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ORIGINAL ARTICLE

Expression of hypoxia-inducible factor-1α and hepatocyte growth factor in development of fibrosis in the transplanted kidney

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Summary

Late renal graft loss is associated with interstitial fibrosis. Hypoxia-inducible factor-1α (HIF-1α) is thought to facilitate fibrosis through interaction with TGF-β1, while hepatocyte growth factor (HGF) may act antifibrotic in the kidney allograft. The aim of this study was to investigate the expression of HIF-1α and HGF in protocol biopsies as possible prognostic biomarkers for renal fibrosis. Thirty-nine renal transplant recipients were included in the study. Protocol biopsies performed 1 and 2 years after transplantation were used for immunohistochemistry analysis. The correlation between HIF-1α/HGF and the Banff score was analysed. In addition, progression in renal fibrosis and graft survival among recipients with high or low expression of HIF-1α/HGF after transplantation was compared. There was no significant correlation between fibrosis and the HIF-1α expression 1 and 2 years after transplantation, but an inverse significant correlation between the HGF expression and the fibrosis score 1 year after transplantation was shown. Even when adjusting for human leucocyte antigen mismatches, there was a significant relationship between fibrosis and HGF expression. Graft survival was not significantly correlated to HIF-1α or HGF at 1 year, although the trend was towards better graft survival with high HGF. HGF may have antifibrotic effects in human renal transplants. (Central.Denmark.Region.Committee number: 1-10-72-318-13)

Introduction

Short-term graft survival after renal transplantation has significantly improved since the introduction of calcineurin inhibitors in the 1980s: long-term graft survival still has to be improved [1–3].

Chronic allograft dysfunction after transplantation is a major cause of renal graft loss and includes both immunologic and nonimmunologic mechanisms [4,5]. As histological signs of glomerulosclerosis, interstitial fibrosis (IF), tubular atrophy (TA), mesangial matrix expansion and arteriolosclerosis are detected already 1 year after transplantation [5,6], these morphological changes may be used as markers to facilitate early diagnosis of allograft nephropathy.

Numerous profibrotic [transforming growth factor-β1 (TGF-β1), hypoxia-inducible factor (HIF), plasminogen activator inhibitor-1 (PAI-1), platelet-derived growth factor, vascular endothelial growth factor (VEGF)] and antifibrotic [hepatocyte growth factor (HGF), bone morphogenetic protein 7] factors are implicated in renal fibrosis [7–12]. Many of these factors have a variety of functions depending on the duration and type of injury, and some of the functions might be very complex.

HIF is a heterodimer composed of one of two alternative oxygen sensitive α-subunits and a constitutive β-subunit. The two α-subunits have almost similar structures and regulation [13,14], but are differently expressed: HIF-1α is primarily expressed in the tubular segments, whereas HIF-2α is expressed in peritubular endothelial cells and fibroblasts as well as in glomerular cells [15]. The HIF system seems to have a protective role in acute ischaemia injuries related to heme oxygenase-1 [16]. In contrast, the influence on other HIF target genes such as tissue inhibitor of matrix metalloproteinase-1, connective tissue growth factor and PAI-1 may play a profibrotic role [17]. A synergistic effect between hypoxia and TGF-β1 concerning production of VEGF and collagens is evident [17]. Furthermore, it is known that TGF-β1 decreases prolyl hydroxylase-2 via a Smad-dependent pathway leading to HIF-1α accumulation [18]. Thus, HIF activation during hypoxia may contribute to renal fibrosis by (i) direct transcriptional regulation of target genes that control extracellular matrix (ECM) turnover and by (ii) interaction with the profibrotic factor TGF-β1 [17,19].

HGF is synthesized by mesenchymal-derived cells as a single-chain precursor and then processed by serine proteases into a two-chain, biologically active form [20]. HGF activates multiple signalling pathways via binding to the c-mesenchymal epithelial transition factor receptor. The kidney is one of the organs in the body in which HGF and its receptor are abundantly expressed [20,21]. HGF has a morphogenic, mitogenic, and anti-apoptotic role in renal tubular cells, podocytes and endothelial cells [22,23]. Furthermore, HGF is a potent antifibrotic factor that prevents progression of chronic renal fibrosis by inhibiting TGF-β1 expression, myofibroblasts activation and epithelial–mesenchymal transition [24–27]. Studies indicate that HGF acts by interrupting the nuclear translocation of Smad2/3 and by upregulating the expression of Smad transcriptional corepressors SnoN and TG-interacting factor [26,28]. In rats developing chronic allograft nephropathy treatment with recombinant HGF for 4 weeks after engraftment protects against early allograft injury [29]. Although most studies indicate that HGF is an antifibrotic factor [21,22], Laping *et al.* [30] reported that chronic exposure to HGF reduced creatinine clearance and increased microalbuminuria in diabetic mice.

HIF-1α and HGF seem to have opposite roles in the development of renal fibrosis; thus, it is interesting to investigate their expression level in the transplanted kidney over time. The aims of this study were to investigate the expression of HIF-1α and HGF in renal protocol biopsies 1 and 2 years after transplantation and relate it to the amount of and development of fibrosis during 1 year and to graft survival.

