Recipient Age and Weight Affect Chronic Renal Allograft Rejection in Rats

SHANYING LIU,* JENS LUTZ,* BALAZS ANTUS,* YOUSHENG YAO,* SOHYUN BAIK,* FRIEDERIKE ILLIES,† and UWE HEEMANN*

Departments of *Nephrology and Hypertension and † Pediatric Nephrology, University Hospital Essen, Essen, Germany.

Abstract. Nephron doses and immune responses change with age. Therefore, age is a potential risk factor for graft survival after kidney transplantation. The aim of this study was to determine whether age-related differences are of importance for long-term outcomes after renal transplantation. Kidneys from Fisher 344 rats were orthotopically transplanted into nephrectomized Lewis rats. Kidneys were transplanted using donors and recipients of three age levels, i.e., young (8 wk of age), adult (16 wk of age), and old (40 wk of age). Rats were killed 24 wk after transplantation, and functional, morphologic, and molecular evaluations were performed. Recipient age, rather than donor age, determined graft survival rates. No significant correlation was observed between donor kidney weight on the day of transplantation and morphologic results.

As the major cause of late renal allograft loss, chronic rejection has been extensively studied in recent years. Chronic rejection of kidney grafts is characterized by slow but inexorable functional impairment, with nonspecific histopathologic features such as tubular atrophy, interstitial fibrosis, intimal thickening of arteries, and various glomerular lesions (1,2). Therefore, the term chronic allograft nephropathy has been proposed. Both alloantigen-dependent and alloantigen-independent factors have been implicated (3,4).

The increasing number of patients awaiting kidney transplantation, in association with the worldwide donor shortage, has led to more extensive use of suboptimal donors, specifically very young and very old donors. Moreover, the relative proportion of elderly patients among kidney recipients has sharply increased. Therefore, the effects of both donor and recipient ages on renal transplantation results have garnered more attention.

Age is considered to be an alloantigen-independent risk factor for kidney transplantation (5,6). Donor age may affect allograft function in at least two ways. On one hand, the nephron dose decreases with advancing age (7–9); on the other hand, pediatric kidneys are unaccustomed to the workload demanded by adult recipients (10–13). In such situations, it has been proposed that the reduced functional transplanted renal mass may lead to hyperfiltration-induced glomerular injury and inferior long-term outcomes. Furthermore, recipient age may be of importance. Young age is thought to be associated with greater immune responsiveness (14,15) and advanced age with attenuated immune responsiveness (16).

It is difficult to evaluate the effects of donor and recipient ages in clinical settings, because a large number of other factors influence allograft outcomes. Clinical surveys differ in the number of patients studied, the inclusion criteria, and the length of follow-up monitoring. In addition, several centers tend to transplant older kidneys into older recipients, according to an age-matching policy. Therefore, donor- and recipient-related differences cannot be investigated separately. The inferior results that were observed with very young donors may be attributable to surgical problems during transplantation (17). Therefore, it is not surprising that some studies concluded that increased donor age is associated with reduced allograft survival rates (16,18), whereas others noted no differences (19–21). Whether recipient age has an effect on long-term outcomes is even more controversial (22–24).

This study was designed to determine the effects of donor and recipient ages on the development of chronic renal allograft rejection, in the absence of other, well-known risk factors. Therefore, we transplanted kidneys from young, adult, and old donors into young, adult, and old recipients and evaluated...
functional and morphologic changes typically associated with chronically rejecting renal allografts.

Materials and Methods

Animals

Inbred male rats (Charles River, Sulzfeld, Germany) were used throughout the experiments. Lewis (RT1) rats acted as graft recipients and Fisher 344 (RT1/v) rats as donors. Animals were maintained under standard conditions and were fed rat chow and water ad libitum. All experiments were approved by the local Animal Care and Research Committee.

Transplantation

The left donor kidney was isolated, cooled, and positioned orthotopically in the host, for which the left renal vessels had been dissected free and clamped and the native kidney removed. Donor and recipient arteries, veins, and ureters were then anastomosed end-to-end with 10-0 Prolene sutures. No ureteral stent was used. The ischemic time ranged from 25 to 30 min. All animals were treated with low-dose cyclosporin A (1.5 mg/kg per d for 10 d), for suppression of initial episodes of acute rejection. The contralateral native kidney was removed on the 10th postoperative day. Rats with any overt signs of unsuccessful surgery were discarded from the experiment.

