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TRANSLATIONAL IMPLICATIONS OF ENDOTHELIAL CELL DYSFUNCTION IN ASSOCIATION WITH CHRONIC ALLOGRAFT REJECTION

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Abstract

Advances in therapeutics have dramatically improved short-term graft survival, while the incidence of chronic rejection has not changed in the past 20 years. New insights into mechanism are sorely needed at this time and it is hoped that the development of predictive biomarkers will pave the way for the emergence of preventative therapeutics. In this review, we discuss a paradigm suggesting that sequential changes within graft endothelial cells (EC) lead to an intragraft microenvironment that favors the development of chronic rejection. Key changes include EC injury, activation and uncontrolled leukocyte-induced angiogenesis. We propose that all of these changes may lead to abnormal blood flow patterns, local tissue hypoxia and an associated overexpression of HIF-1α-inducible genes including *Vascular Endothelial Growth Factor*. We also discuss how regulators of mTOR-mediated signaling within EC are of critical importance in microvascular stability and the inhibition of chronic rejection. Finally, we discuss recent findings indicating that miRNAs regulate EC stability, and their potential as novel non-invasive biomarkers of allograft rejection. Altogether, this review provides insights into molecular

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events, genes and signals that promote chronic rejection and their potential as biomarkers for the future development of interruption therapeutics.

Introduction

Renal transplantation is widely recognized as the treatment of choice for children with end stage renal disease (ESRD) [1, 2]. The life expectancy benefit compared with chronic dialysis is significant [3], and graft survival can be superior to adult recipients [2]. However, the human immune response changes with increasing age, and some studies suggest that children are predisposed to an increased risk for both late acute rejection as well as chronic rejection [4]. Indeed, recent analyses of the NAPRTCS registry data for 11,603 pediatric renal transplants performed between 1987 and 2010 indicate that chronic rejection is the leading cause of graft failure, accounting for ~40% of all index pediatric graft failures [4]. Current therapeutics are most efficient to target acute rejection but have no effect on the progression of graft failure due to chronic rejection. Several groups [5–7], including our own group [8, 9] have strongly advocated for the development of mechanistic biomarkers to define the status of the alloimmune response at different times post transplantation or to monitor indices of graft injury. This approach will support the prediction of chronic rejection such that new therapeutic interventions can be tested in clinical trials. However, the development and use of biomarkers demands an understanding of chronic rejection pathogenesis, which is likely driven by multiple factors, especially in a growing child.

In this review, we discuss a paradigm in which the evolution of sequential endothelial-derived molecular events within the graft characterizes the progression of chronic rejection. We also review distinct cell intrinsic signals within endothelial cells that function to sustain vascular stability and thus, the intragraft microenvironment. The concepts described in this review open up avenues for translational research including the development of biomarkers to predict disease initiation and possible therapeutic targets for intervention in the future.

An Endothelial Cell-Based Paradigm To Define Chronic Rejection Pathogenesis

The Banff classification [10–13] adopted terminology that defines chronic rejection based on pathology, but also takes into account active immune processes. In this manner, it is possible to define injury based on alloantibody-mediated rejection (AMR) and/or T-cell-mediated rejection (TCR). Chronic rejection can thus be classified as ‘chronic active antibody-mediated rejection’, and/or ‘chronic active T-cell-mediated rejection’ and/or ‘interstitial fibrosis and tubular atrophy’ (IFTA) based on whether the exact mechanism is understood [12, 13]. The purpose of this review is to discuss a new paradigm for pathogenesis that involves the effect of immunity on the local intragraft microenvironment. We focus our discussion on the identity of events, and molecules that drive the remodeling of the microvasculature and EC-dependent responses to injury. Since we do not discriminate between cellular or humoral responses, we will use the term ‘chronic rejection’ to describe this process throughout the review.