Patients and methods

Patients

Between 1985 and 1988, renal transplant recipients in Jutland, Denmark, were included in a study concerning different immunosuppressive regimens [31,32]. Patients were treated with cyclosporine (CyA) and prednisolone during the first year after transplantation. At the start of the second year, the recipients were randomized to treatment with either azathioprine (Aza) or CyA in combination with prednisolone. Characteristics of the renal transplant patients and the donor background are shown in Table 1. Renal protocol biopsies performed 1 year (T1) and 2 years (T2) after transplantation were embedded in paraffin and used for the following study. Patients that met the following inclusion criteria qualified for this study: (i) both renal transplant protocol biopsies should be available, (ii) no rejection at the time the biopsies were taken, (iii) no change of immunosuppressive agent between the first and second year after the renal transplantation and (iv) patients should be alive at least 2 years after transplantation with a functioning graft. Thirty-nine transplanted recipients and 78 biopsies were examined at Institute of Pathology, Aarhus University Hospital, Denmark and included in this study (Fig. 1). The study was approved by the Central Denmark Region Committee on Biomedical Research Ethics (1-10-72-318-13).

Renal function

The renal allograft function was evaluated 1 and 2 years after transplantation by the estimated glomerular filtration rate (eGFR) according to the modification of diet in renal disease (MDRD) formula based on the serum creatinine level, gender and age and expressed in ml/min/1.73 m^2 [33–35].

Routine histology

Biopsy sections were stained with Masson trichrome for routine histology. The renal biopsies were scored blinded by a pathologist (N.M.) according to the Banff07 chronic grading system [36]: mild IF/TA below 25% of the cortical area was classified grade 1, moderate IF/TA 26–50% of the cortical area was classified grade 2, and severe IF/TA above 50% of the cortical area was classified grade 3. The Banff classification includes scores for IF (ci), TA (ct) and arteriolar hyaline thickening (ah). Due to lack of cortical tissue, three of the renal biopsies were excluded (Fig. 1). The assessed percentage of fibrosis was used in the correlation analysis.

Immunohistochemistry

The analyses were performed on 4-μm paraffin-embedded renal tissue sections stained with mouse anti-HIF-1α

Table 1. Recipient and donor background.

Patient characteristic	All patients (n = 39) No. (%)
Recipient age (years)	
Median	44.0
Interquartile	35.0–59.0
Recipient sex	
Female	22 (56.4)
Male	17 (43.6)
Human leucocyte antigen mismatches, total (A + B + DR)	
0 mismatches	4 (10.2)
1–3 mismatches	15 (38.5)
4–6 mismatches	20 (51.3)
Transplant number	
1st	30 (76.9)
2nd	6 (15.4)
3rd	3 (7.7)
Treatment in the 2nd year after Tx	
Azathioprine + prednisolone	21 (53.8)
Cyclosporine + prednisolone	18 (46.2)
Patients treated with ACE inhibitor	6 (15.4)
Native kidney disease	
Glomerulonephritis	9 (23.1)
Interstitial nephritis	5 (12.8)
Diabetic	5 (12.8)
ADPKD	3 (7.7)
Renal hypoplasia	2 (5.1)
Other	5 (12.9)
Unknown	10 (25.6)
Donor age (years)	
Median	36.0
Interquartile	21.0–51.0
Donor sex	
Female	16 (42.1)
Male	23 (57.9)
Donor type	
Living	2 (5.3)
Deceased	37 (94.7)

ADPKD, autosomal dominant polycystic kidney disease; Tx, transplantation.

monoclonal antibody (ab16066; Abcam, Cambridge, UK) and rabbit anti-human HGF polyclonal antibody (LS-B4957; LifeSpan BioSciences, Seattle, WA, USA).

The sections were deparaffinized, rehydrated in serial dilutions of alcohol and washed in running water. Endogenous peroxidase activity was blocked with 10% hydrogen peroxide. Heat-induced epitope retrieval was carried out in TEG buffer (pH 9.0) for HIF-1α and in sodium citrate buffer (pH 6.0) for HGF. After cooling, sections were incubated with primary antibodies diluted in TBS buffer supplemented with Triton-X for HIF-1α (1:400 dilution for 60 min) and with bovine serum albumin (1%) for HGF (1:50 dilution for 45 min). EnVision FLEX+ Mouse linker (Dako, Glostrup, Denmark) was used to amplify the mouse HIF-1α primary antibody before incubation with EnVison/HRP-conjugated secondary antibody (Dako). Diaminobenzidine (Sigma-Aldrich, St. Louis, MI, USA) as chromogen visualized the reaction. HGF was incubated with biotinylated goat anti-rabbit secondary antibody (1:300 dilution for 30 min; Dako) and detected by alkaline phosphatase streptavidin using Liquid Permanent Red (Dako) as a chromogen. Mayer's haematoxylin was used for nuclear counterstaining in both protocols. HIF-1α stain sections were dehydrated and mounted with Pertex Mounting Media (Leica Biosystems, Ballerup, Denmark). An alternative mounting process was used for HGF; the stain slides were air-dried overnight and mounted with permanent mounting media (Dako).

Internal and control sections for each staining were included to check the variation and the staining protocol. Tissue samples from the small intestine and tonsils served as positive controls of HIF-1α expression. Liver tissue and kidney tissue served as positive control for HGF. Negative control slides were performed using the same protocol without primary antibodies. To test the specificity of the HGF staining, we performed an immunizing peptide blocking experiment. Here, the HGF antibody was neutralized with an excess of control peptide (LS-PB4957; LifeSpan BioSciences), before proceeding with the staining protocol above.