Experimental Groups

Animals were defined as young, adult, or old on the basis of their age at the time of transplantation, i.e., 8, 16, or 40 wk of age, respectively. Animals were assigned to the following nine experimental groups (n = 7/group) according to the ages of the donor and recipient: young donor/young recipient (group Y → Y), young donor/adult recipient (group Y → A), young donor/old recipient (group Y → O), adult donor/young recipient (group A → Y), adult donor/adult recipient (group A → A), adult donor/old recipient (group A → O), old donor/young recipient (group O → Y), old donor/adult recipient (group O → A), and old donor/old recipient (group O → O).

Functional Measurements

Every 4 wk, body weights were measured and 24-h urine samples were collected, using metabolic cages with urine-cooling systems. Urinary protein concentrations were quantitatively determined nephelometrically (Boehringer Mannheim, Mannheim, Germany). Serum and urinary creatinine levels were measured and creatinine clearance was calculated at the end of the study.

Collection

After 24 wk, rats were anesthetized with diethyl ether and intra-aortic BP was measured (Sirecust 404; Siemens, München, Germany). Animals were then exsanguinated, and the transplanted kidney was removed. Hematocrit values were determined using capillary microcentrifugation. Representative portions of the kidneys were snap-frozen in liquid nitrogen and stored at −80°C for immunohistologic and PCR analysis or fixed in buffered formalin (4%) for histologic evaluation.

Histologic Evaluation

For histologic assessments, kidney tissues were fixed in 4% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for evaluation of inflammatory infiltrates as well as tubulointerstitial fibrosis and tubular atrophy. Periodic acid-Schiff staining was performed to evaluate the extent of glomerulosclerosis and graft vasculopathy. Glomerulosclerosis was defined as the collapse of capillaries, the adhesion of the obsolescent segment of Bowman’s capsule, and the entrapment of hyaline in the mesangium (25). At least 200 glomeruli were counted in each kidney section, and the proportion of sclerosed glomeruli was expressed as a percentage of total glomeruli. Glomerulopathy, tubular atrophy, interstitial fibrosis, and vascular intimal proliferation were quantified according to the Banff97 classification (26) and were scored from 0 to 3+, to yield numerical coding (0 to 12+) of kidney damage.

Antibodies and Immunohistologic Assays

Monoclonal antibodies against macrophages (ED1) and CD5+ T lymphocytes (OX19) were purchased from Serotec (Camon Labor-Service, Wiesbaden, Germany). The secondary rabbit anti-mouse IgG antibody and the alkaline phosphatase-anti-alkaline phosphatase complex were obtained from Dako A/S (Hamburg, Germany). Representative portions of kidney grafts were snap-frozen in liquid nitrogen, cut with a cryostat (4 μm), fixed in acetone at 4°C for 5 min, air-dried, and stained with the respective antibodies. After incubation with the primary antibody, the sections were incubated with rabbit anti-mouse IgG and the alkaline phosphatase-anti-alkaline phosphatase complex. Positive cell counts for macrophages (ED1) and T lymphocytes (OX19) were expressed as the mean ± SEM of the number of cells per field of view; >20 fields of view/specimen were evaluated at ×400 magnification.

PCR

Total RNA Isolation. Total RNA was extracted and used for reverse transcription-PCR (RT-PCR). Kidney tissue was stored in 500 μl of cold lysis solution containing 4 M guanidine isothiocyanate (Sigma Chemical Co., St. Louis, MO), 25 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, and 0.5% sarcosyl and was frozen in liquid nitrogen. Total RNA was extracted from the kidneys according to the modified guanidine-isothiocyanate procedure (27). Briefly, frozen tissues were mixed with 4 ml of 4 M guanidine isothiocyanate (Sigma) and acid phenol-chloroform (pH 4; Roth, Karlsruhe, Germany) and homogenized. The samples were centrifuged at 1500 × g for 10 min at 20°C. The supernatant was added to an equal volume of isopropanol, followed by centrifugation. The RNA was purified with an RNaseasy total RNA isolation kit (Qiagen, Hilden, Germany) and stored at −80°C until further processing. The RNA concentration was measured spectrophotometrically.

