kidney.

Conclusions. This proof-of-principle clinical observation demonstrates that extrarenal cells can participate in the regenerative response following ATN. These findings provide rationale for the cellular therapy of acute renal failure.

Key words: stem cell, regeneration, acute tubular necrosis, tubular epithelium, transplantation, cellular therapy.

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Nephrotoxic and ischemic insults to the kidney lead to acute renal failure and most often manifest as acute tubular necrosis. Recovery of renal function following acute renal failure is dependent on the replacement of necrotic tubular cells with functional tubular epithelium. The source of these new tubular cells is thought to be resident renal tubular cells. Recapitulating developmental paradigms, these cells dedifferentiate, proliferate, and eventually reline denuded tubules restoring the structural and functional integrity of the kidney [1–5]. The molecular events defining this renal regeneration have been well characterized and strategies to accelerate the repair process tested in both experimental models and in humans [1–6].

The discovery of bone marrow-derived stem cells that possess the ability to differentiate into different cell lineages has led to a reexamination of the cellular source and processes involved in recovery from organ injury [7–23]. We hypothesized that non-renal cells, likely of bone marrow origin, participate in the tubular regeneration following acute tubular necrosis (ATN). To test this hypothesis in humans, we examined the origin of tubular cells in male patients with resolving acute tubular necrosis who had received a kidney transplant from a female donor. This strategy has been successfully used to demonstrate repair of injured liver by extrahepatic cells, to define the origin of mesenchymal cells in patients with chronic renal transplant rejection, to demonstrate chimerism of the transplanted human heart, and to prove that blood-derived stem cells could differentiate into liver, skin and gastrointestinal tract cells [20, 21, 24–26].

METHODS

Patients

Frozen kidney tissue from archived samples of kidney biopsies were obtained from a male patient who received a kidney transplant from a male donor and developed acute rejection (positive control); a female patient with minimal change disease (negative control); two male re-

cipients of female kidneys who had developed acute tubular necrosis (ATN) following transplantation; and two male recipients of female kidneys who underwent renal biopsy for elevated serum creatinine but did not have biopsy evidence for ATN. Approval for this study was granted by the Human Subjects Committee at the University of Minnesota.

Staining procedure

The general strategy was to use immunohistochemistry to identify renal tubular epithelial cells (cytokeratin positive) and leukocytes (CD45 positive); fluorescent in-situ hybridization (FISH) to identify Y chromosome containing cells; and 4′6-diamidino-2-phenylindole dihydro chloride (DAPI) as a nuclear stain. All staining was performed on the same section. Five-micrometer (μm) sections were placed on plain glass slides and stored at −70°C. Preliminary studies demonstrated that the protease digestion and heating required for FISH resulted in loss of the CD45 epitope. To circumvent this problem, sections were first stained for cytokeratin and CD45, cover slipped, and images captured. The cover slips were then removed and FISH performed. For the technical reasons outlined above, CD45 staining was not available for all sections.

Immunohistochemistry for cytokeratin and CD45. The sections were fixed in acetone for 10 minutes, and washed three times in phosphate buffered saline (PBS). Fifty microliters (μL) of R-phycoerythrin-cy5 (RPE-cy5) mouse monoclonal anti-human CD45, clone T29/33 (cat# C7099; Dako, Carpenteria, CA, USA) at 1:25 dilution was applied to the tissue, and incubated for 90 minutes in humidified chamber in dark at room temperature. Sections were then washed in PBS and incubated for 90 minutes with 50 μL of fluorescein isothiocyanate (FITC)-labeled mouse monoclonal anti-human cytokeratin peptide 18, clone CY-90 (Cat# F4772; Sigma Chemical Co., St. Louis, MO, USA) at a 1:25 dilution. Sections were washed in PBS and one drop of Gel/Mount™ (cat# M01; Biomeda Corp, Foster City, CA, USA) was placed on the sections followed by cover slips. Pictures were taken immediately as described below. After obtaining the pictures, the slides were immediately placed in 1× PBS at room temperature and processed for FISH.

Fluorescent in situ hybridization (FISH). The sections were dehydrated by sequentially placing the slides for three minutes in 70%, 85%, 90% and 100% ethanol. Sections were then air dried and incubated in 2 × SSC (sodium chloride/sodium citrate) at 70°C for ten minutes. The tissue was denatured in 70% formamide/2 × SSC at 75°C for five minutes followed by the dehydration protocol described above. To detect the Y chromosome, a Spectrum Orange™ fluorophore labeled, CEP Y (satellite III) DNA probe (cat# 32-130024; Vysis Inc., Downers Grove, IL, USA), which hybridizes to satellite III sequence of the Y chromosome (band Yq12, locus DYZ1) was used. The probe was denatured at 75°C for five minutes, the sections hybridized at 42°C for 90 minutes, followed by immersion in 4 × SSC/0.3% NP-40 at 75°C for two minutes. The slides were then washed at room temperature in 2 × SSC/0.1% NP-40 for 90 seconds followed by three three-minute washes in PBS. To stain nuclei, the sections were incubated for 30 minutes at room temperature with 50 μL of 30 nmol/L DAPI (cat# D-1306; Molecular Probe, Eugene, OR, USA) followed by three PBS washes. One drop of Gel/Mount™ was applied and sections were cover slipped.

