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Original Articles

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Severe venous neointimal hyperplasia prior to dialysis access surgery

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Abstract

Background. Venous neointimal hyperplasia is the most common cause of arteriovenous (AV) fistula and graft dysfunction following dialysis access surgery. However, the pathogenetic impact of pre-existing venous neointimal hyperplasia at the time of AV access creation on final clinical success is currently unknown in the setting of advanced chronic kidney disease (CKD) and end-stage renal disease (ESRD) patients. The aim of this study was to perform a detailed histological, morphometric, and immunohistochemical analysis of vein specimens in advanced CKD and ESRD patients collected at the time of new vascular access placement.

Methods. Vein samples from 12 patients were collected at the time of AV access creation near the site of AV anastomosis. Histological, immunohistochemistry and morphometric studies were performed on these vein samples.

Results. Examination of the tissue specimens obtained at the time of surgery showed neointimal hyperplasia in 10 of 12 specimens, ranging from minimal to very severe. The majority of cells within the neointima were myofibroblasts with a minority of contractile smooth muscle cells present.

Conclusion. Our work represents a detailed description of the morphometric and cellular phenotypic lesions present in the veins of CKD and ESRD patients, prior to dialysis access placement. These studies (i) suggest the future possibility of a new predictive marker (pre-existing venous neointimal hyperplasia) for AV dialysis access dysfunction and (ii) open the door for the future development of novel local therapies for optimization of the venous substrate on which the dialysis access is created.

Keywords: arteriovenous access; cellular phenotypes; vascular access stenosis; venous neointimal hyperplasia

Introduction

Vascular access is truly the lifeline for the hemodialysis patient [1–3]! Currently, approximately 1 billion US dollars are spent annually treating complications from vascular access dysfunction [4]. Both arteriovenous (AV) fistulae and AV grafts currently have dismal primary failure rates [5,6] and primary patency rates at 1 year [6,7], respectively, because of the development of venous stenosis. While we and others have carefully characterized the lesion of venous stenosis following AV fistula and graft placement as being secondary to aggressive venous neointimal hyperplasia [8–15], there is currently no good information about either the phenotype or the possible future impact of pre-existing venous neointimal hyperplasia on AV access success or failure.

The aim of this study, therefore, was to perform a detailed histological, immunohistochemical and morphometric examination of venous tissue samples from advanced chronic kidney disease (CKD) and prevalent hemodialysis patients, collected prior to dialysis access creation.

Materials and methods

Study population

Over a 3-month period, we screened 24 patients for entry into the study. Two patients were excluded because of pre-existing narrowing of the venous system on the pre-operative access mapping and 10 patients were excluded because of an inability to obtain adequate tissue at the time of surgery or because they refused to participate in this study. A total of 12 patient samples were collected for this study. Prior to each new access evaluation, a pre-operative access mapping examination of both extremities with ultrasound or venograms was performed to evaluate vein and artery diameters. A minimal diameter of 2.0 mm for arteries and 2.5 mm for veins were required for construction of an AV fistula [16]. Demographic data were collected at the time of recruitment. Data pertaining to the site of access placement and specific vessel obtained were collected at the

time of surgery. University of Cincinnati Institutional Review Board approval was obtained to conduct this study.

Specimen collection

Venous tissue specimens were collected at the time of surgical creation of vascular access. During the surgery, an ~8 to 10 mm circumferential segment of vein was removed from the same vein used to create the AV access, near the planned anastomosis site in each patient, and immediately fixed in formalin.

Specimen processing

Each venous tissue sample, fixed in formalin, was embedded and cut into two to three tissue blocks of 3–4 mm thickness using previously described techniques [8,10,17]. Following paraffin embedding, each piece was sliced into 5-µm sections for histological, immunohistochemistry and morphometric studies.

Histological and immunohistochemistry studies

Sections from each tissue block were evaluated with a hematoxylin and eosin stain (H&E) and for expression of α-smooth muscle actin (SMA, a smooth muscle cell and myofibroblast marker; DAKO; 1A4, 1:200), desmin (a marker of differentiated contractile smooth muscle cells; DAKO; 1:100), vimentin (a marker of fibroblasts; DAKO V9, 1:200), CD3 (a marker of T cells; DAKO 1:200), CD31 (a marker of endothelial cells; DAKO; 1:40) and Ki-67 (Becton Dickinson; 1:50, a marker of cellular proliferation) and a macrophage marker (PGM-1, DAKO; 1:50) as previously described [8,10]. Routine immunohistochemistry was performed and reported in detail as described previously [8,10]. A brown color on the specimen indicated a positive stain. Negative controls were performed with each assay, by omitting the primary antibody. In addition, positive control tissue (lymph node, small bowel, tonsil or artery) was used to document the efficacy of each antibody.