Reverse Transcription. RNA was amplified by RT with an oligo(dT)12-18 primer (Life Technologies/BRL, Karlsruhe, Germany). One μg of total RNA was added to 0.5 μg of primer. A reaction mixture containing buffer solution (50 mM Tris-HCl, pH 8.3, 75 mM potassium chloride, 5 mM magnesium chloride, 5 mM dithiothreitol; Life Technologies/BRL), ATP, TTP, GTP, and CTP (each in a concentration of 0.2 mM; Boehringer Mannheim), 0.5 μl of 40 U/μl recombinant ribonuclease inhibitor (Promega, Madison, WI), and 0.5 μl of 200 U/μl Moloney murine leukemia virus reverse transcriptase (Life Technologies/BRL) was added, and the first chain reaction was allowed to proceed (36°C, 1 h). The reaction was stopped by heating to 95°C for 5 min, followed by cooling on ice.

Amplification of Specific cDNA. Specific cDNA products corresponding to mRNA for transforming growth factor-β1 (TGF-β1) (28), platelet-derived growth factor (PDGF) A chain (29), PDGF B chain (30), and β-actin (31) were amplified using PCR. One microliter from the RT reaction was taken for PCR, which was performed in PCR buffer [750 mM Tris-HCl, pH 9.0, 200 mM (NH4)2SO4, 0.1%,
Table 1. Kidney weights and body weights\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Donor Body Weight (g)</th>
<th>Donor Right Kidney Weight (mg)</th>
<th>Recipient Body Weight at Tx (g)</th>
<th>Kidney Body Weight/Body Weight at Tx</th>
<th>Graft Weight at Collection (mg)</th>
<th>Recipient Body Weight at Collection (g)</th>
<th>Kidney Weight/Body Weight at Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y→Y</td>
<td>6</td>
<td>175 ± 9</td>
<td>722 ± 35</td>
<td>172 ± 8</td>
<td>4.22 ± 0.24</td>
<td>1530 ± 98</td>
<td>422 ± 34</td>
<td>3.79 ± 0.44</td>
</tr>
<tr>
<td>Y→A</td>
<td>6</td>
<td>168 ± 3</td>
<td>677 ± 16</td>
<td>345 ± 7</td>
<td>1.96 ± 0.62</td>
<td>1817 ± 51</td>
<td>435 ± 8</td>
<td>4.19 ± 0.18</td>
</tr>
<tr>
<td>Y→O</td>
<td>7</td>
<td>198 ± 6</td>
<td>761 ± 36</td>
<td>438 ± 1</td>
<td>1.15 ± 0.12</td>
<td>1730 ± 41</td>
<td>501 ± 15</td>
<td>3.46 ± 0.12</td>
</tr>
<tr>
<td>A→Y</td>
<td>7</td>
<td>281 ± 7</td>
<td>929 ± 25</td>
<td>165 ± 4</td>
<td>5.62 ± 0.17</td>
<td>1654 ± 37</td>
<td>418 ± 16</td>
<td>3.98 ± 0.10</td>
</tr>
<tr>
<td>A→A</td>
<td>6</td>
<td>289 ± 4</td>
<td>967 ± 30</td>
<td>372 ± 8</td>
<td>2.60 ± 0.10</td>
<td>1815 ± 84</td>
<td>470 ± 4</td>
<td>3.87 ± 0.18</td>
</tr>
<tr>
<td>A→O</td>
<td>7</td>
<td>296 ± 5</td>
<td>1011 ± 41</td>
<td>448 ± 12</td>
<td>2.26 ± 0.94</td>
<td>1730 ± 59</td>
<td>483 ± 12</td>
<td>3.59 ± 0.15</td>
</tr>
<tr>
<td>O→Y</td>
<td>7</td>
<td>348 ± 6</td>
<td>1197 ± 35</td>
<td>200 ± 8</td>
<td>6.01 ± 0.25</td>
<td>1759 ± 28</td>
<td>454 ± 8</td>
<td>3.88 ± 0.11</td>
</tr>
<tr>
<td>O→A</td>
<td>7</td>
<td>349 ± 8</td>
<td>1204 ± 24</td>
<td>361 ± 9</td>
<td>3.34 ± 0.98</td>
<td>1727 ± 43</td>
<td>465 ± 8</td>
<td>3.71 ± 0.91</td>
</tr>
<tr>
<td>O→O</td>
<td>7</td>
<td>336 ± 8</td>
<td>1057 ± 26</td>
<td>445 ± 10</td>
<td>2.38 ± 0.57</td>
<td>1776 ± 53</td>
<td>473 ± 10</td>
<td>3.77 ± 0.17</td>
</tr>
</tbody>
</table>

