

NEOINTIMAL AND TUBULOINTERSTITIAL INFILTRATION BY RECIPIENT MESENCHYMAL CELLS IN CHRONIC RENAL-ALLOGRAFT REJECTION

PAUL C. GRIMM, M.D., PETER NICKERSON, M.D., JOHN JEFFERY, M.D., RASHMIN C. SAVANI, M.B., CH.B., JAMES GOUGH, M.D., RACHEL M. MCKENNA, PH.D., ELZBIETA STERN, M.Sc., AND DAVID N. RUSH, M.D.

ABSTRACT

Background Tissue remodeling depends on mesenchymal cells (fibroblasts and myofibroblasts) and is a prominent feature of chronic renal-transplant rejection. It is not known whether the mesenchymal cells that participate in remodeling originate locally or from circulating precursor cells.

Methods We obtained biopsy specimens of renal allografts from six male recipients of an allograft from a female donor, four female recipients of an allograft from a male donor, two male recipients of an allograft from a male donor, and two female recipients of an allograft from a female donor. All the allografts were undergoing chronic rejection. We used immunohistochemical methods to identify mesenchymal cells with smooth-muscle α -actin and in situ hybridization to identify mesenchymal cells with Y-chromosome DNA.

Results No Y-chromosome bodies were identified in the case of the two renal-allograft specimens in which both the donor and the recipient were female. In the case of the two renal-allograft specimens in which both the donor and the recipient were male, approximately 40 percent of mesenchymal cells contained a Y-chromosome body. In the case of the six specimens in which the donor was female and the recipient was male, a mean (\pm SD) of 34 ± 16 percent of mesenchymal cells in the neointima, 38 ± 12 percent of such cells in the adventitia, and 30 ± 7 percent of such cells in the interstitium contained the Y-chromosomal marker, indicating that they originated from the recipient rather than the donor. In the case of the four renal-allograft specimens in which the donor was male and the recipient was female, the respective values were 24 ± 15 percent, 33 ± 9 percent, and 23 ± 8 percent, indicating a persistent population of donor mesenchymal cells.

Conclusions The presence of mesenchymal cells of host origin in the vascular and interstitial compartments of renal allografts undergoing chronic rejection provides evidence that a circulating mesenchymal precursor cell has the potential to migrate to areas of inflammation. (N Engl J Med 2001;345:93-7.)

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TISSUE remodeling is central to the pathogenesis of many chronic illnesses. In the vascular narrowing of atherosclerosis,¹ the proliferation of smooth-muscle cells and the synthesis of matrix lead to the formation of a neointima. The same events in the adventitia² cause a constrictive fibrosis that prevents the vessel from dilating

in response to the encroachment of the neointima into the vascular lumen.¹ Tissue remodeling is also important in chronic allograft rejection, which is the chief limitation to the long-term success of organ transplantation. The principal lesions in chronic rejection are vascular fibrointimal hyperplasia and interstitial fibrosis.³ The mesenchymal cells that synthesize matrix in the vascular neointima are smooth-muscle cells, whereas in the interstitium they are myofibroblasts.⁴⁻⁶

It is commonly accepted that in allograft rejection the mononuclear inflammatory cells derive from the recipient,⁷ whereas the mesenchymal cells that participate in remodeling originate in the graft itself. However, it has been shown that collagen vascular grafts are rapidly infiltrated and remodeled by smooth-muscle cells into physiologically responsive neovessels, suggesting that circulating smooth-muscle cells (or their mesenchymal precursors) migrate to areas of tissue remodeling and take part in the process.⁸ Moreover, studies in animal models⁹⁻¹¹ indicate that mesenchymal cells involved in chronic rejection may originate from the recipient.

To investigate the origin of mesenchymal cells in tissue remodeling, we studied the infiltrating mesenchymal cells in sex-mismatched renal allografts that were undergoing chronic rejection. We used combined immunohistochemical techniques to identify mesenchymal cells with smooth-muscle α -actin and in situ hybridization to determine whether Y-chromosome DNA was present in biopsy specimens of renal allografts.

METHODS**Patients and Biopsies**

Our group routinely performs renal-allograft biopsies 1, 2, 3, 6, and 12 months after transplantation; the characteristics of 76 patients who were studied under this protocol have been described previously.^{12,13} All patients received cyclosporine, azathioprine, and prednisone for immunosuppression after renal transplantation and were in clinically stable condition at the time of biopsy. The biopsy protocol was approved by the faculty committee on the use of human subjects in research at the University of Manitoba, and all patients gave written informed consent to participate.