Quantification of Immunohistochemistry

Blinded and objective quantification was performed for both HIF-1α and HGF staining. To lower bias based on the subjectivity and interobserver variability, the following specific criteria and stereological methods were used [37]:

HIF-1α: Whole slide images were captured by Nanozoomer (Hamamatsu Phototonics KK, Hamamatsu City, Japan) at a magnification of 20× and saved as image files; images were used for counting HIF-1α positive profiles. Only nuclear staining in tubules was counted as positive HIF-1α profiles. Areas with inflammation were excluded from the total area and point counting. Using point counting, the total area of tubules and renal tissue was estimated (NewCAST software; Olympus, Ballerup, Denmark). The total area of tubules was estimated as:

$$A(tubules) = a/p(kidney) \cdot \Sigma P(kidney) \cdot \frac{p(kidney)}{p(tubules)} \cdot \frac{\Sigma P(tubules)}{\Sigma P(kidney)}$$

In this formula, $a/p(kidney)$ is the area associated per test point, p is the number of test points, and P is the number of points hitting tubules or kidney. The total number of positive HIF-1α counted profiles per area (Qa(HIF-1α/*tubules*)) was calculated as:

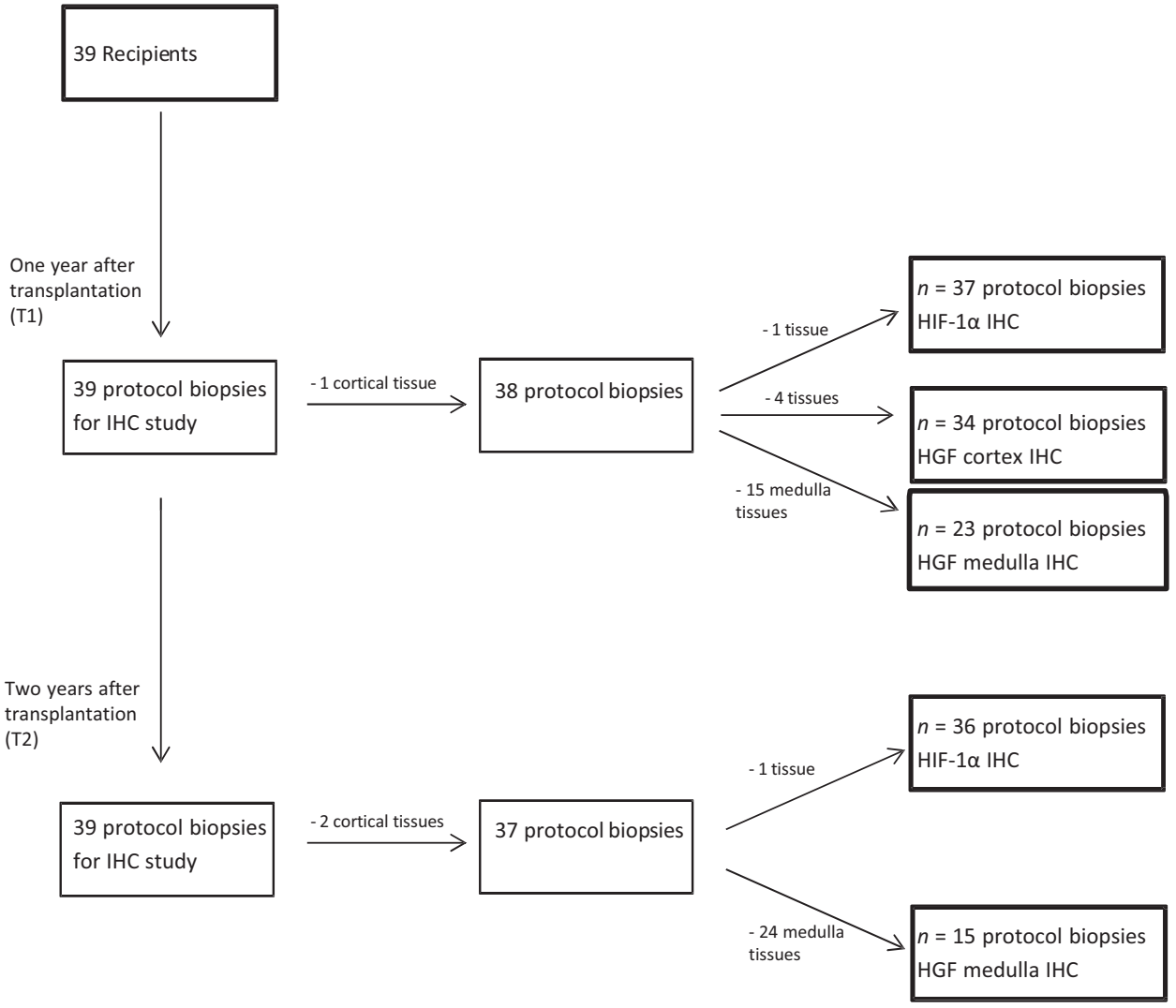

Figure 1 Details of biopsies included for histology and immunohistochemistry (IHC).

$$Qa\left(\frac{\text{HIF-1}\alpha}{tubules}\right) = \frac{\Sigma Q(\text{HIF-1}\alpha)}{A(tubules)}$$

Here, Q(HIF-1α) is the number of positive HIF-1α cell profiles counted in the whole kidney (medulla and cortex, contemporary). The objective used was 10×.

HGF: Systematic uniform random sampling (NewCAST software) was used to count HGF positive cell profiles in cortex and medulla, and the total area of renal tissue. All tubular segments in cortex were included in the analysis. Positive HGF staining in medulla and medulla rays were only counted when located in distal tubules and collecting ducts. The counting frame was 11 100 μm^2, and the objective used was 20×.