\(^a\) Donor body weight, donor right kidney weight, recipient body weight at transplantation, and kidney body weight/body weight ratio at transplantation were determined at the time of transplantation; graft weight, recipient body weight, and kidney weight/body weight ratio at collection were determined 24 wk after transplantation. Y→Y, young donor/young recipient; Y→A, young donor/adult recipient; Y→O, young donor/old recipient; A→Y, adult donor/young recipient; A→A, adult donor/adult recipient; A→O, adult donor/old recipient; O→Y, old donor/young recipient; O→A, old donor/adult recipient; O→O, old donor/old recipient; Tx, transplantation. Results are given as mean ± SD.

Table 2. Functional parameters at the time of collection (24 wk after transplantation)\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Proteinuria (mg/d)</th>
<th>Creatinine Clearance (ml/min per 100 g body wt)</th>
<th>Mean Arterial BP (mmHg)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y→Y</td>
<td>6</td>
<td>12.5 ± 2.4</td>
<td>0.39 ± 0.05</td>
<td>85.5 ± 7.2</td>
<td>42.6 ± 0.7</td>
</tr>
<tr>
<td>Y→A</td>
<td>6</td>
<td>25.2 ± 3.6</td>
<td>0.36 ± 0.02</td>
<td>71.8 ± 5.5</td>
<td>41.6 ± 1.2</td>
</tr>
<tr>
<td>Y→O</td>
<td>7</td>
<td>39.5 ± 12.1</td>
<td>0.25 ± 0.03</td>
<td>84.7 ± 9.4</td>
<td>37.9 ± 1.9</td>
</tr>
<tr>
<td>A→Y</td>
<td>7</td>
<td>28.5 ± 2.7</td>
<td>0.35 ± 0.01</td>
<td>91.6 ± 3.6</td>
<td>43.2 ± 1.4</td>
</tr>
<tr>
<td>A→A</td>
<td>6</td>
<td>34.7 ± 4.0</td>
<td>0.33 ± 0.01</td>
<td>75.2 ± 8.3</td>
<td>36.1 ± 3.8</td>
</tr>
<tr>
<td>A→O</td>
<td>7</td>
<td>24.1 ± 5.9</td>
<td>0.33 ± 0.01</td>
<td>90.1 ± 11.9</td>
<td>39.7 ± 0.8</td>
</tr>
<tr>
<td>O→Y</td>
<td>7</td>
<td>30.8 ± 6.1</td>
<td>0.36 ± 0.01</td>
<td>80.9 ± 10.4</td>
<td>39.2 ± 1.2</td>
</tr>
<tr>
<td>O→A</td>
<td>7</td>
<td>27.8 ± 2.9</td>
<td>0.31 ± 0.02</td>
<td>84.0 ± 10.4</td>
<td>42.5 ± 0.7</td>
</tr>
<tr>
<td>O→O</td>
<td>7</td>
<td>27.4 ± 7.2</td>
<td>0.37 ± 0.04</td>
<td>94.0 ± 14.4</td>
<td>38.5 ± 1.9</td>
</tr>
</tbody>
</table>

\(^a\) Results are given as mean ± SD.
contrast, kidney weights did not differ among the groups at the time of collection (Table 1).

At the time of transplantation, the donor kidney weight/recipient body weight ratio was considerably higher for young recipients, compared with adult and old recipients, irrespective of whether young recipients received grafts from young, adult, or old donors. Moreover, there was a trend toward higher kidney weight/recipient body weight ratios for groups $A\rightarrow A$, $A\rightarrow O$, $O\rightarrow A$, and $O\rightarrow O$, compared with groups $Y\rightarrow A$ and $Y\rightarrow O$. At the end of the follow-up period, kidney weight/recipient body weight ratios did not differ among the groups (Table 1).