Changes in the tubules, interstitium, glomeruli, and vessels were

From the Department of Pediatrics, University of California at San Diego, San Diego (P.C.G.); the Departments of Internal Medicine (P.N., J.J., R.M.M., E.S., D.N.R.) and Pathology (J.G.), University of Manitoba, Winnipeg, Canada; and the Department of Pediatrics, University of Pennsylvania, Philadelphia (R.C.S.). Address reprint requests to Dr. Grimm at the Department of Pediatrics, UCSD, 9500 Gilman Dr., MC 0831, La Jolla, CA 92093-0831, or at pgrimm@ucsd.edu.

assessed by a pathologist who was unaware of the clinical condition of the patients. The pathologist assigned semiquantitative scores for these changes using the standardized Banff classification.¹⁴ The Banff criteria have accepted validity and reproducibility and are widely used in the histologic analysis of transplants. We scanned our data base of renal biopsies and selected samples from all sex-mismatched grafts with a Banff grade of CV2 (indicating the presence of chronic rejection with a moderately vascular neointima) or CV3 (indicating the presence of chronic rejection with severe vascular changes, including narrowing of the vascular lumen by more than 50 percent) and adequate tissue available for study. We identified such biopsy specimens from six male recipients who had received an allograft from a female donor and four female recipients who had received an allograft from a male donor.

We then selected biopsy specimens from two female recipients of an allograft from a female donor as a negative control and biopsy specimens from two male recipients of an allograft from a male donor as a positive control. As an additional control, we studied nine sex-mismatched biopsy specimens with no evidence of rejection (from five male recipients of an allograft from a female donor and four female recipients of an allograft from a male donor). In no case did we study biopsy specimens that had been obtained from a single patient at more than one time.

Combined Immunohistochemical Analysis and in Situ Hybridization

We performed all immunohistochemical procedures using the Microprobe apparatus (Fisher Scientific) with semiautomated capillary staining to ensure that the results of staining were consistent and reproducible. Formalin-fixed, paraffin-embedded blocks were cut into sections that were 4 μ m thick, and the sections were placed on microscope slides (ProbeOn Plus, Fisher Scientific) and incubated. Immunohistochemical analysis with use of an antibody against smooth-muscle α -actin (M0851, Dako) as a marker for smooth-muscle cells¹⁵ was performed essentially as described previously.¹⁶ DNA in situ hybridization for the human Y chromosome was immediately performed with use of pHY2.1, a Y-chromosome repeat marker labeled with digoxigenin (1558196, Boehringer Mannheim). The pHY2.1 fragment hybridizes with a 2000-copy tandem repeat on the long arm of the Y chromosome.¹⁷ The slides were dehydrated in ethanol, air dried, then incubated with hybridization solution containing 2 \times saline sodium citrate (1 \times saline sodium citrate is 0.15 M sodium chloride and 0.015 M sodium citrate), 50 percent formamide, 10 percent dextran sulfate, 1 mg of salmon-sperm DNA per milliliter, and 1 μ g of digoxigenin-labeled probe per milliliter. Denaturation for 10 minutes at 72°C was followed by incubation overnight at 37°C. The following morning, unbound probe was washed off with 2 \times saline sodium citrate at 37°C. Subsequent detection steps were performed as described previously.¹⁸ Nuclei were counterstained with methyl green, then the slides were cover-slipped with light mineral oil in order to preserve both solvent-soluble and water-soluble components.

Statistical Analysis

Each blue-stained nucleus was interpreted as indicating a mesenchymal cell (myofibroblast or smooth-muscle cell) if it was surrounded by red-stained smooth-muscle α -actin. Staining for the Y chromosome was considered to be positive if a clear dark blue-black signal in the nucleus could be detected. The analysis was performed by an observer who was unaware of the patients' histories. The presence or absence of smooth-muscle α -actin and the Y chromosome was recorded for each nucleus counted in the neointima, perivascular adventitia, and peritubular interstitium, for a total of up to 1000 cells. Differences between groups were analyzed with use of a two-tailed unpaired t-test.