Morphometric analysis

We have previously detailed the approach for measuring luminal stenosis [8]. Briefly, digital H and E and SMA-stained photographs of one section from each block were projected at final magnifications of ×40, ×80, ×100, ×200 and ×400. Image J software (National Institutes of Health) was used for morphometric analyses. As described in Figure 1, measurements were made of the luminal area (enclosed by the black line, region A), neointimal area (enclosed by the blue line, region B) and medial area (enclosed by the red line, region C). Percentage luminal stenosis was calculated using the formula $(1 - A/C) \times 100$. The ratio of intimal area (Ia) to medial area (Ma) was calculated using the formula $Ia/Ma = (B - A)/(C - B)$. The results for these parameters from each specimen were averaged to obtain mean values for percentage luminal stenosis and intimal to medial area ratios for individual patients, which were then averaged to obtain a mean value for all patients [8,18].

After the above measurements, a point was visually placed in the center of the lumen. Four lines extending from the center of the lumen to the endothelial border were drawn at each of the four quadrants (0°, 90°, 180° and 270°). At the point where each one of these points intersected with the lining of the lumen, a line that was approximately perpendicular to the black and blue lines at that point was drawn. This was used as a measure of intimal thickness (line It). A second line was drawn perpendicular to the blue and red lines starting at the point where 'It' touches the red line. This was used as a measure of medial thickness (line Mt). These lines were used to derive an intimal thickness (It) to medial thickness (Mt) ratio (It/Mt ratio) at each of the 4 quadrants; the mean of these four values was defined as average intima to media thickness for a particular section (Avg It/Mt). Finally, a line was drawn visually for each section at the point of maximal intimal thickness (Max It), and the ratio of maximal intimal thickness to medial thickness (Mt) was measured in an identical manner to It/Mt. This ratio was referred to as the maximal intima to media thickness (Max It/Mt). Comparison of average intima to media thickness with maximal intima to media thickness allows us to perform a scientific assessment of the eccentricity of neointimal distribution within a single cross section. Finally, the maximal intima + media thickness (line Max It and Mt) was measured in order to get an estimate of overall wall thickness. Results of these parameters from all tissue blocks of each specimen were averaged to obtain mean values for average intima–media thickness ratio (It/Mt), maximal intima–media thickness ratio and maximal intima + media thickness for each patient. The values obtained from each patient were averaged to calculate a mean value for patients studied in the analysis.

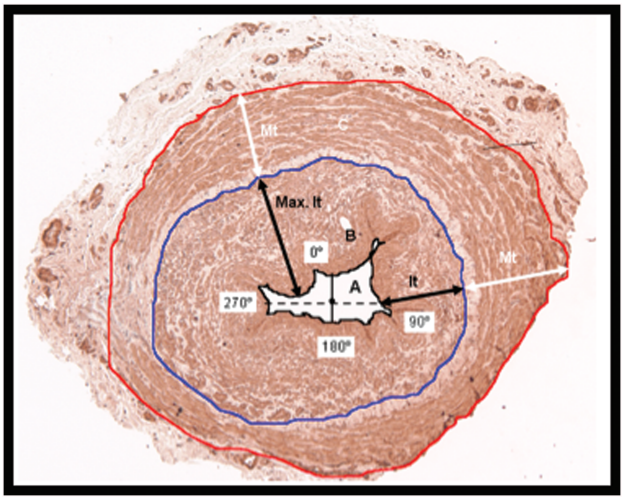

Fig. 1. Morphometric analyses. Methods used for morphometric analyses as described in text. Area enclosed by the solid black line is the luminal area, area enclosed by the blue line is the combined luminal plus neointimal area and the area enclosed by the red is the luminal + intima–media area. Double-headed black arrows show intimal thickness (It) and maximal intimal thickness (Max It) The white double-headed arrows represent medial thickening measurements for average and maximal intimal to medial thickness (Mt) ratios.