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Our proposed paradigm is based on the well-established observation that the graft microvascular endothelial cell (EC) represents a critical interface between the recipient immune response and the donor allograft. The unique anatomic location of the graft EC ensures that it is the primary target of alloimmune-mediated injury [14, 15]. To this end, it is not surprising that microvascular loss occurs as a consequence of all forms of rejection [16, 17]. Furthermore, an increasing body of evidence indicates that the response of the microvasculature to injury is critical in both the initiation of, as well as the progression of chronic rejection. Microvascular EC respond to cell-mediated and humoral alloimmune responses by undergoing a characteristic process of activation. Several studies have shown that select mRNA and protein expression profiles within EC [18–20], and/or select intracellular EC activation responses may serve as an indicator of immune targeting of the graft [8, 21–23]. Also, cytokines produced by mononuclear cell infiltrates induce the expression of MHC class I and II as well as adhesion molecules and chemokines on EC that in turn result in the recruitment and activation of leukocytes within the graft. Growth factors produced by mononuclear cell infiltrates, including *Vascular Endothelial Growth Factor* (VEGF) [9, 24–27], elicit a 'leukocyte-induced' angiogenesis response [28] that is a characteristic event in all cell-mediated immune responses [29]. Thus, dynamic changes occur within the microvascular EC in the initial course of an alloimmune inflammatory reaction.

We [22] and others [16, 30, 31] hypothesize that all these microvascular responses initiate the process of chronic rejection by creating abnormal microvascular networks and chaotic blood flow patterns within the local inflamed tissue [31–34] (illustrated in Figure 1). The associated shunting of blood causes local tissue hypoxia, which can result in tissue injury and microvascular loss. Thus, similar to that described within tumors [35, 36], we have proposed that hypoxia, and hypoxia-stimulated overexpression of growth and survival factors are key events in the initiation and progression of chronic rejection ([22] and Figure 1). Indeed, Nicolls group has demonstrated that local tissue hypoxia occurs within allografts at early times in association with leukocyte infiltration [33]. Further, they demonstrated that the induced expression of hypoxia-inducible factor (HIF-1α) is a physiological response to alloimmune inflammation, and that HIF-1α-inducible responses result in the expression of multiple growth factors [37], most notably VEGF. While these factors function to sustain microvascular integrity, at the same time they serve as leukocyte chemoattractants and as angiogenesis factors. We have thus proposed that the overexpression of VEGF and related growth factors are of central importance to the biology of an intragraft microenvironment that sustains chronic rejection.

Although beyond the scope of this review, several other events contribute to microvascular disease in association with chronic rejection. For instance, neutrophils participate in many aspects of the transplant rejection process, including ischemia-reperfusion injury, acute and chronic rejection [38–40]. Importantly, following activation neutrophils release their DNA, histones and neutrophil antimicrobial proteins, resulting in the formation of Neutrophil Extracellular Traps (NETs) [41] that bind within microcapillaries and promote thrombosis [42] and/or the development of local vasculitis [43]. Elevated serum NET levels have been reported to be associated with organ injury, due in part to changes within the

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microcirculation [44, 45]. Neutrophil NETs have also been shown to damage endothelial cells and expose immunostimulatory molecules in the context of lupus, and similar mechanisms may be responsible for organ injury after transplantation [46]. Furthermore, it has been noted that reduced levels of NETs in the circulation following transplantation can be associated with improved graft function [47]. Thus, we speculate that the activation of neutrophils and NETosis within intragraft microcapillaries has potential to augment hypoxia-induced changes in the microvasculature and disease that is associated with chronic rejection.

Another event that is worthy of note relates to the role of endothelial-to-mesenchymal transition (EndMT) in chronic rejection [48]. Data suggest that the presence of inflammatory cytokines in association with endothelial proliferation precedes EndMT, where EC and/or pericytes dedifferentiate into collagen-secreting fibroblasts [49, 50] (and data not shown). In this manner, the pathological intragraft microenvironment, as illustrated in Figure 1, that is associated with inflammatory changes within EC, local tissue hypoxia and the overexpression of intragraft VEGF, may promote EndMT-dependent fibrosis and scarring. As will be discussed below, these collective findings suggest that the monitoring of molecular events associated with microvascular injury and repair, and/or intragraft expression of hypoxia-inducible growth factors may serve as predictive biomarkers of chronic rejection.