### Functional Parameters

Donor and recipient ages did not significantly affect proteinuria, serum creatinine levels, mean arterial BP, or hematocrit values. However, a correlation between recipient body weight at the time of transplantation and creatinine clearance was observed ($r = 0.284, P = 0.028$). No significant correlation between creatinine clearance and donor kidney weight or kidney weight/recipient body weight ratio at the time of transplantation was observed. There was a trend toward increased proteinuria and decreased creatinine clearance for $Y\rightarrow O$ animals, compared with $Y\rightarrow Y$ animals and recipient groups of the same age (groups $A\rightarrow O$ and $O\rightarrow O$), but the differences did not reach statistical significance. Interestingly, group $Y\rightarrow Y$ developed the lowest proteinuria and the highest creatinine clearance, compared with the other groups (Table 2).

### Histologic Assessments

At the end of the follow-up period, histologic evaluation revealed glomerulosclerosis, tubular atrophy, interstitial fibrosis, inflammatory cellular infiltration, and intimal thickening of graft arteries in all groups. The severity of glomerulosclerosis tended to increase with recipient age, regardless of donor age. In animals with a kidney allograft derived from an old donor, the recipient age determined the allograft outcome. In particular, the glomerulosclerosis index was significantly higher for old and adult recipients (groups $O\rightarrow O$ and $O\rightarrow A$, respectively) than for young recipients (group $O\rightarrow Y$). The profound glomerular injury in these animals was accompanied by a significantly higher degree of interstitial fibrosis and moderate tubular atrophy, which was observed in approximately 25% of the cortical tubules. In addition, luminal obliteration of graft arteries (approximately 25%) was more obvious in these allografts. We noted similar effects of recipient age on the overall findings of chronic rejection among animals that had received grafts from adult donors, with mild interstitial fibrosis, mild tubular atrophy, and mild/moderate intimal thickening. However, these differences did not reach statistical significance. Accordingly, among animals that had received kidneys from young donors, the percentage of sclerosed glomeruli was significantly higher in old recipients (group $Y\rightarrow O$), compared with young recipients (group $Y\rightarrow Y$).

No significant differences in interstitial fibrosis, tubular atrophy, or intimal proliferation were noted among recipients with respect to donor age, although old recipients with grafts from old donors tended to exhibit relatively more glomerulosclerosis than did old recipients with grafts from young or adult donors. However, the differences were not significant (Figure 1 and Table 3).

Although the effect of recipient age was weak, the recipient body weight and kidney weight/body weight ratio on the day of transplantation were significantly correlated with the percentage of sclerotic glomeruli (Table 4). However, at the time of collection, we observed no significant correlation between recipient body weight or kidney weight/recipient body weight ratio and morphologic results.

![Figure 1](image1.png)

**Figure 1.** Representative photomicrographs of periodic acid-Schiff–stained renal allograft sections: (A) old donor to old recipient; (B) young donor to old recipient; (C) young donor to young recipient.
The number of infiltrating cells was low for young recipients compared with donor age and was most pronounced for old recipients of old kidney grafts (group O→O) (Table 3). Infiltration of T lymphocytes and macrophages was significantly more pronounced in group O→O than in groups O→A and O→Y. Similar effects of recipient age were noted for animals that had received kidneys from adult donors. However, the differences did not reach statistical significance. When young donors were used, cellular infiltration was most pronounced in adult recipients (group Y→A). The number of infiltrating cells was low for young recipients with young kidney grafts (group Y→Y) and old recipients with young kidney grafts (group Y→O) (Table 3).