Immunohistochemical analysis

Immunohistochemistry was performed to assess cellular phenotypes within the neointima by staining for SMA, desmin, vimentin, PGM-1, Ki-67, CD31 and CD3. Sections were graded using a semiquantitative scoring scale from 0 to 4+, which indicated the percentage of total cells that were positive for the specific marker in different regions of the vessel wall (0 indicates 0–10% positive; 1+ = 11–25% positive; 2+ = 26–50% positive; 3+ = 51–75% positive and 4+ = 76–100% positive) [8,10]. Mean values for the cellular markers for all patients were calculated. These markers were used to identify the relative contributions of myofibroblasts, fibroblasts, contractile smooth muscle cells, proliferating cells, lymphocytes and macrophages within the neointima, media and adventitia using the schema described in Table 2. PGM-1 and Ki-67 could not be graded due to minimal staining.

Statistics

The distribution of study variables was characterized according to means ± SEs, medians and proportions. Associations between degree of pre-existing intimal hyperplasia and maturation failure were determined by univariate logistic regression. All statistical analyses were performed using JMP® 8.0 (Cary, NC) statistical software package.

Results

Demographics

Twelve patients in total were recruited into this study (eight males and four females). The median age of the patients was 67.7 years, five were black, seven had diabetes, seven were pre-emptive (not yet on dialysis) and 11 patients had no previous vascular access placements. Seven patients had an AV fistula placed and five patients had an AV graft

placed. Six cephalic vein, three axillary vein, one brachial vein, one basilic vein and one antecubital vein specimen(s) were collected.

Histomorphometric analysis

All 12 patients had suitable tissue blocks for analysis. Examination of the tissue specimens showed neointimal hyperplasia in 10 of 12 specimens. Among the 10 specimens with neointimal hyperplasia present, the degree of neointimal hyperplasia ranged from minimal to severe (Figure 2b–d). Table 1 shows data representing the percentage of luminal stenosis, intimal to medial area ratio (I/M area), average intima–media thickness and maximal intima–media thickness. The degree of luminal stenosis (4–93%) varied between the 12 samples as did the average intimal to medial thickening (range 0.04–1.49), highlighting the broad spectrum of pre-existing neointimal hyperplasia between patients. All 12 specimens had medial hypertrophy present as compared to the three to four-layer-thick venous media in normal vein (Figure 2a). To demonstrate the aggressiveness of neointimal hyperplasia and medial hypertrophy in vessels obtained at the time of access surgery, Figure 2a shows a vein from a healthy individual without CKD and a normal intima–media thickness. The intimal region, within the normal vein, is only one to two cell layers thick and the media is only three to four cell layers thick. To further demonstrate the aggressiveness of neointimal hyperplasia at the time of surgery, Figure 2e shows stenotic vein obtained at the time of access revision in a prevalent hemodialysis patient. Note that the magnitude of pre-existing venous neointimal hyperplasia (Figure 2d) in some cases is as much as that in a stenotic dysfunctional dialysis access.

Immunohistochemical staining and cellular phenotyping

Figure 3 describes representative samples from patients who had vein tissue obtained at the time of vascular access surgery. In addition to standard H&E staining, these samples were stained with antibodies against alpha SMA, desmin, vimentin, Ki-67, CD3, CD31 and PGM-1. Figure 4 shows the semiquantitative cellular phenotype scores for the neointima, media and adventitia, with Table 2 describing the schema that was used to determine the cellular phenotype. Although the majority of cells within the neointima were positive for SMA and vimentin and negative for desmin (Figure 5) (suggesting a myofibroblast phenotype), a number of sections also had desmin-positive cells suggesting that these are contractile smooth muscle cells (Figure 6). Furthermore, several sections showed the presence of both myofibroblasts and contractile smooth muscles cells adjacent to each other within the same cross section (Figure 5), suggesting the possibility of ongoing phenotypic switching and differentiation/dedifferentiation within the neointima [10]. Within the media, the majority of cells were SMA-positive and desmin-positive contractile smooth muscle cells. However, a number of SMA-positive, desmin-negative and vimentin-positive myofibroblasts were seen within the muscle bundles. Similarly, the majority of cells within the adventitia were SMA-negative, desmin-negative and vimentin-positive fibroblasts. Staining for PGM-1, CD3