Cell Intrinsic Regulation of Microvascular Stability within an Allograft

Understanding the regulation of key signals within the EC that are critical for microvascular stability may pave the way for the development of new interruption therapeutic strategies to target the initiation of the chronic rejection process. To this end, the mammalian target of rapamycin (mTOR) signaling pathway has emerged as a critical mediator of EC stability [51–55]. Furthermore, the regulation of signals mediated through this pathway is likely of great importance in both the initiation and the progression of chronic rejection [56–58]. Cytokines produced by leukocytic infiltrates within the graft, and the binding of donor specific antibodies (DSA) to the microvasculature induce mTOR-dependent intracellular signals [59, 60] (illustrated in Figure 2). Responses include the characteristic expression of adhesion molecules and chemokines [53–55], and an activated EC phenotype that promotes mononuclear cell recruitment [21, 55, 58–61]. mTOR/Akt induced responses may also disrupt the allograft microenvironment through their potent effects to elicit EC proliferation and angiogenesis [22, 51]. Thus, while a physiological response to injury and inflammation, excessive mTOR activity within EC drives many molecular processes within the microenvironment as key events in the biology of chronic rejection.

mTOR is a highly conserved serine/threonine kinase that constitutes the catalytic subunit of two structurally and functionally distinct protein complexes called mTOR complex (mTORC)-1 and mTORC2 [62]. mTORC1 integrates signals from growth factors, energy status, oxygen and amino acids to regulate a variety of metabolic processes involved in cell growth and proliferation [62, 63]. mTORC1 is composed of mTOR, raptor (rapamycin-sensitive protein of TOR) [64] and mLST8 (mammalian lethal with SEC13 protein 8) [65]; it also associates with PRAS40 (proline-rich Akt substrate) [66] and DEPTOR (DEP

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domain-containing mTOR-interacting protein) [67] that function to negatively regulate the activity of the complex. Once activated, mTORC1 up-regulates protein synthesis by phosphorylating 4E-BP1 and S6K1, key regulators of mRNA translation, ribosome synthesis, cell proliferation and cellular growth [68]. mTORC1 has been found to be dysregulated in ageing, and several diseases including cancer, diabetes and obesity [63], and it is likely of great importance in chronic inflammatory disease states.

In contrast to mTORC1, little is known about upstream activation of mTORC2 or how it functions to facilitate downstream cellular function. mTORC2 activity classically results in the phosphorylation/activation of Akt (Ser473) and SGK1, well established to function in cell survival [69]; it also functions in actin and cytoskeletal organization [70, 71]. The mTORC2 complex is composed of mTOR, rictor (rapamycin-insensitive companion of mTOR) [70], mSIN1 (mammalian stress-activated protein kinase interacting protein) [72], Protor (protein observed with rictor)-1 [73] and mLST8 [71], and associates with the endogenous negative regulator DEPTOR [67]. There is crosstalk between both mTOR complexes in as much as mTORC1/S6K1-mediated activity may result in the phosphorylation of rictor, to inhibit mTORC2 complex formation [74]. In this manner, mTOR activity is maintained at a strict ”level” and it may be oscillatory in nature [63, 75] such that survival, cell growth and proliferation are maintained.

However, it is important to note that the mTOR pathway interacts with many other signaling cascades and promotes significant crosstalk among intracellular signals. Thus, the cell-intrinsic regulation of mTOR is potentially important in the deregulation of other signaling networks involved in EC biological responses (Figure 2). For instance, we have recently reported that the adaptor protein DEPTOR, an intrinsic inhibitor of mTOR signaling [67], is also potent to regulate MAPK- and STAT-induced signaling in EC [58]. Interestingly, the treatment of EC with TNFα markedly reduces intracellular levels of DEPTOR (within mins-hrs) presumably by changing its state of activity such that it is targeted for ubiquitination [76, 77]. Consistent with its cell intrinsic regulatory effects, we also find that reduced levels of intracellular DEPTOR (via siRNA knockdown) is associated with marked EC activation characterized by a massive release of T cell chemoattractant chemokines and the induction of cell surface adhesion molecules (e.g., VCAM-1, ICAM-1) analogous to activation in response to cytokines. Reduced levels of cell intrinsic DEPTOR promotes leukocyte-EC adhesion and enhanced angiogenesis *in vitro*, which can be attenuated by combined inhibition of mTOR and ERK activity. Our findings support the possibility that sustaining DEPTOR expression will stabilize the microvasculature and thus have a notable effect to inhibit EC activation and pathological angiogenesis. To this end, in pilot experiments we have found that overexpression of DEPTOR in EC is efficient to prevent TNFα-induced expression of adhesion molecules in vitro; and, using a transgenic mouse, we have found that forced overexpression of DEPTOR within cardiac transplants prolongs graft survival in vivo in an established fully MHC mismatched mouse model (unpublished data). One could therefore hypothesize that novel small molecule inhibitors of DEPTOR degradation [78] or agents that increase DEPTOR activity [79] will be anti-inflammatory and have potential to prevent the initiation of chronic rejection. Interestingly, recent studies have shown that DEPTOR is also functional in non-EC including proximal tubular epithelial cells [80]

