Immunohistochemical Detection of Hypoxia-Inducible Factor-1α in Human Renal Allograft Biopsies

Christian Rosenberger,* Johann Pratschke,† Birgit Rudolph,‡ Samuel N. Heyman,§ Ralf Schindler,* Nina Babel,* Kai-Uwe Eckardt,‖ Ulrich Frei,* Seymour Rosen,¶ and Petra Reinke*

**Nephrology and Medical Intensive Care, †Department of General, Visceral and Transplantation Surgery, and ‡Pathology, Charité Universitätsmedizin, Berlin, Germany; §Department. of Medicine, Hadassah Mt. Scopus and the Hebrew University Medical School, Jerusalem, Israel; ‖Department of Nephrology and Hypertension, University of Erlangen-Nuremberg, Erlangen, Germany; and ¶Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts*

Although it generally is accepted that renal hypoxia may occur in various situations after renal transplantation, direct evidence for such hypoxia is lacking, and possible implications on graft pathophysiology remain obscure. Hypoxia-inducible factors (HIF) are regulated at the protein level by oxygen-dependent enzymes and, hence, allow for tissue hypoxia detection. With the use of high-amplification HIF-1α immunohistochemistry in renal biopsies, hypoxia is shown at specific time points after transplantation with clinicohistologic correlations. Immediately after engraftment, in primarily functioning grafts, abundant HIF-1α is present and correlates with cold ischemic time >15 h and/or graft age >50 yr ($P < 0.04$). In contrast, a low HIF-1α score correlates with primary nonfunction, likely reflecting loss of oxygen consumption for tubular transport. Protocol biopsies at 2 wk show widespread HIF-1α induction, irrespective of histology. Beyond 3 mo, both protocol biopsies and indicated biopsies are virtually void of HIF-1α, with the only exception being clinical/subclinical rejection. HIF-derived transcriptional adaptation to hypoxia may counterbalance, at least partly, the negative impact of cold preservation and warm reflow injury. Transient hypoxia at 2 wk may be induced by hyperfiltration, hypertrophy, calcineurin inhibitor–induced toxicity, or a combination of these. Lack of detectable HIF-1α at 3 mo and beyond suggests that at this time point, graft oxygen homeostasis occurs. The strong correlation between hypoxia and clinical/subclinical rejection in long-term grafts suggests that hypoxia is involved in such graft dysfunction, and HIF-1α immunohistochemistry could enhance the specific diagnosis of acute rejection.

J Am Soc Nephrol 18: 343–351, 2007. doi: 10.1681/ASN.2006070792

It generally is accepted that in renal transplants, hypoxia occurs in association with cold storage followed by warm reflow or as part of calcineurin inhibitor (CNI) toxicity (1). However, direct evidence for such allograft hypoxia is lacking.

Hypoxia-inducible transcription factors (HIF) allow for hypoxia detection at a single cell resolution (for reviews, see references [2–6]). HIF are heterodimers of a constitutive β-subunit and one of at least two alternative α-subunits. HIFα is regulated by oxygen-dependent proteolysis by so-called HIF prolyl hydroxylases (7,8). These key enzymes of HIFα degradation are oxygen dependent and can be considered cellular oxygen sensors, because their activity varies in the range of physiologic/pathologic oxygen tensions (9). HIF regulate transcriptional activity of a host of genes that are involved in metabolism, vascular tone, angiogenesis, cell cycle, iron metabolism, radical scavenging, erythropoiesis, inflammation, *etc.* (for reviews, see references [6,10]). There is sufficient amount of evidence to support the view that the HIF system is ubiquitous, instantaneously upregulated upon hypoxia, and short-lived upon reoxygenation and that many HIF target genes confer cell/tissue protection.

Detection of HIF activation in tissue sections requires *immunohistochemical* detection of nuclear HIFα. Previous studies in rats revealed the need for standardized fixation, special target retrieval, and high-amplification technique to obtain reliable results (11–20). With the use of various experimental models of global or regional renal hypoxia/ischemia, it has

This paper utilizes immunohistochemical techniques to demonstrate widespread hypoxia of multiple causes in the first 3 months after transplantation, but correlates hypoxia only with clinical/subclinical rejection after 3 months. This paper is linked to an article by Kambhan et al. *in this month's issue of* CJASN *(pp. 135–142), which describes a new histologic system for identifying and scoring calcineurin inhibitor toxicity. Both findings have the potential to improve discrimination between rejection and calcineurin toxicity in transplant biopsies and allow more targeted therapy for a failing graft.*

Received July 26, 2006. Accepted November 1, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Christian Rosenberger, Nephrology and Medical Intensive Care, Charité Universitätsmedizin, Virchow-Campus, Augustenburger Platz 1, 13353 Berlin, Germany. Phone: +49-30-450-553232; Fax: +49-30-450-553909; E-mail: chrosenbe@aol.com

ISSN: 1046-6673/1801-0343

been demonstrated that HIFα activation corresponds with expected renal oxygen gradients and that hypoxia and hypoxic adaptation occur in a cell type– and stimulus-specific pattern (12,13,17,20).

In this study, we show such hypoxia and hypoxia adaptation in renal allograft biopsies using HIF-1α immunohistochemistry. Indeed, HIF-1α expression is demonstrated at various stages after engraftment and correlates well with clinical/histologic findings.

Materials and Methods

Patients

All renal transplant patients at the Charité University Clinic Virchow Campus were eligible for the study, provided that their biopsies were fixed as described next and initial immunosuppression included an antibody (daclizumab, basiliximab, anti-thymocyte globulin, or muronomab), a CNI (either cyclosporin A [Sandimmune optoral, Novartis, Basel, Switzerland] or tacrolimus [Prograf, Fujisawa, Osaka, Japan]), and a steroid (prednisolone or methyl-prednisolone). Combined transplants with a nonrenal organ were excluded.

Renal Allograft Biopsies

Timing. Open postengraftment biopsies were obtained between 25 and 40 min after the vascular anastomoses, which corresponded with the time required for ureteral suturing and surgical hemostasis. Thereafter, protocol biopsies were obtained between days 10 and 14 (so-called 2-wk protocol biopsy) and during months 4 to 6 (3-mo protocol biopsy). Indicated biopsies were obtained whenever considered necessary on a clinical basis. In general, for logistic reasons, biopsies were performed at noon or in the early afternoon.