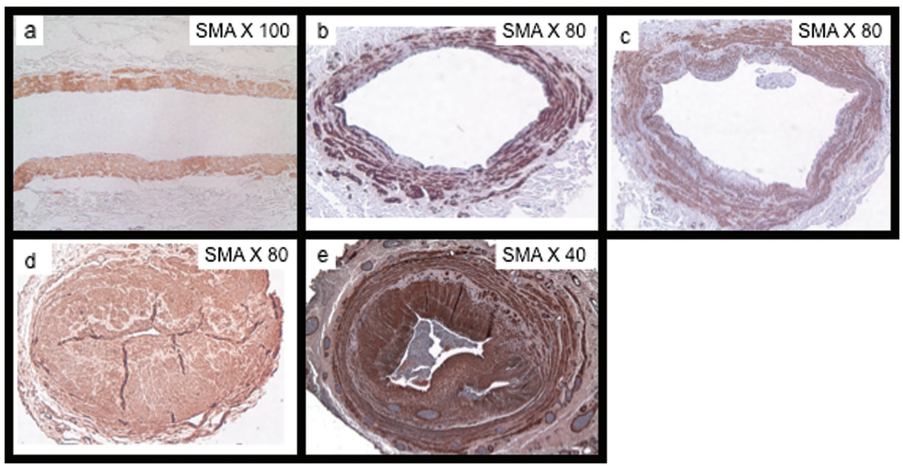

Fig. 2. Histopathology of vein specimens from normal patients to stenosed AVF. (**a**) Shows normal human vein in a patient with no CKD. Note the absence of medial thickness and neointimal hyperplasia (**b**–**d**) shows SMA sections of patients with advanced CKD at the time of AV access placement. Note that neointimal hyperplasia in patients is variable from minimal neointimal hyperplasia to very severe lesions (**e**) shows a human vein in a patient with ESRD with a stenotic AV fistula. Note the aggressive thickening of the neointima and media and significant luminal stenosis that is similar to the lesion prior to access placement in some patients (d).

Table 1. Morphometric analysis values for vein samples

Stenosis (%)	Intimal–medial area ratio	Average intimal–medial thickness	Maximal intimal–medial thickness
46.6 ± 9.3	0.24 ± 0.07	0.34 ± 0.12	1.16 ± 0.30

Table 2. Schemes for cellular phenotyping

	SMA	Vimentin	Desmin	PGM-1
SMC	+	−	+	−
Myofibroblasts	+	+	−	−
Fibroblasts	−	+	−	−
Macrophage	−	+	−	+

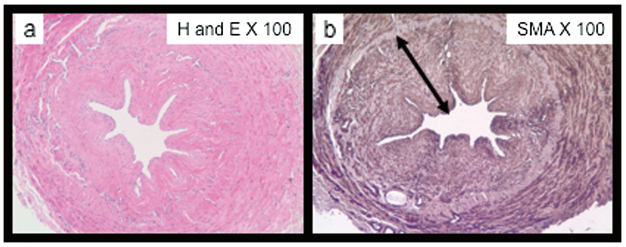

Fig. 3. H and E and SMA of severe pre-existing neointimal hyperplasia. (**A**) H&E) and (**B**) SMA on sequential sections of vein specimen obtained at the time of AV access surgery. Note the very severe degree of neointimal hyperplasia [black double-headed arrows in (B)].

and Ki-67 was minimal or absent within the neointima, media and adventitia in all samples. Figures 7 and 8 show representative staining for CD3 and Ki-67. We saw strong staining for CD31 within the endothelium of the vessel (Figure 9).

Short-term vascular access outcomes

Our data in our accesses after 6 months show that 4/7 fistulas were successfully used for dialysis at 6 months. Three of five grafts were successfully used for dialysis at 2 months. In the fistula group, we found an association between an increasing average It/Mt with maturation failure ($P = 0.03$) and a trend associating increased percentage of luminal stenosis with maturation failure ($P = 0.085$).