Statistical analysis

Data were expressed as mean ± SD. A two-way repeated measures ANOVA was used to analyse the estimated fibrosis related to treatment and time. The Holm–Sidak method was used for all pairwise multiple comparisons. Pairwise comparisons were evaluated using Student's *t*-test. The variable factors were all logarithmically transformed to obtain a normal distribution of data. Pearson's correlation analysis/simple regression was used to examine the relationship between HGF or HIF-1α and the estimated fibrosis score. For more than two variables, a multivariate regression analysis was used. The total human leucocyte antigen (HLA) mismatches (A + B + DR) were treated as a categorical variable; 0: 0 mismatches, 1: 1–3 mismatches, 2: 4–6 mismatches.

Event-censored renal allograft survival was illustrated by Kaplan–Meier plots and calculated by the log-rank test. Patients with a functioning graft at the time of analysis, patients who had died but death was unrelated to functioning graft, or patients who had moved were treated as censored data. Values were considered significant at $P < 0.05$.

Results

The protocol biopsies were performed 1.07 ± 0.12 and 2.11 ± 0.17 years after transplantation. The mean estimated fibrosis score was significantly increased from 24.0 at T1 to 31.2 at T2 ($P = 0.04$, $n = 36$). This effect was independent of the two treatment regimens ($P = 0.31$, Aza vs. CyA). Likewise, the graft survival time was not significantly affected by the treatment group ($P = 0.40$, Aza vs. CyA; data not shown). The two treatment types did not significantly influence the HIF-1α and HGF expression ($P = 0.52$, HIF-1α; $P = 0.96$, HGF). There was no statistical significant interactions between the HIF-1α or HGF expression and treatment and time (HIF-1α: $P = 0.96$, $n = 38$; HGF medulla: $P = 0.63$, $n = 15$). No statistical significant differences in proteinuria (data not shown) or eGFR and the two treatment regimens at T2 were demonstrated (eGFR: Aza mean 41 ± 14 ml/min/1.73 m^2, CyA mean 39 ± 19 ml/min/1.73 m^2, $P = 0.73$, $n = 39$). Furthermore, there was no statistical significant interaction between eGFR and treatments over time (two-way ANOVA repeated: $P = 0.44$, $n = 38$). Thus, the following analyses were performed on the human material as one group independent of treatment regime. Graft survival was 14.1 years with a 95% confidence interval 10.6–17.6 years ($n = 39$). The eGFR was 40 ml/min/1.73 m^2 at T1 and T2 (T1: 95% CI: 34–45 ml/min/1.73 m^2, $n = 38$; T2: 95% CI: 35–45 ml/min/1.73 m^2, $n = 39$) and significantly inversely correlated with the fibrosis score at both time points (T1: $P = 0.001$, $r = -0.53$, $n = 37$; T2: $P = 0.0001$, $r = -0.62$, $n = 37$). The number of biopsies included in the immunohistochemical studies differed depending on which part of the kidney the biopsies represented (Fig. 1).

Histological scores

Mean number of glomeruli and chronic Banff scores is shown in Table 2. Patients were graded as follows: mild IF/TA (T1: $n = 24$; T2: $n = 15$), moderate IF/TA (T1: $n = 2$; T2: $n = 14$) and severe IF/TA (T1: $n = 3$; T2: $n = 8$). Representative images of each group are shown in Fig. 2. Thirteen patients (33%) did progress from T1 to T2 regarding the graded IF/TA score, whereas nineteen patients (49%) were stable. Patients who progressed in IF/TA score did not differ in their HGF expression profile compared with those who stayed stable (HGF medulla: $P = 0.95$, $n = 15$).

Table 2. Histologic Banff scores in the studied biopsies 1 and 2 years after transplantation.

Variable	*n*	Mean ± SD	95% CI
1 year biopsies			
Number of glomerulus	39	16.05 ± 1.88	12.24–19.86
Graded IF/TA	38	1.45 ± 0.11	1.24–1.66
Ci score	38	1.45 ± 0.11	1.24–1.66
Ct score	38	1.45 ± 0.12	1.21–1.69
Ah score	37	0.81 ± 0.17	0.47–1.15
2 year biopsies			
Number of glomerulus	39	13.72 ± 1.94	9.80–17.64
Graded IF/TA	37	1.81 ± 0.13	1.55–2.07
Ci score	37	1.73 ± 0.15	1.43–2.03
Ct score	37	1.57 ± 0.15	1.27–1.87
Ah score	36	1.03 ± 0.14	0.74–1.31

IF/TA, interstitial fibrosis/tubular atrophy.

HIF-1α expression

HIF-1α was expressed in the tubular cells as nuclear staining (Fig. 3b). The amount of HIF-1α positive cell profiles in the tubular segments was low and did not differ significantly between T1 and T2 ($P = 0.11$, the number of positive HIF-1α cell profiles counted per mm^2 tissue at T1: geometric mean HIF-1α: 0.91, 95% CI: 0.65–1.22; and at T2: geometric mean HIF-1α: 1.28, 95% CI: 0.94–1.66). There was no significant correlation between the HIF-1α expression and the estimated fibrosis score at T1 and T2 (T1: $P = 0.63$, $r = -0.08$, $n = 37$; T2: $P = 0.68$, $r = -0.07$, $n = 36$; Table 3). In addition, no significant relationship between fibrosis and HIF-1α expression adjusted for donor age and/or HLA mismatch was found at either 1 or 2 years after transplantation (Table 3). Furthermore, no significant correlation was observed among the eGFR and HIF-1α expression at both time points (T1: $P = 0.47$, $r = 0.12$, $n = 38$; T2: $P = 0.65$, $r = 0.08$, $n = 38$).