RESULTS

We studied 14 renal-biopsy specimens with the typical vascular and interstitial changes of chronic re-

TABLE 1. PERCENTAGE OF MESENCHYMAL CELLS THAT WERE POSITIVE FOR THE Y-CHROMOSOME BODY.*

SEX OF DONOR	SEX OF RECIPIENT	NO. OF SPECIMENS	NEO-INTIMA	ADVENTITIA	INTERSTITIUM	TUBULES
percentage of cells						
Female	Female	2	0	0	0	0
Male	Male	2	38	31	43	41
Male	Female	4	24 \pm 15	33 \pm 9	23 \pm 8	44 \pm 13
Female	Male	6	34 \pm 16	38 \pm 12	30 \pm 7	4 \pm 2†

*Plus-minus values are means \pm SD.

†This value probably represents infiltration by the recipients' mononuclear cells.

jection. Two specimens each were from female recipients of an allograft from a female donor and from male recipients of an allograft from a male donor, four specimens were from female recipients of an allograft from a male donor, and six specimens from male recipients of an allograft from a female donor. The underlying reason or reasons for transplantation were diabetes mellitus in six patients, glomerulonephritis in six, polycystic kidney disease in four, hypertensive nephrosclerosis in three, chronic pyelonephritis in two, obstructive uropathy in one, and unknown disease in one.

The results are summarized in Table 1. In renal-allograft specimens from the four female patients with male donors, we found clear evidence of infiltration of the neointima, adventitia, and interstitium by mesenchymal cells of donor (male) origin. These allografts also demonstrated a population of male mesenchymal cells.

The Y-chromosome body was clearly distinguishable in approximately 40 percent of the tubular, interstitial, and neointimal mesenchymal cells in the grafts from male donors that had been transplanted into male recipients (Fig. 1A and Table 1). Although the analysis was performed in a blinded fashion, a kidney-biopsy specimen from a male donor was immediately obvious on cursory inspection of the tubular-cell nuclei. In renal-allograft specimens in which both the donor and the recipient were female, the Y-chromosome body was always absent (Fig. 1B and Table 1).

In cases in which the donor was female and the recipient was male, the presence of Y-chromosome bodies in cells infiltrating the renal-allograft specimen was also obvious. In such cases, a mean (\pm SD) of 34 \pm 16 percent of the neointimal smooth-muscle cells in the renal-allograft specimens clearly expressed the Y-chromosomal signal (Fig. 1C and Table 1). In cases in which the donor was male and the recipient was female, a mean of 24 \pm 15 percent of neointimal smooth-muscle cells in the renal-allograft specimens were of male origin (Fig. 1D and Table 1).

In cases in which the donor was female and the recipient was male, a mean of 38 ± 12 percent of perivascular interstitial smooth-muscle cells in the renal-allograft specimens were of male origin, whereas in cases in which the donor was male and the recipient was female, a mean of 33 ± 9 percent were of male origin (Table 1). In specimens from male recipients of allografts from female donors, the Y-chromosome body was present in a mean of 30 ± 7 percent of the smooth-

muscle cells in the renal interstitium (Fig. 2A and Table 1). In cases in which the donor was male and the recipient was female, a mean of 23 ± 8 percent of mesenchymal cells in the interstitium of the renal-allograft specimens showed the Y-chromosomal signal (Fig. 2B).

In cases in which both the donor and the recipient were male or were female and in cases in which the donor was male and the recipient was female, the tu-