Image analysis

Images for each fluorophore were sequentially captured at ×100, ×200 and/or ×400 magnifications using a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss Inc., Jena, Germany). For cytokeratin, the FITC signal was excited at 500 nm and emission collected at 525 nm. Positive staining appeared green. The signal for CD45 was detected using a RPE-cy5 labeled probe excited at 488 nm and collected at 670 nm resulting in a red color. The Spectrum Orange™ signal of the Y chromosome was excited at 559 and collected at 588 nm and appeared reddish orange. Finally DAPI signals were excited at 358 nm and collected at 461 nm. The sequentially captured images were digitally combined using Adobe Photoshop version 6.0.1 (San Jose, CA, USA).

RESULTS

Patient characteristics are displayed in Table 1. As a positive control, we examined the kidney of a male patient with moderate acute tubulointerstitial rejection that developed 40 days after he received a transplant from a male donor. The creatinine at the time of the biopsy was 3.1 mg/dL (274 μmol/L). The Y chromosome signal was detected in approximately 40% of tubular epithelial cell nuclei due to partial sampling related to the 5 μm sections missing some portions of the nucleus (Fig. 1A). This result is consistent with what other investigators have found in liver, kidney, heart and skin [20, 24–26]. As expected the Y chromosome signal was also detected in interstitial cells. No Y chromosome signal was detected in the female kidney of a patient with minimal change glomerulonephritis that was used as a negative control (Fig. 1B).

Two male patients who had received female kidneys were studied. The first presented with an elevated serum creatinine 12 months following transplantation. At the time of the biopsy the serum creatinine was 2.7 mg/dL (239 μmol/L), which was elevated from a baseline value of 1.7 mg/dL (150 μmol/L). The etiology of his acute renal failure was unclear. A renal biopsy revealed resolving acute tubular necrosis. Numerous Y chromosome

Table 1. Patient information

Specimen	Age *years*	Days from transplant to biopsy	Creatinine *mg/dL*	Pathologic diagnosis
1 (Positive control)	36	40	3.1	Moderate acute tubulointerstitial rejection
2 (Negative control)	14	NA	0.9	Minimal change disease
3	36	10	9.3	Resolving ATN with mild acute tubulointerstitial rejection
4	43	365	2.6	Resolving ATN
5	50	67	1.9	No pathologic diagnosis
6	42	515	4.9	Mild chronic rejection

Abbreviations are: NA, not applicable; ATN, acute tubular necrosis. To convert creatinine to SI units (μmol/L) multiply by 88.4.