Between June 1, 2003, and April 30, 2005, a total of 197 biopsies were obtained according to the study fixation protocol detailed in the previous paragraph: 39 postengraftment biopsies, 49 2-wk protocol biopsies, 55 3-mo protocol biopsies, and 54 indicated biopsies (owing to a significant rise in serum creatinine, for definition see the Materials and Methods section). There was no biopsy-related graft loss in this series.

Fixation. Immediately after the biopsy, the cores were transferred into plastic vials that contained 4% buffered formalin (Sigma, Deisenhofen, Germany). After 15 to 20 min of fixation at room temperature, the vial was placed in ice to slow/stop fixation. Specimens were kept on ice until transfer into a graded ethanol series for paraffin embedding. We found out that this procedure allowed both HIF staining and satisfactory routine morphology. Paraffin embedding usually was performed at 1 to 3 h after the biopsy but occasionally the morning after.

Morphologic Studies. Three-micron paraffin sections served for either routine histologic stainings or for immunohistochemistry.

Routine Histology. Biopsy sections were stained for routine histology with hematoxylin and eosin, periodic acid-Schiff, Masson, and silver methenamine. Only representative biopsies that contained seven or more glomeruli, renal medulla, and at least one artery were included. Biopsies were scored by the same investigator (B.R.) according to the Banff 97 working classification of renal allograft pathology (21).

Immunohistochemistry for HIF-1α was performed as described previously (12,13). In brief, deparaffinized sections were cooked in target-retrieving solution (TRS; Dako, Hamburg, Germany) in a pressure cooker. Mouse anti-human HIF-1α antibody (α67; Novus Biologicals, Littleton CO; dilution of 1:10,000) and a CSA kit (Dako) were used. Signals were obtained with the help of the peroxidase technique using diaminobenzidine as a chromogen. Kidneys of rats that were kept for 4 h in a hypoxic chamber (12) served as positive controls. Omission of the first antibody or use of mouse-derived non–HIF-directed first antibodies served as negative controls. Only nuclear staining was considered positive. Immunohistochemistry for HIF-1α proved highly reliable in this material in that (*1*) background was virtually absent, (*2*) staining was strictly nuclear, and (*3*) staining was reproducible in parallel sections.

Semiquantitative assessment of HIF-1α signals was performed according to the following HIF score: 0, no HIF signals; 1, one to two HIF-positive tubules per biopsy; 2, three to five HIF-positive tubules per biopsy; 3, six to 10 HIF-positive tubules per biopsy; 4, 11 or more HIF-positive tubules per biopsy. HIF-positive glomeruli and clusters of interstitial cells were counted as positive tubules. In addition, HIF score was adjusted for the length of the biopsy core (in mm) according to the formula $\text{HIF score}_{\text{adjusted}} = \text{HIF score} \times \text{biopsy length}/7$. Because comparative results were achieved with both the adjusted and the nonadjusted HIF scores, data are presented as nonadjusted HIF score only.

Definitions

Histologic Groups. Unremarkable was defined as absence of significant tubulitis, vasculitis, interstitial inflammation, glomerulopathy, interstitial fibrosis, tubular atrophy, mesangial matrix increase, and vascular or arteriolar changes (t0, v0, i0, g0, ci0, ct0, cg0, mm0, cv0, and ah0, according to the Banff 97 classification). Acute rejection was defined as interstitial inflammation with tubulitis (equal to or greater than type IA acute rejection, according to the Banff 97 classification) as the single histologic diagnosis, together with a significant rise in serum creatinine (for definition of the latter see previous paragraph). Subclinical acute rejection was defined as histologic appearance of acute rejection but absence of significant rise in serum creatinine. Other histology was defined as any changes other than acute rejection or unremarkable histology. This group included biopsies with prominent isometric tubular vacuoles, interstitial fibrosis, tubular atrophy, tubular calcifications, arteriolopathy, and glomerulopathy.

Good Quality of Donor Kidneys. Postengraftment Banff 97 chronicity score not greater than ci0, ct0, cg0, mm0, cv0, or ah0.

Primary Graft Function. Primary graft function was determined by graft urine output of at least 30 ml during each of the first 3 h after completion of the vascular anastomoses. Urine was collected through a mono-J silastic ureter stent.

Significant Rise in Serum Creatinine. Significant rise in serum creatinine was determined to be a change in creatinine of either 0.5 mg/dl or 30% above baseline level.

Statistical Analyses

Data were stored and processed using the Crunch 4.0 statistics software (Oakland, CA) and are presented as means ± SEM. Nonpaired *t* test, Pearson χ^2 analysis, one-way ANOVA for multiple comparisons with *post hoc* Newman-Keuls test, and simple correlations between variables were applied as indicated. Statistical significance was set at $P < 0.05$.

Results

Postengraftment Biopsies

All postengraftment biopsies were of good histologic quality (for definition, see the Materials and Methods section). Vascular patency of the transplant was confirmed by Doppler ultrasound in all study patients immediately after surgery. HIF immunostaining varied among biopsies, ranging from 0 to +4. In strongly stained samples, abundant HIF1α appeared in both cortex and medulla (Figure 1), clearly showing that cold ischemia followed by warm reflow causes widespread hypoxia in

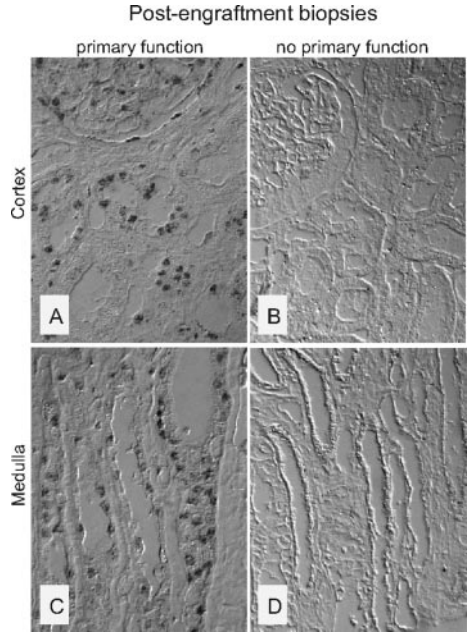

Figure 1. Postengraftment Hypoxia-inducible factor-1α (HIF-1α) in functioning *versus* nonfunctioning grafts. HIF-1α immunohistochemistry in postengraftment renal allograft biopsies. In primary functioning grafts, strong and abundant HIF-1α upregulation occurs in both cortex (A) and medulla (C). Signals mostly locate in glomeruli and tubules. Primarily nonfunctioning grafts are void of HIF-1α signals (B and D). Magnification, ×400.