No significant correlation between the development of fibrosis from 1 to 2 years after transplantation and HIF-1α expression at T1 was found by univariate or multivariate regression analysis (data not shown). Furthermore, we did not find any significant difference in graft survival rate for patients with high HIF-1α in the tubular segments at T1 compared with those with a low level ($P = 0.63$, $n = 37$; data not shown).

HGF expression

By immunohistochemistry, the expression of HGF was located in the cytoplasma of cells lining the distal tubules and collecting ducts of the kidney (Fig. 4). A weak HGF positive staining was demonstrated in the proximal tubules and thin segments of the loop of Henle (Fig. 4). Thus, HGF

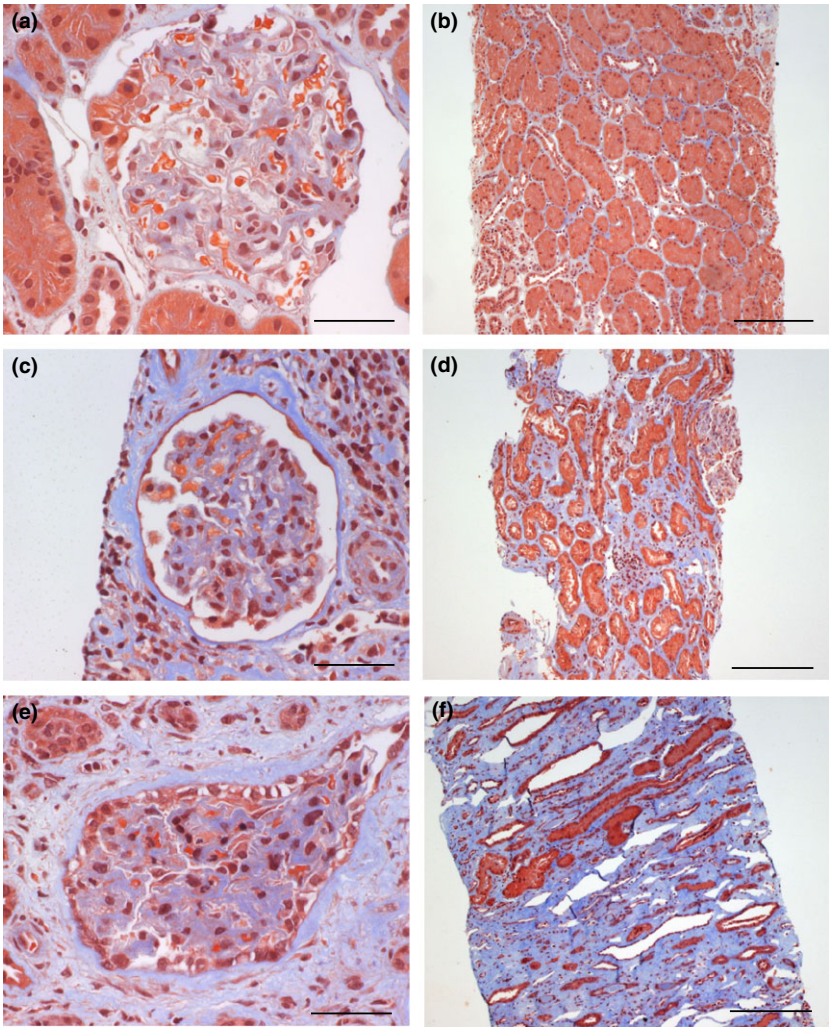

Figure 2 Representative histological images of trichrome-stained sections for each group of patients with mild (biopsies at T1) (a and b), moderate (biopsies at T2) (c and d) and severe (biopsies at T2) (e and f) interstitial fibrosis/tubular atrophy (IF/TA). (a, c, and e) scale bar = 50 μm. (b, d, and f) scale bar = 200 μm.

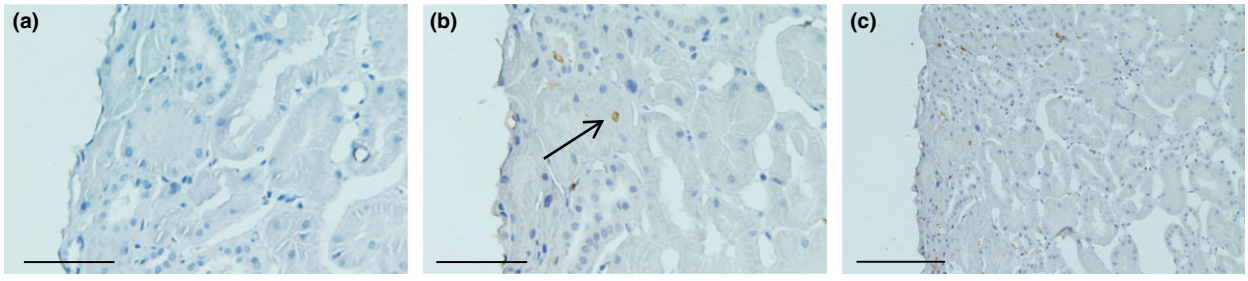

Figure 3 Immunohistochemical staining of hypoxia-inducible factor-1α (HIF-1α) in renal protocol biopsies 1 year after transplantation; (a) Negative control for HIF-1α, performed by substituting the primary antibody with nonimmune wash buffer (scale bar = 75 μm). (b) One HIF-1α positive stained nuclear in a proximal tubuli (arrow) (scale bar = 75 μm). (c) Positive HIF-1α staining (scale bar = 150 μm).