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further supporting the concept that sustaining levels of expression may be therapeutic to maintain intragraft homeostasis.

miRNA-Mediated Regulation of EC Responses and their Potential Role in Chronic Allograft Rejection

It is increasingly apparent that miRNAs function to regulate EC activation, and they are well established to play a critical role in the control of microvascular stability. Furthermore, an increasing number of studies indicate that miRNAs are functional in the maintenance of microvascular stability post transplant [81, 82]. miRNAs have been shown to be secreted in biological fluids within microparticles, where their expression is extremely stable [83, 84] and accumulating data indicate their tremendous potential as non-invasive biomarkers of chronic vascular diseases, including allograft rejection ([81, 85–87] and discussed below).

Micro-RNAs (miRNAs) are small (20–22 nucleotides) endogenous non-coding RNAs that regulate gene expression through their binding to target mRNAs and the subsequent inhibition of the target mRNA translation. miR-126 is the only EC-specific miRNA described to date [88, 89] where it functions to sustain vascular homeostasis in response to injury. In mouse models [89, 90], deletion of miR-126 is associated with defects in EC proliferation, migration and angiogenesis; in zebrafish, knockdown of miR-126 results in loss of vascular integrity during embryonic development [88]. miR-126 augments VEGF-inducible responses within EC by directly repressing negative regulators of PI-3K/Akt/mTOR signaling. Consistent with these findings, miR-126 silencing in mice using a single dose of antagomir-126 was found to impair ischemia-induced angiogenic responses [91]. miR-126 has also been reported to function in the regulation of EC activation and in leukocyte-EC interactions in part by inhibiting the inducible expression of VCAM-1 [92, 93]. In general, these observations suggest that miR-126 regulated responses are of great biological importance in EC-dependent events associated with chronic rejection, and its potential as a biomarker is currently being studied.

In EC, miR-100 has been reported to regulate mTOR activity and attenuate signaling responses including cell proliferation [94]. In accordance with these findings, inhibition of miR-100 *in vivo* using specific antagomirs results in increased angiogenesis [94]. miR-21 was also recently reported to mediate rapamycin-induced suppression of EC proliferation and migration [95]. Treatment of EC with rapamycin significantly increased the expression of miR-21, and blockade of miR-21 (using specific inhibitors) reduced the effects of rapamycin on EC growth and mobility [95]. In addition, miR-21 has been found to play a major role in vascular remodeling in an EC-specific miR-21 knockout mouse model [96, 97].

Several additional miRNAs function as key regulators of EC proinflammatory responses and as mediators of inflammation resolution. Pober's group profiled the expression of miRNAs in TNFα-activated EC and identified miR-31 and miR-17–3p as key regulators of E-selectin and ICAM-1 expression, respectively [98]. Specific antagonism of these miRNAs increased neutrophil binding to cultured EC, whereas transfection of EC with their miRNA mimics decreased leukocyte-EC adhesion [98]. Several other miRNAs have also been found to

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inhibit EC activation responses, including miR-181b [99], miR-10a [100], miR221/222 and miR-155 [101], miR-146a and miR-146b [102], and could therefore also constitute biomarkers of EC stability.

Collectively, these data indicate that miRNAs function in cell-intrinsic regulation of EC responses associated with inflammation, and suggest that they are likely of functional importance in the evolution of chronic rejection. miRNAs are secreted within microparticles as exosomes or shed microvesicles [103, 104] and can be detected in the blood and in biological fluids such as urine. Their expression is extremely stable as they are protected from RNase-mediated degradation within microparticles [83, 84]. Because of this unusual stability, the analysis of miRNAs has emerged as a promising non-invasive biomarker assay for a variety of human disease processes including transplant rejection [105].