renal grafts. Signals located predominantly in tubular profiles (mostly collecting ducts) and glomeruli but some capillary endothelial and tubulointerstitial cells were stained as well. Underlying renal disease of the recipient ($n = 12$ for glomerulonephritis, $n = 6$ for polycystic renal disease, $n = 4$ for ureteral obstruction/reflux, $n = 3$ for interstitial nephritis, $n = 4$ for nephrosclerosis, $n = 7$ for diabetes, and $n = 3$ for unclear renal disease) did not correlate with HIF expression (data not shown).

As expected, cold ischemic time (CIT) inversely correlated with primary function ($R = -0.41$, $P = 0.001$). Primary graft function correlated with HIF-1α score: In nonfunctioning kidneys, HIF-1α score (0.42 ± 0.20, n = 7) was significantly lower than in functioning kidneys (1.59 ± 0.23; $n = 32$; $P < 0.001$, t test). As illustrated in Table 1, all 13 kidneys with strongly positive HIF score (range 2 to 4) were functioning, whereas all seven primarily nonfunctioning kidneys were among the 26 kidneys with low HIF score of 0 to 1. This contradictory pattern fits well with our observations that abolished glomerular filtration paradoxically ameliorates renal hypoxia as tubular workload and, hence, oxygen consumption decreases (22–26).

Although it generally is accepted that donor age and CIT predispose to reflow injury, neither CIT alone nor donor age alone correlated with HIF. Noteworthy, in clinical practice, no clearcut thresholds seem to exist for CIT and donor age. Conceivably, short CIT can compensate, at least partly, for advanced donor age and *vice versa*, thereby reducing postengraftment hypoxia and HIF. We hypothesized that such compensation no longer will exist beyond a certain limit of donor age or CIT, and, consequently, hypoxia and HIF will occur. Indeed, primary functioning grafts with either donor age >50 yr or CIT >15 h exhibited more HIF (HIF score 2.25 ± 0.33 (SEM) *versus* 1.20 ± 0.38 in the group with donor age ≤50 yr and CIT ≤15 h ($P = 0.023$; Table 2). As shown in Table 2 and Figure 2, all of the 12 kidneys with donor age >50 yr or CIT >15 h were HIF positive. By contrast, in the group with donor age ≤50 yr and CIT ≤15 h, six of 20 were HIF negative ($P = 0.035$, Pearson χ^2 analysis).

It is interesting that, as illustrated in Table 1, there was a statistically nonsignificant trend for fewer episodes of acute rejection during the first month after engraftment in strongly HIF-immunostained kidneys. With a greater patient number, the link between early HIF and rejection indeed might show significance.

Two-Week Protocol Biopsies

To avoid possible confounding factors, at the 2-wk time point, we investigated only well-functioning grafts, and clinically indicated biopsies were excluded from this analysis. Strong and abundant HIF-1α appeared in both cortex and medulla of almost all 2-wk protocol biopsies (Figures 3, A and

Table 1. Postengraftment biopsies (overall data summary)[a]

Parameter	Highly Positive HIF-1α (Score 2 to 4)	Low/Negative HIF-1α (Score 0 to 1)	*P*
No. of patients	13	26	
HIF score	2.9 ± 0.2	0.6 ± 0.1	0.0001[b]
Primary function	13/13 (100%)	19/26 (73%)	0.04[c]
CIT (h)	11.5 ± 7.0	12.1 ± 5.6	NS
Donor age (yr)	44.8 ± 9.9	41.85 ± 13.4	NS
Living donor	3/13 (23%)	4/26 (15%)	NS
Acute rejection <1 mo after engraftment	3/13 (23%)	12/26 (46%)	0.16[c] (NS)

[a]CIT, cold ischemic time; HIF, hypoxia-inducible factor.
[b]*T* test.
[c]Pearson χ^2 analysis.

Table 2. Postengraftment biopsies with primary function

Parameter	Donor Age ≤50 yr and CIT ≤15 h	Donor Age >50 yr and/or CIT >15 h	*P*
No. of patients	20	12	
HIF score	1.2 ± 0.4	2.25 ± 0.3	0.023
CIT (h)	9.5 ± 1.0	12.9 ± 2.2	0.17 (NS)
Donor age (yr)	38 ± 3	50 ± 3	0.014
Living donor	4 (20%)	3 (25%)	NS
Acute rejection <1 mo after engraftment	7 (35%)	4 (33%)	NS

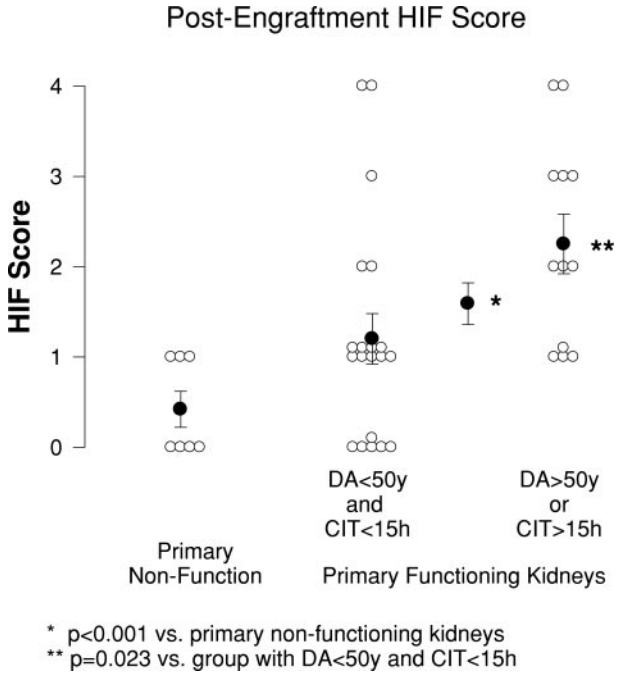

Figure 2. HIF-1α expression in postengraftment renal allograft biopsies. DA, donor age; CIT, cold ischemic time. Grafts with primary function have higher HIF scores when compared with nonfunctioning grafts. Among functioning grafts, DA >50 yr and/or CIT >15 h correlated with higher HIF scores.