expression was compartmentalized; high in medulla and low in cortex. The HGF expression level in the medulla was similar at T1 and T2 ($P = 0.52$, the number of positive HGF cell profiles counted in medulla per mm^2 kidney tissue: geometric mean HGF medulla (T1): 194, 95% CI: 119–316; geometric mean HGF medulla (T2): 153, 95% CI:

Table 3. Correlation between hypoxia-inducible factor-1α (HIF-1α) or hepatocyte growth factor (HGF) expression and fibrosis adjusted for donor age or human leucocyte antigen (HLA) mismatches.

	Univariate regression analysis			Multivariate regression analysis			
	Fibrosis			Fibrosis + donor age		Fibrosis + HLA	
Time after transplantation	β	*r*	*P*-value	β	*P*-value	β	*P*-value
1 year (T1)							
HIF-1α profiles (*n* = 37)	−2.47	−0.08	0.63	−4.92	0.35	−4.72	0.35
HGF (medulla) profiles (*n* = 23)	−9.17	−0.53	**0.009***	−8.45	0.06	−8.73	**0.02****
HGF (cortex) profiles (*n* = 34)	5.54	0.27	0.13	4.80	0.19	5.24	0.14
2 years (T2)							
HIF-1α profiles (*n* = 36)	−3.05	−0.07	0.68	−3.34	0.65	−7.19	0.24
HGF (medulla) profiles (*n* = 15)	−6.39	−0.32	0.25	−0.48	0.94	−0.15	0.98

*between the HGF level in medulla and the estimated fibrosis score 1 year after transplantation.
**between the HGF level in medulla and the estimated fibrosis score adjusted for HLA mismatches 1 year after transplantation.

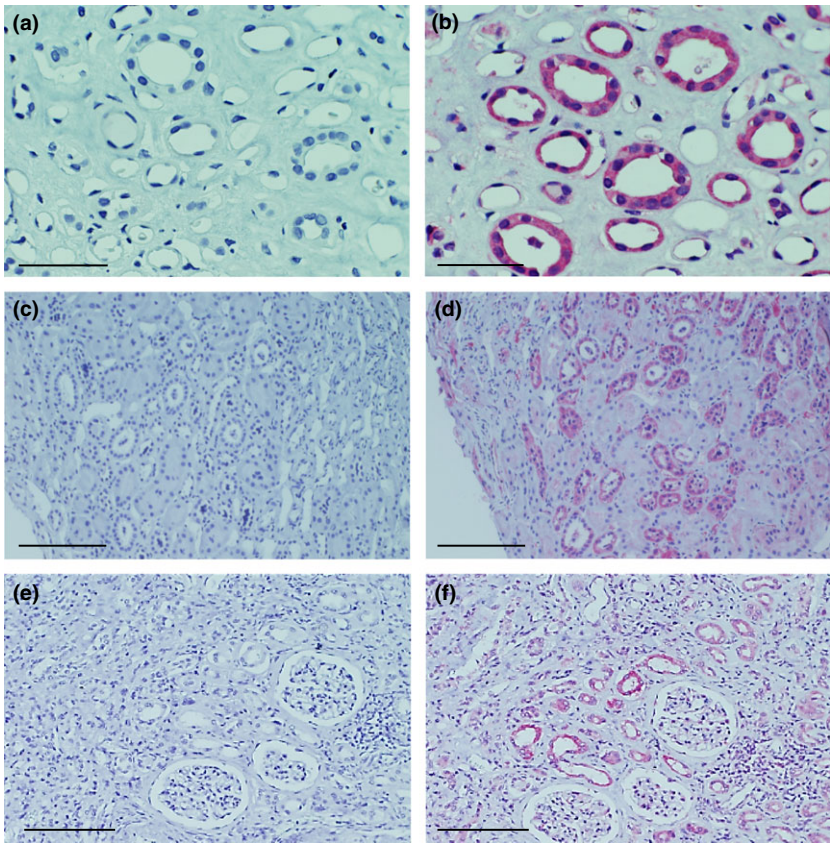

Figure 4 Immunohistochemical staining of hepatocyte growth factor (HGF) in renal protocol biopsies one (a and b) and two (c and d) years after transplantation; (a) Negative control slides for HGF staining (scale bar = 50 μm). (b) Images from medulla (scale bar = 50 μm). (c) Negative control slides for HGF staining (scale bar = 150 μm). (d) HGF staining was detected in the cytoplasma of tubular cells (HGF immunostaining; scale bar = 150 μm). (e and f) negative and positive staining of normal kidney (HGF; scale bar = 150 μm).