Biomarker Strategies

Diagnostic and prognostic assays to assess chronic rejection classically take the form of routine biopsy analyses of the graft in combination with organ-specific assessments of graft function. In addition, recent data suggest that it is possible to use several classes of biomarkers to support the diagnosis of acute rejection and acute graft injury. Nevertheless, little emphasis has been placed on the evaluation of molecular events and biomarkers that are associated with, and/or predict the development of chronic injury and chronic allograft rejection. To compound this issue, it is well known that current biopsy staining techniques, imaging tools and assessments of organ function do not predict the future development of chronic rejection. Ideally, newly developed biomarkers will predict disease before it is established, and thus enable clinicians to develop clinical trials that promote interruption therapies according to the status of the intragraft microenvironment. Many studies have searched to identify such factors using high throughput screens of mRNAs, miRNAs, proteins and cellular phenotypes in transplant biopsies, blood and urine samples. Several promising markers have been identified, mostly through analyzes of immunity-related molecules and genes. However, immune-specific biomarkers usually reflect ongoing inflammation/rejection and are suggested to be unlikely to predict the occurrence of chronic rejection months or years in the future.

The model that we have proposed is highly suggestive that intragraft expression and circulating levels of genes associated with vascular stability, injury and repair will serve as predictive monitors of disease within the allograft microenvironment. Also, our model predicts that local hypoxia and the induced expression of HIF-1α-regulated genes such as VEGF-A and related growth factors will be characteristic of the initiation of the chronic rejection intragraft microenvironment. This model provides a strong rationale to screen for detectable EC injury and repair responses as central biomarkers of the initiation of chronic rejection. For instance, the overexpression of VEGF-A is predicted to serve as a prototype HIF-inducible gene and may serve as the earliest biomarker of a response to any form of immune-mediated injury to the microvascular EC.

To test this possibility, we initially performed cross-sectional analyses among circulating serum levels of angiogenic factors in cardiac transplant patients with/without established

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chronic rejection/disease [8]. Univariate analysis identified 6 proteins, angiopoietin-2, artemin, urinary plasminogen activator, vasohibin, as well as VEGF-A, and VEGF-C that were significantly associated with allograft vasculopathy, the *sine qua non* of chronic rejection. Further analysis identified three proteins, VEGF-A, VEGF-C, and platelet factor 4 (PF-4) as the optimal biomarker set for the diagnosis of chronic rejection in this cohort. In a pilot study, we also tested whether these same factors predict chronic rejection [106]. Plasma levels were measured in a patients cohort who were greater than 2 years post transplant, and all patients were followed for 5 years. At a median follow-up time of 4.7 yrs, we find that high circulating levels of VEGF-A identifies a subgroup of recipients who subsequently develop adverse cardiac events over this time period. Thus, it is possible that low/basal plasma levels of VEGF-A identify a low risk patient population, whereas high levels may identify a cohort that will benefit from an interruption therapeutic, prior to complications from established disease. To further validate these observations, we are currently collaborating with the multicenter CTOT-05 consortium to evaluate whether these angiogenesis-related factors predict the initiation of allograft vasculopathy/chronic rejection within the first post-transplant year [107].

It is also increasingly apparent that miRNAs constitute a new class of promising biomarkers for the prediction of allograft rejection. As discussed above, miRNAs are secreted into body fluids and their expression is extremely stable. Several groups have analyzed miRNA expression profiles in PBMC [108, 109], serum [110], urine [111–113] and allograft biopsies [82] from transplanted patients and have identified candidate miRNA biomarkers of rejection (reviewed in detail in [85]). Anglicheau et al. profiled the expression pattern of miRNAs in kidney graft biopsies and in peripheral blood and identified 7 miRNAs that were upregulated and 10 that were downregulated in biopsies of patients undergoing acute rejection [82]. Among those identified, miR-155 and miR-223 are established to function in the regulation of EC angiogenic and inflammatory responses [114–117], and to increase over the course of immune-mediated EC injury in diabetic nephropathy, atherosclerosis [118, 119] and in association with EndMT [120]. Scian et al. correlated tissue miRNA signatures with profiles identified in paired urine samples [112]. They found that miR-142-3p, miR-204 and miR-211 were expressed in both tissue and urine in association with chronic kidney allograft rejection, suggesting that it is possible to monitor miRNAs in urine as an effective diagnostic of chronic rejection. Maluf et al. also identified 22 urine miRNAs that were selective for chronic rejection [113], and performed a longitudinal analysis to define a subset that were expressed prior to histological evidence of allograft injury. Collectively, these findings suggest that changes in the expression of specific miRNAs within the urine may be reflective of an intragraft phenotype that is associated with chronic rejection. However, large-scale multicenter studies will be required to validate their potential to serve as predictive biomarkers of risk for chronic rejection prior to their future use in clinical trials.