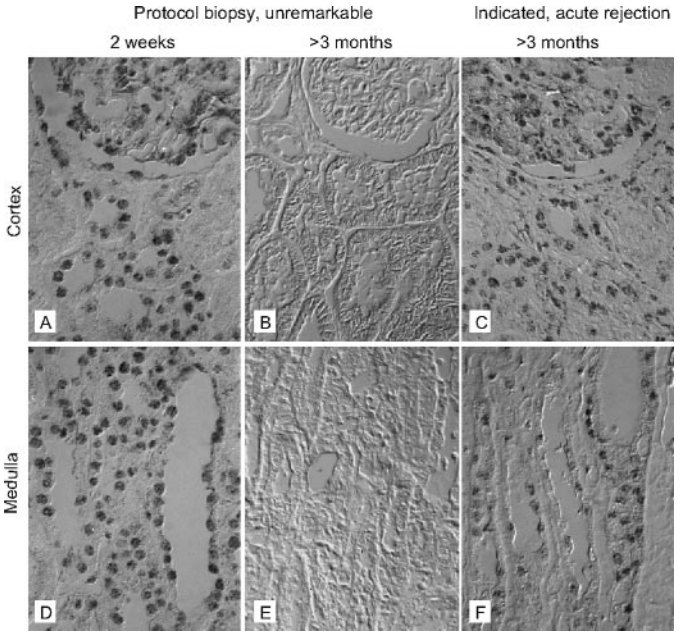

Figure 3. HIF-1α immunohistochemistry in protocol and indicated biopsies of renal allografts. At 2 wk after engraftment, most protocol biopsies exhibit marked HIF-1α upregulation in both cortex (A) and medulla (D). Signals mostly locate in glomeruli and tubules, but some interstitial cells are stained as well. At 3 mo after engraftment and beyond, unremarkable protocol biopsies are void of HIF-1α (B and E). However, at 3 mo after engraftment and beyond, indicated biopsies with acute rejection reveal strong HIF-1α signals in glomeruli, tubules, and interstitial cells (C and F). It is interesting that at the same time point (3 mo after engraftment and beyond), protocol biopsies with *sub*clinical acute rejection but no other histologic entity are positive for HIF-1α as well (data not shown). Magnification, ×400.

D, and 4; Table 3). No significant differences in HIF score occurred between histologic groups. The data clearly show that widespread graft hypoxia occurs at 2 wk after engraftment. Nevertheless, because postengraftment biopsies also exhibit HIF-1α, it remains uncertain whether hypoxia is persistent throughout the first 2 wk or rather appears *de novo*.

Three-Month Protocol Biopsies

This set of biopsies was obtained at 3 mo after engraftment and beyond (mostly between 3 and 6 mo after transplantation). In sharp contrast with the previous time points, 3-mo protocol biopsies in most cases were void of HIF-1α (Figures 3, B and E, and 5; Table 3), with the only exception of subclinical acute rejection. It is interesting that the group of subclinical borderline rejection (according to the Banff 97 classification) was negative as well. HIF-1α was absent in specimens that contained prominent histologic alterations such as vasculopathy, arteriolopathy, interstitial fibrosis, isometric tubular vacuoles, and glomerulopathy (all of which were included in the group of "other histologic changes").

Clinically Indicated Biopsies

For avoidance of possible confounding factors, the analysis included only indicated biopsies that were performed at 3 mo after engraftment and beyond. At this time point, baseline graft function and medication are fairly stable. This group contains biopsies that wee mandated by a significant rise in serum creatinine but obtained within a similar time frame as in the 3-mo protocol biopsies.

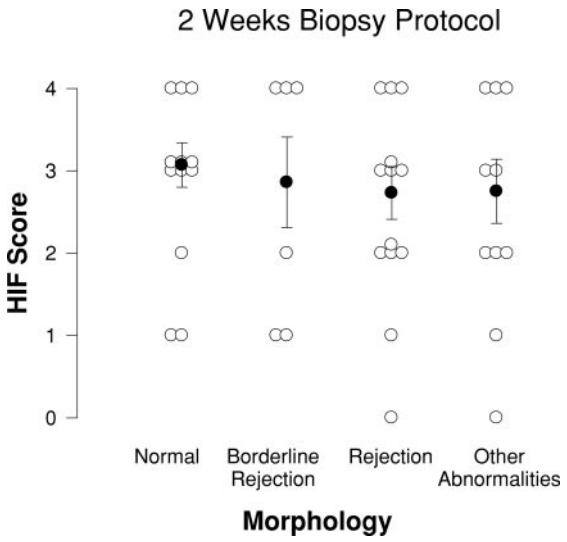

Figure 4. HIF-1α score in 2-wk protocol biopsies. In protocol biopsies (of well-functioning grafts) that were obtained at 2 wk after engraftment, HIF-1α expression is increased irrespective of histology and without significant differences between histologic groups.

HIF scores were comparable with the 3-mo protocol biopsies in that only grafts with acute rejection exhibited strong and abundant HIF-1α (Figures 3, C and F, and 6; Table 3), averaging 3.1 ± 0.2 ($P < 0.001$ *versus* all other groups, ANOVA). Again, both the borderline rejection group and kidneys that were defined as other histologic changes (as outlined previously) mostly were HIF negative.