87–269). A significant negative relationship between the HGF level in medulla and the estimated fibrosis score at T1 was shown ($P = 0.009$, $r = -0.53$, $n = 23$; Table 3). The HGF level accounts for 28% of the variation in the fibrosis score (Fig. 5a). Even when adjusted for HLA mismatches, a significant negative relationship was evident ($P = 0.02$, $n = 23$; Table 3). The relationship between fibrosis and HGF expression in medulla was not statistically significant when adjusting for donor age alone or together with HLA mismatches (Table 3), although the trend was still towards

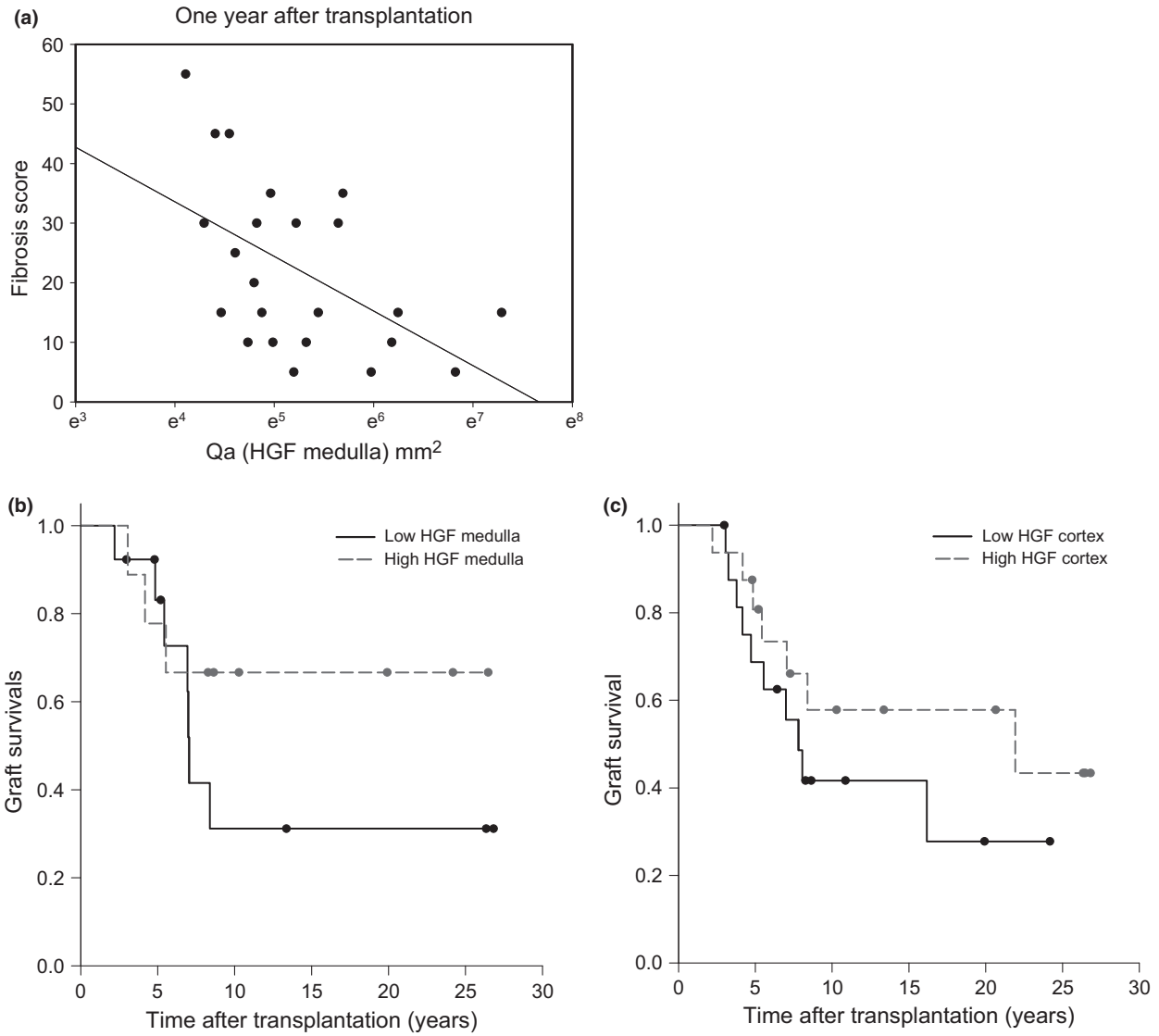

Figure 5 (a) Relationship between fibrosis score and hepatocyte growth factor (HGF) measured in renal medulla 1 year after transplantation for 23 patients drawn using a natural logarithmic scale for HGF. (b and c) Graft survival analysis. (b) HGF measured in medulla, low HGF mean: 12.6 years, 95% CI: 6.1–19.1 years, $n = 14$. High HGF mean: 19.1 years, 95% CI: 10.7–27.5 years, $n = 9$. (c) HGF measured in cortex. Low HGF mean: 12.1 years, 95% CI: 7.4–16.8 years, $n = 17$. High HGF mean: 17.1 years, 95% CI: 11.3–22.9 years, $n = 17$.

a negative correlation. HGF levels seemed to decrease with increasing donor age. Even when the patients treated with ACE inhibitor were excluded from the analysis, there was a significant relationship between the HGF level in medulla and the fibrosis score at T1 ($P = 0.021$, $r = -0.52$, $n = 19$).

No significant correlations were seen between HGF in medulla and fibrosis score at T2 ($P = 0.25$, $r = -0.32$, $n = 15$; Table 3). Furthermore, no significant correlation was found between the development of fibrosis from 1 to 2 years after transplantation and HGF (data not shown). A significant positive correlation was observed between the eGFR and HGF in medulla at T1 ($P = 0.002$, $r = 0.61$, $n = 23$). However, no significant correlation was seen at T2.