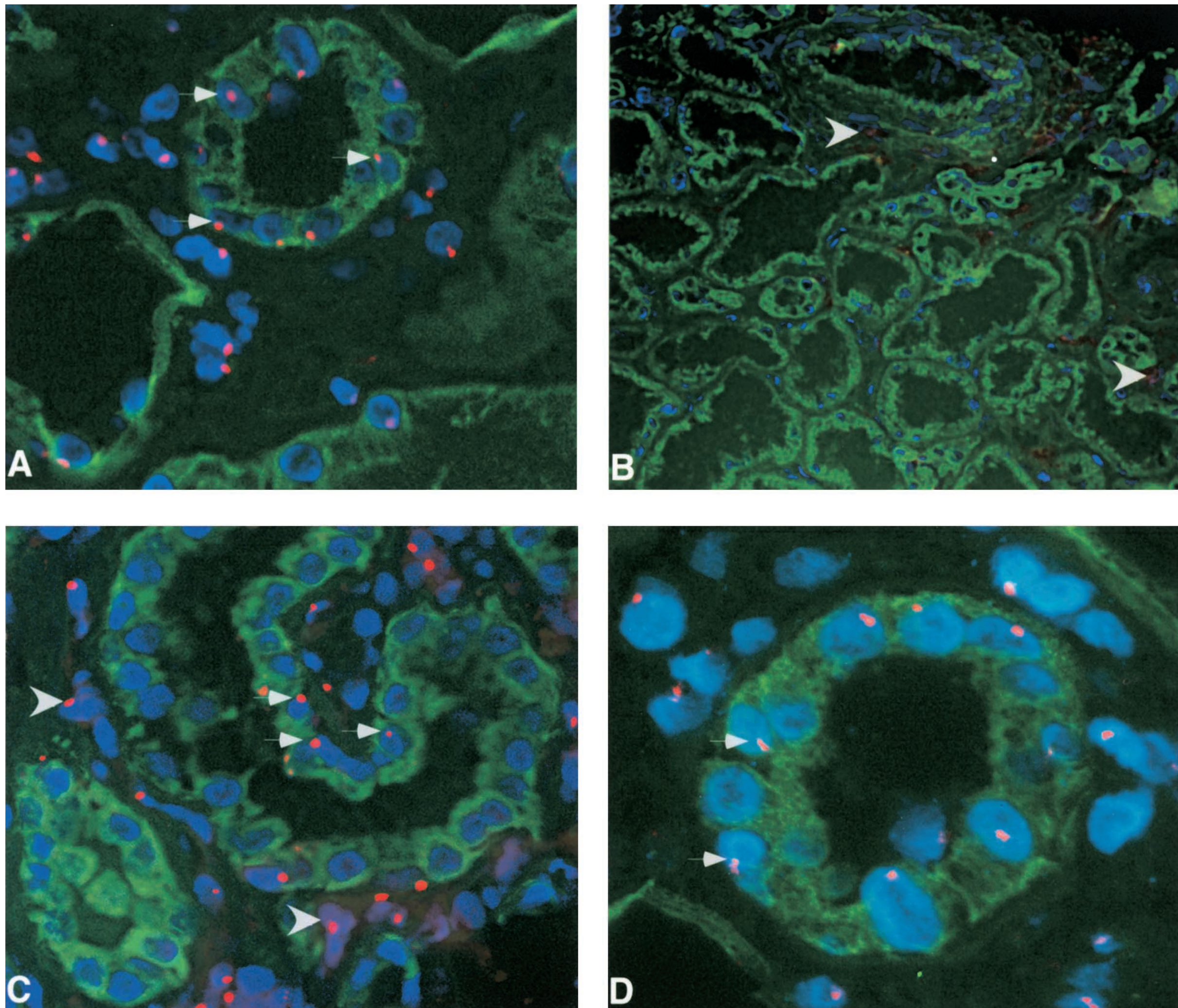

Fig. 1. Immunofluorescent photomicrographs of kidney biopsies. (*A*) Male recipient and male donor (positive control) with moderate tubulointerstitial rejection. Both tubular cells (green) and interstitial cells (unstained) contain the Y chromosome signal (red dot; arrows) in nuclei. Magnification ×200. (*B*) Female patient with minimal change disease (negative control). No Y chromosomes are seen. Some CD45 positive cells are seen in the interstitium (arrowheads). Magnification ×100. (*C*) Male recipient of female kidney with resolving acute tubular necrosis (ATN). Multiple cells in this tubule contain Y chromosomes (arrows). Some interstitial cells also contain Y chromosomes and stain positive for CD45 (red staining; arrowhead). Magnification ×200. (*D*) Male recipient of female kidney with resolving ATN and superimposed mild acute tubulointerstitial rejection. Multiple cells in this tubule contain Y chromosomes (arrows). Some interstitial cells also contain Y chromosomes. Magnification ×400.

containing renal tubular cells were seen, defined by their morphology, positive staining for cytokeratin, and negative staining for CD45 (Fig. 1C). Some Y chromosome positive/CD45 positive cells were seen in the interstitium consistent with mild renal inflammation. The second male recipient was biopsied ten days following a transplant from a female donor that was complicated by delayed graft function. The serum creatinine at the time of biopsy was 9.3 mg/dL (822 μmol/L). The biopsy showed resolving ATN with superimposed mild acute tubulo-interstitial rejection. Y chromosome signal was detected in renal tubular epithelial cells. An example is shown in Figure 1D, where multiple cytokeratin positive cells in a renal tubule contained a Y chromosome, providing evidence that these tubular cells were derived from the male recipient. Although this particular section was not co-stained with CD45, cells in the interstitium also contained the Y chromosome signal.

In both patients the presence of Y chromosome containing tubular cells was a rare event, occurring in less than 1% of renal tubules. When present, multiple cells in a positive tubule stained for the Y chromosome as the examples in Figure 1 C and D illustrate.

As additional controls, we examined the biopsies of two male patients who had received female kidneys but had no evidence for ATN on biopsy. The diagnosis in Patient 5 was dehydration combined with cyclosporine nephrotoxicity. Patient 6 had clinical evidence for dehydration and mild chronic rejection on biopsy. No Y chromosome containing renal tubular cells were seen in either of these biopsies, suggesting that recipient derived cells do not routinely repopulate the transplanted kidney in the absence of ATN.