Discussion

To our knowledge, this is the first report on HIF-1α protein upregulation in human renal biopsies. The transplant situation provides a well-defined focus of hypoxic injury to relatively unremarkable renal tissue and allows repeated studies to define the evolution of the HIF activation in conjunction with clinical parameters. The major findings are as follows: (*1*) HIF-1α is upregulated abundantly in postengraftment biopsies (Figure 1); (*2*) HIF-1α is absent in primarily nonfunctioning grafts (Figure 1); (*3*) in *functioning* grafts, HIF-1α is higher in older kidneys (>50 yr) and/or after prolonged ischemic period (>15 h; Figure 2); (*4*) HIF-1α is activated in protocol biopsies that are obtained at 2 wk after transplantation, irrespective of histology (Figures 3 and 4); (*5*) beyond 3 mo after transplantation, both protocol and indicated biopsies generally are void of HIF-1α (Figures 3 and 5); (*6*) the only exceptions to the observation in (*5*) are both clinical and subclinical acute rejection, which show marked HIF-1α upregulation at 3 mo from transplantation and beyond (Figures 3, 5, and 6).

HIF-1α Is Upregulated in Postengraftment Biopsies

It has been the general belief that cold storage of donor kidneys followed by warm reflow leads to hypoxic graft damage (for review, see reference [27]), but it is not until this study that hypoxia has been demonstrated clearly in this setting. Somewhat surprising, HIF-1α is absent in grafts with primary nonfunction. One might expect that primary nonfunction would associate with greater hypoxic reflow damage and, hence, with more HIF. However, reduced or even abolished glomerular filtration leads to a paradoxic increase in renal oxygen tensions as tubular workload for reabsorption declines. To underscore that during acute injury GFR reduction may serve for renal structural preservation, Thurau and Boylan (28) coined the term "acute renal success," and, certainly, both *in vivo* and *ex vivo* studies of medullary thick ascending limb injury have documented this extensively (22–25). Grafts with primary nonfunction are at greater risk for early acute rejection, supporting the view that nonspecific cell injury promotes specific alloimmunologic events (29–33). It is tempting to speculate that HIF upregulation might reduce immunogenicity of parenchymal cells. Noteworthy, there was a trend for less early acute rejections (within 1 mo) in kidneys with postengraftment HIF-1α activation. Lack of statistical significance may owe to interpatient variation in several acknowledged determinants of early acute rejection (*e.g.*, donor age, CIT, number of HLA mismatches, preexisting recipient HLA antibodies, immunosuppression).

Functioning kidneys of donors beyond 50 yr or with CIT >15 h exhibit more pronounced HIF-1α, indicating enhanced hypoxia adaptational responses. Noteworthy, in clinical practice, successful engraftment may occur far beyond these limits. HIF activation provides a reasonable explanation for this phenomenon in that HIF-derived transcriptional adaptation might ameliorate, at least partly, reflow injury and improve graft outcome.

HIF-1α Activation in 2-Wk Protocol Biopsies, Irrespective of Their Histology

Most of the well-functioning grafts displayed marked HIF activation at 2 wk after transplantation, the reason for which remains unclear. One possible explanation is hyperfiltration of the renal allografts with consequently increased workload for solute reabsorption, increased oxygen consumption, and hypoxia. A second possible explanation is graft hypertrophy. Growth hormones that promote hypertrophy have been shown to modulate HIF *via* phosphatidylinositol-3-kinase and mitogen-activated protein kinase (for review, see reference [34]). A third possible explanation is acute CNI-induced toxicity, which may cause renal hypoxia (35–43). Unfortunately, this plausible explanation cannot be tested by comparison with patients who were not given CNI, because all study patients were receiving this treatment at 2 wk after transplantation. A fourth possible explanation is HIF upregulation by inflammatory cytokines, which were shown to modulate HIF (for reviews, see references [44,45]. Indeed, at 2 wk after transplantation, there is considerable influx of blood-derived cells into the kidney (46,47). However, because at this time point HIF-1α is independent of histology, we suggest that inflammation is unlikely to be the cause for HIF upregulation. Location of tubular HIF-1α showed a predilection for collecting ducts throughout the clinical/histologic groups, which is in accordance with our previous observations in several experimental models (12,13,17,19,20).

Table 3. HIF-1α score in protocol and clinically indicated biopsies

Histology	2-Wk Protocol Biopsy	3-Mo Protocol Biopsy	3-Mo Indicated Biopsy
Unremarkable	3.07 ± 0.27 (n = 15)	0.97 ± 0.18 (n = 32)	— (n = 0)
Acute rejection	2.73 ± 0.32 (n = 15)	2.9 ± 0.50[a] (n = 10)	3.14 ± 0.22[b] (n = 21)
Borderline rejection	2.86 ± 0.55 (n = 7)	0.75 ± 0.48 (n = 4)	0.6 ± 0.60 (n = 5)
Others	2.75 ± 0.39 (n = 12)	0.78 ± 0.36 (n = 9)	0.62 ± 0.16 (n = 26)

[a] $P < 0.003$ *versus* other groups, ANOVA.
[b] $P < 0.0001$ *versus* other groups, ANOVA.

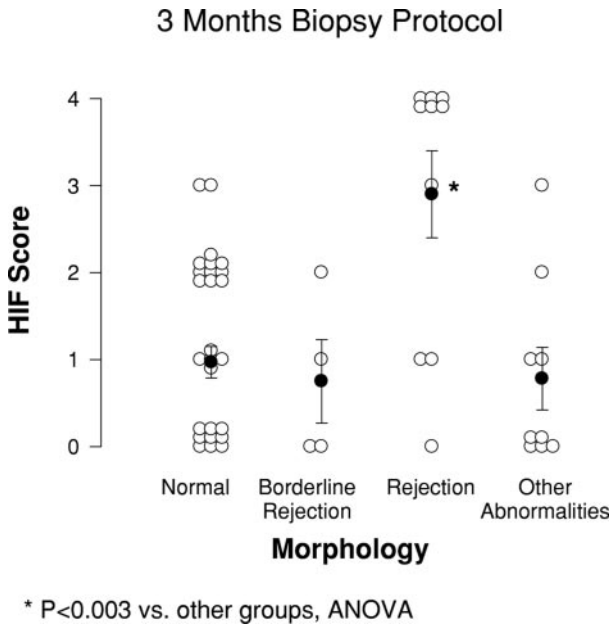

Figure 5. HIF-1α score in 3-mo protocol biopsies. In protocol biopsies (of well-functioning grafts) that were obtained at 3 mo after engraftment and beyond, HIF-1α expression is significantly increased only in the histologic subgroup of (subclinical) acute rejection.