Discussion

Our group has previously demonstrated that the most common histologic lesion in both AV fistula non-maturation [8,10] and AV graft stenosis [9,10] is aggressive venous neointimal hyperplasia. Although there have been occasional reports describing pre-existing neointimal hyperplasia in the veins of humans with advanced CKD or end-stage renal disease (ESRD) at the time of vascular access surgery [19–22], none of these prior studies performed a detailed histological, immunohistochemical and morphometric examination of this venous tissue. Our study clearly demonstrates that severe neointimal hyperplasia is in fact present in the veins of advanced CKD and ESRD patients prior to AV access surgery. Interestingly, in our study, we found a spectrum of intima–media thickening and luminal stenosis, as some patients had very little to no neointimal hyperplasia, while other patients had very severe neointimal hyperplasia with near complete luminal occlusion (Figure 2d). In the fistula group, we found an association between increasing average It/Mt and maturation failure and a trend toward association between increasing luminal stenosis and maturation failure but remain cautious of these results given the small sample size. While we believe that some patients may be more prone to developing baseline venous neointimal hyperplasia as a result of an exaggerated vascular (endothelial) response to the effects of uremia, hypertension and other insults, which may affect successful AV use, it is likely also that vascular remodeling (positive versus negative remodeling) after AV creation, particularly in fistulae, plays a key role in determining successful vascular dilatation, despite the amount of pre-existing neointimal hyperplasia or luminal stenosis present [1,2]. Therefore, it is crucial to better understand the pathophysiology and mechanisms of AV access maturation and dysfunction in the setting of mild, moderate and severe venous neointimal hyperplasia prior to access creation.

We found that the predominant cellular phenotype within the region of venous neointimal hyperplasia was the SMA-positive, vimentin-positive and desmin-negative myofibroblast, consistent with our previous studies where we demonstrated that the myofibroblast was the predominant cell type within the neointima in stenotic AV fistula and grafts [8–11]. However, we also found that a smaller (albeit significant) proportion of neointimal cells were SMA-positive, desmin-positive and vimentin-negative contractile smooth muscle cells. The predominant cell type within the media was the SMA-positive, desmin-positive and vimentin-negative contractile smooth muscle cell, but we also were able to identify some SMA-positive, vimentin-positive and desmin-negative myofibroblasts, which presumably had intercalated between the smooth muscle bundles. Finally, within the adventitia, the predominant phenotype present was the SMA-negative, desmin-negative and vimentin-positive fibroblast. Our results suggest several possibilities with regard to cellular migration, differentiation and dedifferentiation. One possibility is that due to uremic and inflammatory insults, there may be a migration of fibroblast cells from the adventitia, through the media and into the intima where they acquire SMA expression and transform into myofibroblasts [1]. Similarly, it is possible that some contractile smooth muscle cells within the media may migrate into the intima and differentiate/dedifferentiate into myofibroblasts by losing desmin expression [23–25]. Alternatively, the mix of cellular phenotypes within the media and intima could also suggest that phenotypic switching from contractile smooth muscles to myofibroblasts and fibroblasts and vice versa is actively occurring within the media and intima. While it is not clear how myofibroblasts migrate into the intima, the predominance of myofibroblasts in the intima is similar to stenotic AV fistulae and likely the most important cellular phenotype within the lesion of neointimal hyperplasia [8]. Therefore, future therapies targeting myofibroblasts and the pathways of phenotypic switching could provide targets for drug therapies to prevent pre-existing neointimal hyperplasia.

While we speculate that inflammation plays a key role in fibroblast migration from the adventitia, our samples showed very limited staining for PGM-1 and CD3. One possible explanation is that the downstream effects of cytokines may be playing the most important inflammatory

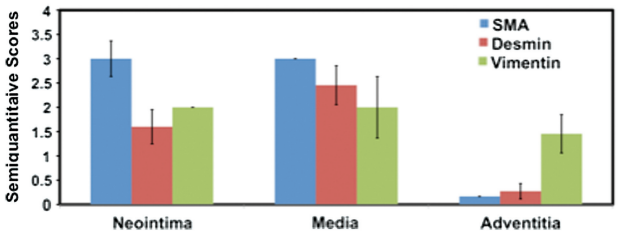

Fig. 4. Cellular phenotyping of venous specimens. Note that the majority of cells in the neointima appear to be SMA-positive, vimentin-positive myofibroblasts, albeit with a reasonable number of SMA-positive, desmin-positive contractile smooth muscle cells. The majority of the cells within the media appear to be SMA-positive, desmin-positive contractile smooth muscle cells with a smaller number of SMA-positive, vimentin-positive myofibroblasts. The adventitia is primarily composed of vimentin-positive, SMA-negative, desmin-negative fibroblasts.