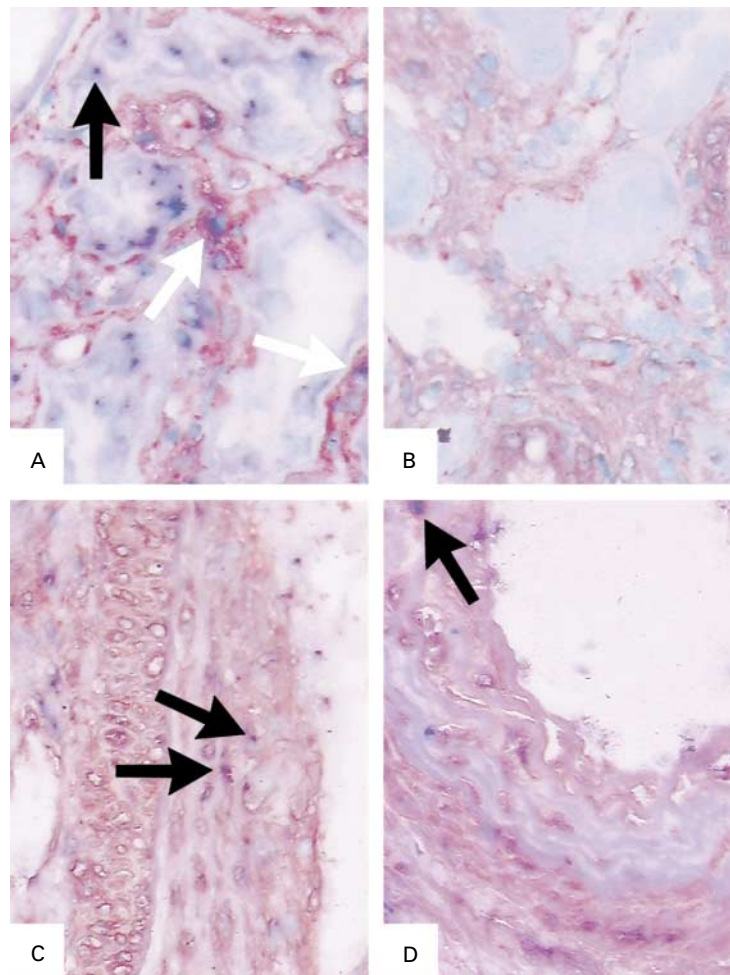


Figure 1. Results of Combined Immunostaining for Smooth-Muscle α -Actin (Red Cytoplasm) and in Situ Hybridization for the Y-Chromosome Body (Black Nuclear Signal) ($\times 325$).

In Panel A, a renal-allograft specimen in which both the donor and the recipient were male shows the Y-chromosome signal in tubular cells (black arrow) and interstitium (white arrows). Some cells did not show Y-chromosome staining because the Y-chromosome body was not included in the section. In Panel B, a renal-allograft specimen in which both the donor and the recipient were female shows no Y-chromosome signal. In Panel C, a renal-allograft specimen in which the donor was female and the recipient was male shows neointimal smooth-muscle cells (stained red), expressing a Y-chromosome signal of host origin (arrows). In Panel D, a renal-allograft specimen in which the donor was male and the recipient was female shows neointimal smooth-muscle cells (stained red), a few of which express a Y-chromosome signal (stained black, arrow).

bular cells in the renal-allograft specimens reflected the sex of the donor. In cases in which the donor was female and the recipient was male, a small number of male cells (4 ± 2 percent) were seen within the confines of the tubular basement membrane in the renal-allograft specimens. These cells were not positive for smooth-muscle α -actin and resembled mononuclear inflammatory cells morphologically. This result probably represents invasion of the tubule by the recipient's immune cells (tubulitis).

In biopsy specimens obtained from patients without chronic rejection, there was no vascular neointima to examine. In cases in which the donor was male and the recipient was female, 30 percent of smooth-muscle cells in the interstitium of the renal-allograft specimens demonstrated a Y-chromosome body. In cases in which the donor was female and the recipient was

male, 10 percent of the smooth-muscle cells in the interstitium of the renal-allograft specimens demonstrated a Y-chromosome body; this value was significantly less than the value found in the corresponding group with chronic rejection (10 percent vs. 30 percent, $P < 0.001$).

DISCUSSION

We found that recipient-derived mesenchymal cells infiltrated the neointima, adventitia, and tubulointerstitial compartments of renal transplants undergoing chronic rejection. These results provide evidence that a circulating mesenchymal cell has the potential to colonize an allograft. This information may lead to the development of ways to prevent and treat chronic rejection.

In chronic renal-allograft rejection, the vascular neointima consists of an infiltrate of lymphoid and mesenchymal (smooth-muscle) cells. As is the case in atherosclerosis in native organs, medial smooth-muscle cells are thought to migrate through breaks in the internal elastic lamina to the neointima.¹⁹ In animal models of aortic and femoral-artery transplants, the neointimal smooth-muscle cells found during chronic rejection of these grafts are of host origin.^{10,11} Our study shows that in humans with chronic rejection, smooth-muscle cells of host origin are also present in the vascular neointima of the renal allograft. Whether these cells derive from precursors that migrate directly into the neointima or initially colonize the adventitia or perivascular interstitium before they migrate through the media to the neointima is unknown.