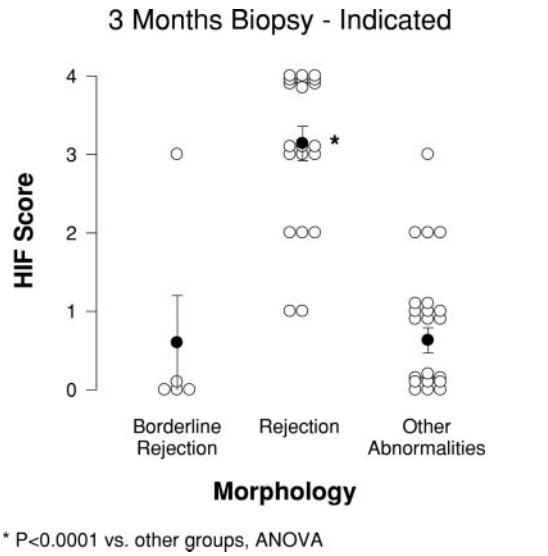

Figure 6. HIF-1α score in indicated biopsies at 3 mo after engraftment and beyond. In indicated biopsies (as a result of a significant rise in serum creatinine of either 0.5 mg/dl or 30% above baseline level) that were obtained at 3 mo after engraftment and beyond, HIF-1α expression is increased significantly only in the histologic subgroup of (clinical) acute rejection.

Finally, it is uncertain whether 2-wk graft hypoxia occurs *de novo* or persists since transplantation.

Stable Long-Term Allografts Are Void of HIF-1α

At 3 mo after transplantation, most renal allografts have reached a functional and structural equilibrium. In general, CNI as one possible cause of renal toxicity/hypoxia are being delivered at lower dosages, as compared with the early post-transplantation period. Not surprising, such grafts are virtually void of HIF-1α, which is in concordance with our results in normal rat kidneys (12–20).

Low or even very low oxygen tensions physiologically exist in kidney zones such as the papilla, the outer medulla, or the medullary rays (for review, see reference [48]. Sophisticated counterbalance mechanisms help to restore renal oxygen homeostasis (49), and some researchers have proposed that HIF physiologically takes part in this fine-tuning network (50,51). However, evidence for HIF upregulation in normal kidneys is inconsistent (for review, see reference [6]). Possible explanations for such conflicting results are differences between species, tissue fixation, or detection methods used.

In this study, we use a HIFα detection technique that has proved sensitive, robust, and reliable in a considerable number of studies on various rat and human tissues. With this method, HIFα immunosignals were undetectable in normal tissues (12–20). Therefore, we propose that either oxygen homeostasis is preserved in normal rat and human kidneys or our detection technique is insensitive for potentially physiologic HIFα. With strict definition of hypoxia as an oxygen imbalance (rather than simply low oxygen tension), we believe that our detection method may help to identify pathologic oxygen imbalance, as it has done so in multiple experimental situations. Consequently, absence of HIF1α in long-term stable grafts may indicate that their oxygen homeostasis is preserved.

Marked HIF-Activation in Clinical/Subclinical Rejection

Unexpected, beyond 3 mo after transplantation, the only histologic conditions that were associated with HIF activation were clinical and subclinical acute rejection (following the Banff

1997 classification). Noteworthy, in terms of HIF-1α expression, borderline changes were comparable with histologically unremarkable grafts. We suggest that tubulitis with subsequently increased tubular oxygen consumption can cause renal hypoxia in both clinical and subclinical acute rejection.

Nevertheless, we cannot rule out that factors unrelated to hypoxia may have activated HIF-1α in rejecting grafts. The specificity of HIFα protein for hypoxia has been challenged, because hypoxia-unrelated factors such as inflammatory cytokines and growth factors were shown to induce HIF in cell cultures that were kept under 21% oxygen (for reviews, see references [44,45]).

The concept of rejection-induced renal hypoxia is backed by two independent observations: First, Sadowski *et al.* (52) recently showed that renal medullary oxygenation is reduced during acute rejection, using blood oxygen level–dependent magnetic resonance imaging. Second, in experimental arthritis—a T cell–mediated inflammation that shares some common features with acute transplant rejection (53)—Peeters *et al.* (54) showed HIF-1α upregulation in conjunction with the hypoxia marker pimonidazole.

Acute tubular rejection is widespread but not diffuse, meaning that because of sampling error, histology sometimes may miss the diagnosis (55). Furthermore, clinical evolution and response to steroid pulses is hardly predictable in subclinical acute rejection, as well as with borderline changes (56). It is tempting to assume that complementary HIF-1α immunohistochemistry could enhance the diagnostic yield of renal allograft biopsies and help to decide for antirejection therapy.

Conclusion

In human renal transplants, hypoxia and hypoxic adaptation seem widespread during the first 2 wk after engraftment. By contrast, graft hypoxia is uncommon in the long term and is associated with clinical/subclinical rejection. HIF-1α immunohistochemistry could be a complementary diagnostic tool in subclinical or ambiguous cases of acute cellular rejection.

Acknowledgments

This study was supported by funds from Harvard Medical Faculty Physicians at Beth Israel Deaconess Medical Center (Boston, MA) and by the Russell Berrie Foundation and D-Cure, Diabetes Care (Jerusalem, Israel).

The technical skills of Gertrud Gruber, Yvonne Naumann, and Anna Skrobecka are greatly appreciated.

Disclosures

None.