The HGF expression level in cortex was only measured at T1. No significant correlation was found between the amount of fibrosis and the HGF expression in cortex ($P = 0.13$, $r = 0.27$, $n = 34$; Table 3). Furthermore, no significant relationship between fibrosis score and HGF level in cortex adjusted for donor age and/or HLA mismatches was detected (Table 3). No significant correlation was observed between the eGFR and HGF in cortex ($P = 0.43$, $r = -0.14$, $n = 34$).

Although there was a trend towards higher graft survival in grafts with high HGF at T1, there was no statistically significant difference in graft survival for patients with high compared with low HGF amount measured in medulla or in cortex ($P = 0.27$ medulla, $n = 23$; $P = 0.26$ cortex, $n = 34$; Fig. 5).

Discussion

Identification of early biomarkers for progression of renal graft loss can facilitate improvements of therapy and graft survival. Especially markers for IF/TA could be useful, since loss of kidney graft function is strongly related to IF/TA, which we also have demonstrated here. This study is the first to demonstrate an inverse correlation between the expression of tubular HGF in protocol biopsies and fibrosis score 1 year after transplantation. However, HGF at 1 year did not predict fibrosis development in the subsequent year or graft survival. HGF measured in the medulla accounts for 28% of the variation in fibrosis score but, notably, HGF expression drops with increasing donor age. ACE inhibitors are demonstrated to enhance HGF expression and reduce fibrosis in the kidney in animal models [38,39]. However, even when the patients treated with ACE inhibitors were excluded, we found a significant inverse correlation between HGF expression in medulla and the fibrosis score. This may be due to the type of ACE inhibitor or the differences between human and animal studies.

We found HGF located in the cytoplasm of tubular cells, primarily in distal and collecting ducts, and only weak staining in the proximal tubules. This result confirms previous HGF staining patterns in humans [40–42]. However, animal studies demonstrate HGF localization in interstitial cells, endothelial cells, mesangial cells and macrophages [43–46]. In the tubular region, HGF may act on the tubular epithelial cells nearby in a paracrine manner [47]. Our findings suggest that the tubular segments may be a source of HGF in humans. Many studies have shown that HGF is upregulated in the kidney following renal injury [47]. Therefore, a local HGF source would make sense. In another study, increased HGF mRNA transcripts were detected in tubular epithelial cells and in mesenchymal cells, although to a minor extent, in patients with a rejecting kidney [48].

This study investigated the expression of HIF-1α and not HIF-2α. HIF-2α is not detected in epithelial cells of any tubular segment, but is expressed in glomeruli and interstitial cells [15]. The staining pattern of HIF-1α is particular in tubular epithelial cells, and therefore, we counted all positive profiles in the tubular segments [15,49]. Only few HIF-1α positive cell profiles were detected in the protocol biopsies 1 and 2 years after transplantation, and there was no significant correlation between fibrosis and the HIF-1α expression. In addition, no significant relationship between eGFR and the HIF-1α expression at any time points was observed. Thus, HIF-1α would not be a good marker for fibrosis. A study by Rosenberger *et al.* [50] demonstrated high levels of HIF-1α expression in biopsies 2 weeks after transplantation. However, they did not find any HIF-1α expression in protocol biopsies 3 months after transplantation, although these patients had histological changes characterized as IF. In addition, HIF-1α was only expressed in patients with acute rejection [50]. Another study showed that HIF-1α was upregulated in the area with IF/TA, but this finding was not obtained in protocol biopsies [51]. We chose to use protocol biopsies without acute rejection, and in this setting, HIF-1α staining was very weak; this is in accordance with the findings of Rosenberger [50].

Could protocol biopsies contribute to tell whether recipients will develop fibrosis? In a recent publication, Dosanjh *et al.* [52] demonstrated that HGF mRNA was significantly upregulated in early IF/TA, and further upregulated in biopsies with severe IF/TA. In addition, serum HGF levels seem to be increased in patients with end stage renal disease [21,53]. In two rodent models with chronic renal injury, HGF seemed beneficial in preserving normal kidney structure and function because blockage of HGF signalling with neutralizing antibody markedly promoted the onset and progression of tissue fibrosis and renal dysfunction [44,54]. HGF exposure enhances the ECM catabolism in human proximal tubular epithelial cells and in glomerular cells [55,56], which could antagonize the profibrotic effect of TGF-β1. Thereby, HGF can potentially retard ECM accumulation and IF in chronic kidney disease. Mahmoud *et al.* [57] showed that HGF measured in plasma during the early post-transplant period might be a useful marker for detection of acute renal allograft rejection. Our study identified a significant correlation between high levels of HGF in the tubules and low fibrosis score in protocol biopsies 1 year after transplantation, but HGF was not a prognostic biomarker for progression of fibrosis or graft survival. The high HGF level might act as a compensatory mechanism protecting the progression of fibrosis by its mitogenic and anti-apoptotic activities in endothelial and tubular cells and/or by stimulation of proteases involved in ECM remodelling. HGF is highly expressed initially after injury together with the profibrotic factor TGF-β1, but if the injuries continue, HGF gradually declines because of the progressively increased expression of TGF-β1 [24–27]. Thus, the protecting role of HGF seems to be in the initial phase after injury. This may explain why we find a significant correlation between HGF expression and fibrosis score at T1, whereas this correlation was absent 2 years post-transplant. Although our results may be paraphenonema, HGF as a biomarker should be further studied.

Authorship

TK, NM, LW and BJ: designed the study. TK and BJ: collected data. TK and NM: performed the study. TK, JRN, LW and BJ: analysed data. JRN: contributed with analytic tools. TK: wrote the paper. TK, NM, JRN, LW and BJ: revised the paper.

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