Increasing donor age did not affect the number of CD5\(^+\) T lymphocytes and macrophages in young or adult recipients. However, among old recipients, cellular infiltration increased with donor age and was most pronounced for old recipients of old kidney grafts (group O→O) (Table 3).
with more severe allograft injury. The best outcomes were observed for young recipients, irrespective of whether allografts were derived from young, adult, or old donors. In contrast, kidney grafts in old recipients developed serious renal damage regardless of donor age. Moreover, initial recipient body weight was correlated with the development of chronic rejection. A significant but less profound correlation was observed between the donor kidney weight/recipient body weight ratio at the time of transplantation and the development of allograft injury. Donor kidney weight by itself exhibited no significant correlation with long-term allograft injuries, which may have led to the less profound effects of the kidney weight/recipient body weight ratio on long-term allograft outcomes in our experiments. Therefore, factors other than kidney weight, including donor and recipient ages, must be taken into consideration. In other words, the growth in recipient body weight could reflect greater metabolic demands with aging, whereas increased donor kidney weight might reflect tubular but not nephron mass; the increased kidney weights of aging rats may mask the reduced number of functioning nephrons. Therefore, when we use the kidney weight/recipient body weight ratio as a reference for renal transplantation, aging-related nephron loss or functional deterioration should be taken into consideration.

In our experiments, the effect of donor age on the development of chronic renal rejection was weak. Donor age affected allograft outcomes only for old recipients, i.e., increased donor age was associated with more rapid progression of chronic rejection. For young and adult recipients, donor age had no effect on allograft outcomes. Such findings were unexpected, because lower survival rates have been observed in a number of clinical studies using allografts from old donors (16,18).

Experimental studies in rats and human subjects have clearly suggested that aging is accompanied by several morphologic changes in the kidney. In rats, the earliest indications of age-related kidney damage occur at 3 mo. Typical changes include thickening of the glomerular basement membrane (34,35), mesangial proliferation (36), fusion of foot processes of podocytes (37–39), and glomerular sclerosis (36,38). Moreover, it has been suggested that old kidneys are more susceptible to ischemia/reperfusion injury and renal damage (39). These injuries could contribute to a cascade of inflammatory events, further reducing the nephron mass. In light of our findings, these age-related morphologic changes in the kidney may not trigger the rejection process. However, there was a trend toward more pronounced allograft injuries among old recipients of old donor kidneys. Therefore, it is reasonable to assume that small differences among donors become more obvious in animals with high metabolic demands, i.e., old recipients.

It has been well established that protein restriction ameliorates, whereas high dietary protein intake exacerbates, glomerular injury (40). Because the rats in our study were not fed according to a protein-restriction diet, it is likely that their growth was accompanied by increased protein intake. The high-protein diet of older recipients might have contributed to the more severe allograft injuries among these groups. Similarly, low protein intake might be responsible for the better outcomes of grafts in young recipients.

In human subjects and rodents, immune functions decrease with age (41–43); the involution of the thymus may be responsible (42). In our experiments, we did not observe any beneficial effects of lower alloresponsiveness among old recipients. The best outcomes were observed for young recipients of kidneys from young donors. Clinically, poor outcomes have been observed for young recipients (44). There are a few possible explanations for this difference. First, young recipients tend to exhibit lower compliance, compared with older recipients, in clinical settings. Second, we examined chronic, rather than acute, effects. In this setting, it may be important that age-related changes in subsets of CD4⁺ T cells in the periphery produce shifts from Th1 to Th2. Although the Th2 cells are helpful for acute rejection, they may be of importance for the initiation and maintenance of chronic allograft nephropathy (45,46).

PDGF and TGF-β are likely to be involved in the development of chronic allograft nephropathy (47,48). TGF-β has the potential to induce pathologic changes such as fibrosis and
arteriosclerosis (47). The effects of PDGF include promotion of hypertrophy and proliferation, stimulation of chemotaxis and contraction in vascular beds, and stimulation of TGF-β production (49). In rats, it has been demonstrated that decreases in renal mass accelerate and intensify changes resembling chronic rejection, in parallel with increased expression of PDGF and TGF-β, adhesion molecules, and endothelin (48,50). In our experiments, greater expression of PDGF-A and -B, as well as TGF-β, in old recipients of adult or old grafts paralleled the immunologic and histologic results. Therefore, the increased expression of these growth factors may, to some extent, mediate age-related allograft injuries. In conclusion, the effects of recipient age and body weight on allograft injuries were more pronounced than those of donor kidney weight in our experiments.

References

Access to UpToDate on-line is available for additional clinical information at [http://www.jasn.org/](http://www.jasn.org/)