References

1. Nankivell BJ, Chapman JR: Chronic allograft nephropathy: Current concepts and future directions. *Transplantation* 81: 643–654, 2006
2. Maxwell PH: HIF-1's relationship to oxygen: Simple yet sophisticated. *Cell Cycle* 3: 156–159, 2004
3. Metzen E, Ratcliffe PJ: HIF hydroxylation and cellular oxygen sensing. *Biol Chem* 385: 223–230, 2004
4. Semenza GL: Hydroxylation of HIF-1: Oxygen sensing at the molecular level. *Physiology* 19: 176–182, 2004
5. Nangaku M: Chronic hypoxia and tubulointerstitial injury: A final common pathway to end-stage renal failure. *J Am Soc Nephrol* 17: 17–25, 2006
6. Rosenberger C, Rosen S, Heyman SN: Current understanding of HIF in renal disease. *Kidney Blood Press Res* 28: 325–340, 2005
7. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr: HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for O2 sensing. *Science* 292: 464–468, 2001
8. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ: Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 292: 468–472, 2001
9. Epstein ACR, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Hamilton DL, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ: *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43–54, 2001
10. Wenger RH: Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *FASEB J* 16: 1151–1162, 2002
11. Wiesener MS, Seyfarth M, Warnecke C, Jürgensen JS, Rosenberger C, Morgan NV, Maher ER, Frei U, Eckardt KU: Paraneoplastic erythrocytosis associated with an inactivating point mutation of the von Hippel-Lindau gene in a renal cell carcinoma. *Blood* 99: 3562–3565, 2002
12. Rosenberger C, Mandriota S, Jurgensen JS, Wiesener MS, Horstrup JH, Frei U, Ratcliffe PJ, Maxwell PH, Bachmann S, Eckardt KU: Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys. *J Am Soc Nephrol* 13: 1721–1732, 2002
13. Rosenberger C, Griethe W, Gruber G, Wiesener MS, Frei U, Bachmann S, Eckardt KU: Cellular responses to hypoxia after renal segmental infarction. *Kidney Int* 64: 874–886, 2003
14. Warnecke C, Griethe W, Weidemann A, Jurgensen JS, Willam C, Bachmann S, Ivashenko Y, Wagner I, Frei U, Wiesener MS, Eckardt KU: Activation of the hypoxia-inducible factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors. *FASEB J* 17: 1186–1188, 2003
15. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU: Widespread hypoxia-inducible expression of HIF-2α in distinct cell populations of different organs. *FASEB J* 17: 271–273, 2003
16. Jurgensen JS, Rosenberger C, Wiesener MS, Warnecke C, Horstrup JH, Grafe M, Philipp S, Griethe W, Maxwell PH, Frei U, Bachmann S, Willenbrock R, Eckardt KU: Persistent induction of HIF-1alpha and -2alpha in cardiomyocytes and stromal cells of ischemic myocardium. *FASEB J* 18: 1415–1417, 2004
17. Rosenberger C, Heyman SN, Rosen S, Shina A, Goldfarb