Another critical lesion in chronic rejection is interstitial fibrosis, the development and progression of which require smooth muscle thought to derive from local sources.^{5,20} Our data show that mesenchymal cells of host origin infiltrate not only the neointima but also the perivascular and interstitial components of an allograft undergoing chronic rejection.

Whether the mesenchymal cells of the donor are entirely replaced by those of the recipient over time, as has been shown in untreated animal allografts,²¹ is unknown. We found that a large proportion of the mesenchymal cells in allografts undergoing chronic rejection were of donor origin. The persistence of donor mesenchymal cells may be due to immunosuppression with cyclosporine.²¹ In our study, all but two of the biopsy specimens were obtained within six months after transplantation, at which time cyclosporine levels are relatively high. Alternatively, the persistence of donor cells in the vessels and interstitium may be related to the short interval between transplantation and the biopsy.

Our findings suggest the existence of a circulating mesenchymal precursor cell. Indeed, Bucala et al. have provided evidence of the existence of a circulating fibroblastic stem cell.²² In their study, subcutaneous wound-viewing chambers became colonized with a

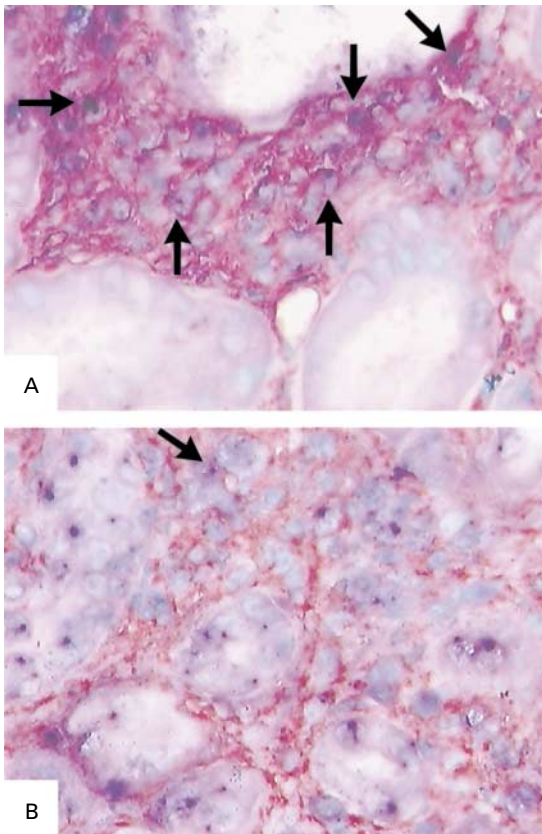


Figure 2. Results of Combined Immunostaining for Smooth-Muscle α -Actin and in Situ Hybridization for the Y-Chromosome Body in Tubulointerstitium ($\times 400$).

In Panel A, a renal-allograft specimen in which the donor was female and the recipient was male shows many mesenchymal cells (stained red) expressing a Y-chromosome signal (black arrows) of host origin. In Panel B, a renal-allograft specimen in which the donor was male and the recipient was female shows a few interstitial mesenchymal cells (stained red) expressing a Y-chromosome signal (black arrow).

circulating population of CD34+ fibrocytes. Moreover, other investigators have shown that smooth-muscle cells can be generated by human stromal cell lines derived from bone marrow,²³ and human mesenchymal progenitor cells have been transferred by bone marrow transplantation.²⁴ Myofibroblasts that appear soon after the onset of an episode of asthma are thought to originate from a circulating pool²⁵ or to be the result of the activation or proliferation of a local precursor cell.²⁶

Our study raises the question whether controlling the migration of smooth-muscle cells may prevent or ameliorate chronic rejection. An interesting possibility in this regard is the inhibition of local signaling of hyaluronan to smooth-muscle cells. These signals control proliferation and motility mediated through CD44 and the receptor for hyaluronan-mediated motility in smooth-muscle cells.^{27,28} This receptor is widely expressed in renal allografts undergoing chronic rejection, and in these allografts, the sequestration of hyaluronan alters the fibrotic response that follows tissue injury.^{29,30} These mesenchymal signaling molecules may represent targets for the blockade of smooth-muscle-dependent remodeling processes.

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