Summary

An increasing body of data indicates that vascular injury and repair and homeostatic angiogenic responses following organ transplantation occur in a dynamic manner post transplantation. Clearly, events associated with efficient repair of the microvascular

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endothelium after ischemia-reperfusion injury and alloimmune-mediated damage will ensure long term graft survival [17, 32]. However, sustained and uncontrolled microvascular injury and repair responses including leukocyte-induced angiogenic responses ultimately result in local tissue hypoxia and the induced expression of HIF-1α regulated genes [31–34]. The paradigm described in this review identifies all these events as key features of the initiation of chronic rejection. In this manner, these microvascular changes can induce growth factors and gene profiles that alter the intragraft microenvironment, and many events can be assessed as biomarkers. To date ongoing studies have validated the use of this paradigm for the development of early disease biomarkers. Ongoing studies have demonstrated that the overexpression of angiogenic factors including VEGF-A and VEGF-C and miRNAs associated with vascular stability serve as biomarkers of disease initiation and progression.

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Key summary points

1. Microvascular endothelial cells (EC) respond to alloimmune targeting of the graft by sequential changes including EC activation and leukocyte-induced angiogenesis.
2. These changes in EC phenotype result in a proinflammatory intragraft microenvironment that favors the development of chronic rejection.
3. Regulators of mTOR-mediated signaling within EC are of critical importance in the regulation of microvascular stability within this microenvironment and the inhibition of chronic rejection.
4. miRNAs also constitute a major class of regulators of EC stability post-transplantation.
5. Monitoring intragraft EC molecular events that participate in the establishment of a proinflammatory microenvironment may provide biomarkers for the early detection of chronic rejection.

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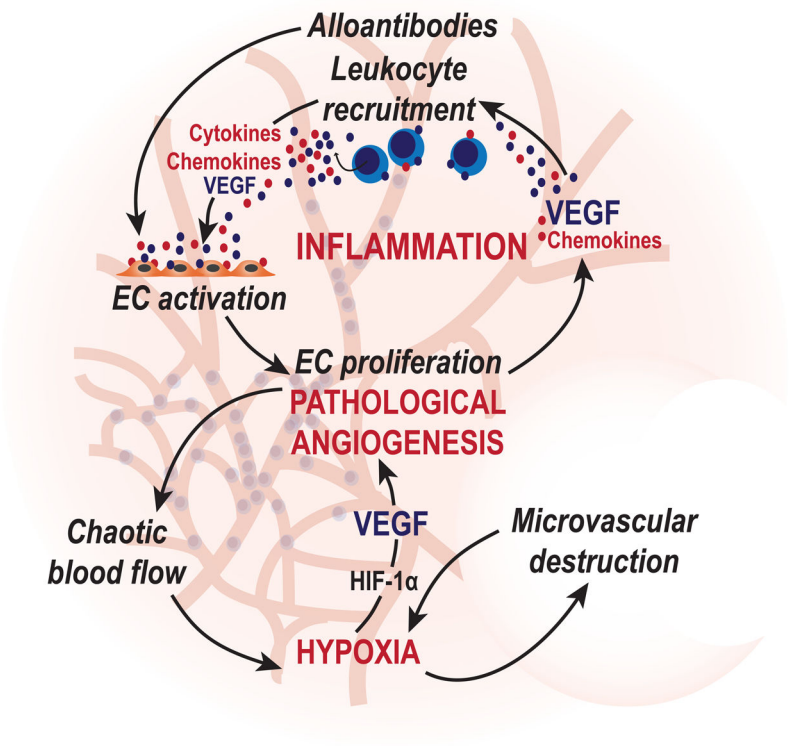

Figure 1. Cartoon illustrating how changes within the microvasculature may define the initiation and progression of chronic allograft rejection