M, Griethe W, Frei U, Reinke P, Bachmann S, Eckardt KU: Up-regulation of HIF in experimental acute renal failure: Evidence for a protective transcriptional response to hypoxia. *Kidney Int* 67: 531–542, 2005
18. Bernhardt W, Schmitt R, Rosenberger C, Munchenhagen PM, Grone HJ, Frei U, Warnecke C, Bachmann S, Wiesener MS, Willam C, Eckardt KU: Expression of hypoxia-inducible transcription factors in developing human and rat kidneys. *Kidney Int* 69: 114–122, 2006
19. Goldfarb M, Rosenberger C, Abassi Z, Shina A, Zilbersat F, Eckardt KU, Rosen S, Heyman SN: Acute-on-chronic renal failure in the rat: Functional compensation and hypoxia tolerance. *Am J Nephrol* 26: 22–33, 2006
20. Rosenberger C, Rosen S, Shina A, Bernhardt W, Wiesener MS, Frei U, Eckardt KU, Heyman SN: Hypoxia-inducible factors and tubular cell survival in isolated perfused kidneys. *Kidney Int* 70: 60–70, 2006
21. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffmann EO, Hunsicker LG, Lindblad AS, Yamaguchi Y, *et al*.: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55: 713–723, 1999
22. Brezis M, Heyman SN, Epstein FH: Determinants of intrarenal oxygenation. II. Hemodynamic effects. *Am J Physiol* 267: F1063–F1068, 1994
23. Brezis M, Agmon Y, Epstein FH: Determinants of intrarenal oxygenation. I. Effects of diuretics. *Am J Physiol* 267: F1059–F1062, 1994
24. Heymann SN, Rosen S, Epstein FH, Spokes K, Brezis M: Loop diuretics reduce hypoxic damage to proximal tubules of the isolated perfused rat kidney. *Kidney Int* 45: 981–985, 1994
25. Brezis M, Rosen S, Silva P, Epstein FH: Transport activity modifies thick ascending limb damage in the isolated perfused kidney. *Kidney Int* 25: 65–72, 1984
26. Shanley PF, Brezis M, Spokes K, Silva P, Epstein FH, Rosen S: Transport-dependent cell injury in the S3 segment of the proximal tubule. *Kidney Int* 29: 1033–1037, 1986
27. Perico N, Cattaneo D, Sayegh MH, Remuzzi G: Delayed graft function in kidney transplantation. *Lancet* 364: 1814–1827, 2004
28. Thurau K, Boylan JW: Acute renal success. The unexpected logic of oliguria in acute renal failure. *Am J Med* 61: 308–315, 1976
29. Hoffmann SC, Kampen RL, Amur S, Sharaf MA, Kleiner DE, Hunter K, John Swanson S, Hale DA, Mannon RB, Blair PJ, Kirk AD: Molecular and immunohistochemical characterization of the onset and resolution of human renal allograft ischemia-reperfusion injury. *Transplantation* 74: 916–923, 2002
30. Penfield JG, Wang Y, Li S, Kielar MA, Sicher SC, Jeyarajah DR, Lu CY: Transplant surgery injury recruits recipient MHC class II-positive leukocytes into the kidney. *Kidney Int* 56: 1759–1769, 1999
31. Lu CY, Penfield JG, Kielar ML, Vazquez MA, Jeyarajah DR: Hypothesis: Is renal allograft rejection initiated by the response to injury sustained during the transplant process? *Kidney Int* 55: 2157–2168, 1999
32. Kouwenhoven EA, de Bruin RW, Bajema IM, Marquet RL, Ijzermans JN: Cold ischemia augments allogeneic-mediated injury in rat kidney allografts. *Kidney Int* 59: 1142–1148, 2001
33. Halloran PF, Homik J, Goes N, Lui SL, Urmson J, Ramassar V, Cockfield SM: The "injury response": A concept linking nonspecific injury, acute rejection, and long-term transplant outcomes. *Transplant Proc* 29: 79–81, 1997
34. Bardos JI, Ashcroft M: Hypoxia-inducible factor-1 and oncogenic signalling. *Bio Essays* 26: 262–269, 2004
35. Rosen S, Greenfeeld Z, Brezis M: Chronic cyclosporine-induced nephropathy in the rat. A medullary ray and inner stripe injury. *Transplantation* 49: 445–452, 1990
36. Binet I, Wallnofer A, Weber C, Jones R, Thiel G: Renal hemodynamics and pharmacokinetics of bosentan with and without cyclosporine A. *Kidney Int* 57: 224–231, 2000
37. Cattaneo D, Perico N, Gaspari F, Remuzzi G: Nephrotoxic aspects of cyclosporine. *Transplant Proc* 36[Suppl]: 234S–239S, 2004
38. Ballardie FW, Edwards BD, Hows J, Jones L, Peters AM: Disturbance in renal haemodynamics and physiology in bone marrow transplant recipients treated with ciclosporin A. *Nephron* 60: 17–24, 1992
39. Nowack C, Garke-Nowack F, Pretschner DP, Frei U, Wonigeit K, Pichlmayr R, Hundeshagen H: The engymetric determination of acute functional impairment in kidney graft function caused by cyclosporine A. *Nucl Med Commun* 9: 389–397, 1988
40. Pichler RH, Franceschini N, Young BA, Hugo C, Andoh TF, Burdmann TA, Shankland SJ, Alpers CE, Bennett WM, Couser WG, *et al*.: Pathogenesis of cyclosporine nephropathy: Roles of angiotensin II and osteopontin. *J Am Soc Nephrol* 6: 1186–1196, 1995
41. Randhawa PS, Shapiro R, Jordan ML, Starzl TE, Demetris AJ: The histopathological changes associated with allograft rejection and drug toxicity in renal transplant recipients maintained on FK506. Clinical significance and comparison with cyclosporine. *Am J Surg Pathol* 17: 60–68, 1993
42. Thomsen HS, Nielsen SL, Larsen S, Lokkegaard H: Renography and biopsy-verified acute rejection in renal allotransplanted patients receiving cyclosporine A. *Eur J Nucl Med* 12: 473–476, 1987
43. Zhong Z, Areel GE, Connor HD, Yin M, Frankenberg MV, Stachlewitz RF, Raleigh JA, Mason RP, Thurman RG: Cyclosporin A increases hypoxia and free radical production in rat kidneys: Prevention by dietary glycine. *Am J Physiol* 275: F595–F604, 1998
44. Haddad JJ, Harb HL: Cytokines and the regulation of the hypoxia-inducible factor (HIF)-1alpha. *Int Immunopharmacol* 5: 461–483, 2005
45. Hellwig-Burgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann V: Review: Hypoxia-inducible factor-1 (HIF-1)—A novel transcription factor in immune reactions. *J Interferon Cytokine Res* 25: 297–310, 2005
46. Kooijmans-Coutinho MF, Bruijn JA, Hermans J, Schindler R, Frei U, Schrama E, van Es LA, Daha MR, van der Woude FJ: Evaluation by histology, immunohistology and PCR of protocollized renal biopsies 1 week post-transplant in relation to subsequent rejection episodes. *Nephrol Dial Transplant* 10: 847–854, 1995
47. Andersen CB, Ladefoged SD, Larsen S: Acute kidney graft rejection. A morphological and immunohistological study on "zero-hour" and follow-up biopsies with special em-

phasis on cellular infiltrates and adhesion molecules. *APMIS* 102: 23–37, 1994
48. Brezis M, Rosen S: Hypoxia of the renal medulla: Its implications for disease. *N Engl J Med* 332: 647–655, 1995
49. Heyman SN, Rosen S, Brezis M: The renal medulla: Life at the edge of anoxia. *Blood Purif* 15: 232–242, 1997
50. Monotham K, Tanaka T, Ohse T, Kojima I, Miyata T, Inagi R, Tanaka H, Sassa R, Fujita T, Nangaku M: A biologic role of HIF-1 in the renal medulla. *Kidney Int* 67: 1428–1439, 2005
51. Zou AP, Yang ZZ, Li PL, Cowley AW Jr: Oxygen-dependent expression of hypoxia-inducible factor-1a in renal medullary cells of rats. *Physiol Genomics* 6: 159–168, 2001
52. Sadowski EA, Fain SB, Alford SK, Korosec FR, Fine J, Muehrer R, Djamali A, Hofmann RM, Becker BN, Grist TM: Assessment of acute renal transplant rejection with blood oxygen level-dependent MR imaging: Initial experience. *Radiology* 236: 911–919, 2005
53. Fournier C: Where do T cells stand in rheumatoid arthritis? *Joint Bone Spine* 72: 527–532, 2005
54. Peeters CL, Morris CJ, Mapp PI, Blake DR, Lewis CE, Winrow VR: The transcription factors hypoxia-inducible factor 1alpha and Ets-1 colocalize in the hypoxic synovium of inflamed joints in adjuvant-induced arthritis. *Arthritis Rheum* 50: 291–296, 2004
55. Sorof JM, Vartanian RK, Olson JL, Tomlanovich SJ, Vincenti FG, Amend WJ: Histopathological concordance of paired renal allograft biopsy cores. Effect on the diagnosis and management of acute rejection. *Transplantation* 60: 1215–1219, 1995
56. Roberts IS, Reddy S, Russel C, Davies DR, Friend PJ, Handa AI, Morris PJ: Subclinical rejection and borderline changes in early protocol biopsy specimens after renal transplantation. *Transplantation* 77: 1194–1198, 2004

See the related editorial, "The Heat Is On: An Expanding Role for Hypoxia-Inducible Factors in Kidney Transplantation," on pages 13–15.