DISCUSSION

The presence of Y chromosome containing tubular epithelial cells in female kidneys of male recipients with resolving ATN supports the hypothesis that extrarenal cells can participate in the regeneration following acute renal failure. Evidence that these cells were tubular and not infiltrating white cells was their tubular morphology, positive staining for cytokeratin, and negative staining for CD45, a marker for white cells. This finding of male tubular cells in a female kidney was a rare event, occurring in approximately 1% of tubules. The relative scarcity of these cells is consistent with what has been seen following liver injury, where male cells were seen in 0.5 to 4% of liver cells [20, 21]. Nonetheless, the presence of these Y chromosome containing tubular cells is an important proof-of-principle that extrarenal cells can repopulate the injured kidney.

Our results confirm the findings of Poulsom and colleagues who demonstrated Y chromosome positive tubular cells comprised 0.6 to 6.8% of tubular cells in biopsies of eight sex-mismatched transplants with a variety of pathologies [27]. Most of the patients in that study had more than one biopsy, all of which showed persistence of Y chromosome positive tubular cells. A limitation of that study was the lack of staining for white cell markers. A similar strategy of sex-mismatched transplants was used to define the origin of mesenchymal cells in renal biopsies of patients with chronic rejection [24]. Mesenchymal cells derived from the recipient were found in the vascular and interstitial compartments of these kidneys. Interestingly, these investigators detected Y chromosome cells "within the confines" of the tubular basement membrane in 4% of tubules of female kidneys transplanted into male recipients. These were felt to be inflammatory cells although no tubular or leukocyte markers were used. Since these kidneys undergo tubular injury, the Y chromosome positive cells could be recipient-derived non-renal cells that differentiate into tubules similar to the results of our study.

The origin of these recipient-derived tubular cells is unclear. Possible sources include the bone marrow, circulating stem cells, or other tissues such as the liver, spleen, lymph nodes, or native kidneys. We speculate the cells arose from pluripotent bone marrow stem cells that either take up residence in the kidney prior to injury and function as tissue stem cells, or are recruited to the kidney at the time of injury [28, 29]. The ability of bone marrow derived stem cells to differentiate into other cell lineages has been demonstrated both experimentally and in humans. For example, in mice and rats bone marrow-derived cells can differentiate into hepatocytes and/or cholangiocytes following hepatic injury and have been used to treat experimental liver disease [7–9]. Bone marrow-derived cells also have been demonstrated to differentiate into skeletal muscle, cardiomyocytes, blood vessels, brain, bone and mesenchyme [10–18, 22, 23, 25]. More recently, Krause and colleagues have shown that a single bone marrow-derived cell can differentiate into cells of the liver, lung, GI tract and skin [19]. The plasticity of adult cells is further illustrated by the ability of skeletal muscle to turn into bone marrow, brain to turn to blood, and adult cells have been used to clone whole mammals [30–33].

The kidney possesses the ability to regenerate following ischemic or nephrotoxic injury. This process of self-renewal restores kidney function in most cases. Although a population of renal stem cells capable of clonal expansion and differentiation into tubules has been demonstrated in vitro [34], it is generally held that the source for regenerating cells are many if not all of the less injured tubular cells [1–5]. These cells undergo dedifferentiation, proliferation and redifferentiation into cells that replace the lost necrotic and/or apoptotic cells that had lined the tubules prior to injury [4]. Our findings that extrarenal

cells also participate in the renal regenerative response are consistent with these well-described cellular events of dedifferentiation, proliferation and redifferentiation with the starting point being a dedifferentiated cell. The relative contributions of renal versus extrarenal cells remains to be determined as does the mechanism whereby these cell home to the injured kidney.

Renal recovery following ATN is often a slow process requiring many days to weeks to occur. During this time patients are dependent on dialysis support. Attempts to accelerate recovery have focused on the administration of growth factors such as epidermal growth factor, hepatocyte growth factor, or insulin-like growth factor-1 [35, 36]. While growth factor therapy has been successful in animal models, no beneficial effects have been observed in limited clinical trials [37, 38]. The ability of extrarenal cells to participate in the regenerative response following post-transplant acute renal failure may hold true for acute renal failure that develops in native kidneys. This concept raises the possibility that autologous adult-derived bone marrow cells, or cells from other tissue sources, could be administered and enhance the recovery from renal injury. It also may be possible to engineer these cells to deliver specific factors to the site of renal injury to aid in the regenerative response.

In conclusion, we have demonstrated the presence of male recipient-derived renal tubular cells in female kidneys of two patients with histologically-proven resolving acute tubular necrosis. This proof-of-principle clinical observation supports a role for extrarenal cells in the regenerative response following acute renal failure.

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