Following organ transplantation, alloimmune targeting of microvascular endothelial cells (EC) is the earliest event in the initiation of a proinflammatory intragraft microenvironment. Once targeted by cellular or humoral immunity, EC become activated and express MHC class I and II molecules, adhesion molecules and chemokines that promote ongoing recruitment and activation of leukocytes in the graft. These events also result in EC proliferation and the process of leukocyte-induced angiogenesis. Uncontrolled EC proliferation that occurs at early times in association with mononuclear cell infiltration leads to the formation of abnormal microvascular networks that in turn can result in chaotic blood flow patterns and local tissue hypoxia. Microvascular loss resulting from persistent injury to the EC amplifies tissue hypoxia, and all these events drive hypoxia-inducible factor (HIF)-1α-dependent activation of growth factors and chemokines. We suggest that the overexpression of VEGF-A by infiltrating leukocytes and by the graft itself in response to hypoxia is central to this cycle of events within the microenvironment.

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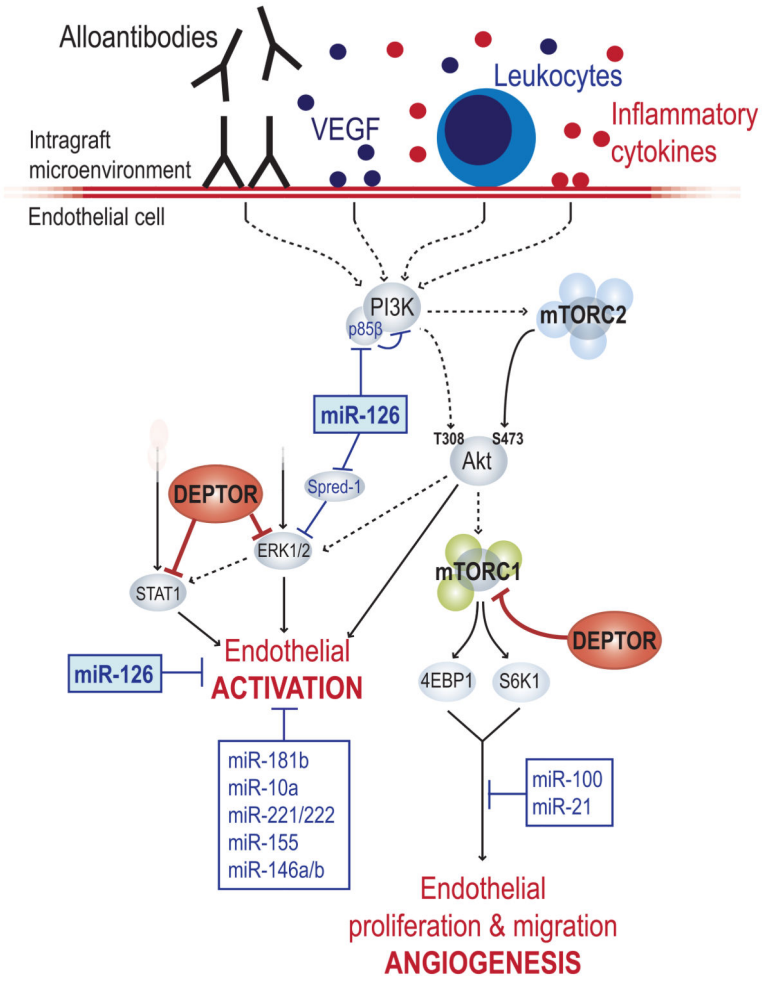

Figure 2. Cartoon illustrating a central role for mTOR signaling in EC in the pathogenesis of chronic allograft rejection

Upon binding to graft microvascular endothelial cells (EC), leukocytes, inflammatory cytokines, growth factors (incl. VEGF, Figure 1) and alloantibodies activate PI3K/Akt/mTOR-mediated signals. Assembly of mTORC2 complex results in the phosphorylation and activation of the Akt kinase, that in turn activates mTORC1 and its substrates 4EBP1 and S6K1. Collectively, these signals result in EC activation, migration and proliferation. In addition, Akt-mediated signals enhance the activity of ERK1/2, which also promotes EC activation. The endogenous protein DEPTOR inhibits mTORC1 and ERK1/2, as well as STAT-1 signaling in EC, highlighting its major function as a cell-intrinsic regulatory of EC activation responses. Several miRNAs have been found to control the activity of the mTOR pathway as well as endothelial cell activation. Notably, endothelial specific miR-126 functions to inactivate both PI3K/Akt- and ERK1/2-mediated signals and thus maintain EC quiescence.