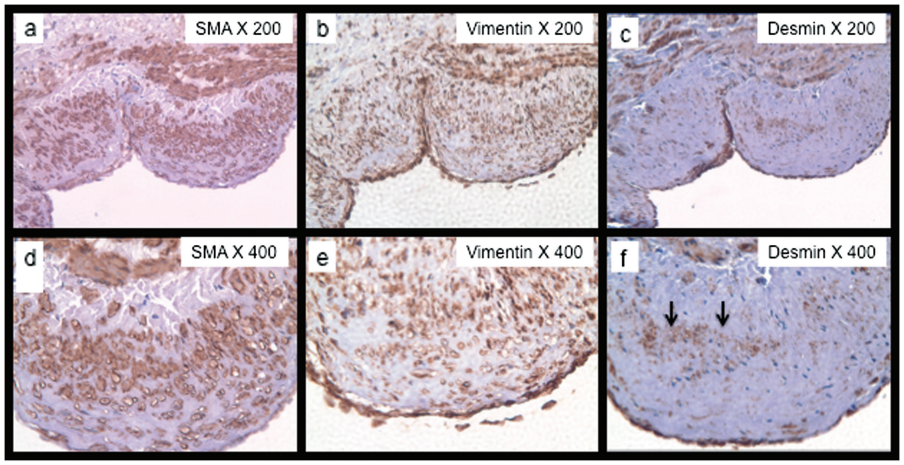

Fig. 5. Cellular phenotyping of neointimal cells representing predominantly myofibroblasts: the expression of smooth muscle cell actin (**a**), vimentin (**b**) and desmin (**c**) within the sequential sections of a patient with neointimal hyperplasia. The majority of cells seen on high-power view are SMA-positive (**d**), vimentin-positive (**e**) myofibroblasts. However, note that a high-power view does show some desmin-positive cells (arrows) (**f**) within the intima–media thickening.

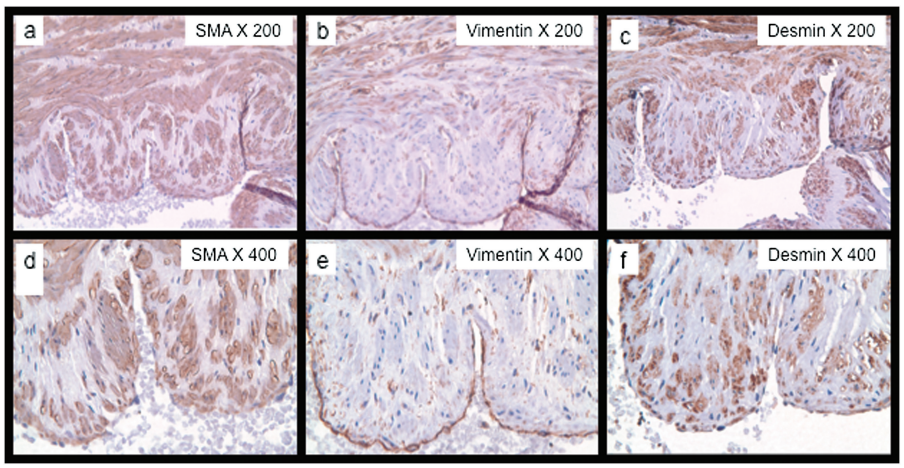

Fig. 6. Cellular phenotyping of neointimal hyperplasia representing predominantly contractile smooth muscle cells. The expression of alpha smooth muscle actin (**a**), vimentin (**b**) and desmin (**c**) within the same section of a patient with neointimal hyperplasia. The majority of cells seen on high-power view in this section as opposed to Figure 5 are SMA-positive (**d**), vimentin-negative (**e**) and desmin-positive (**f**) contractile smooth muscle cells.

role, which assist in smooth muscle cell and fibroblast proliferation and migration. However, we found strong staining for CD31 (an endothelial cell marker).

While many of our samples had extensive intimal hyperplasia, we found minimal staining for Ki-67, a marker of cellular proliferation. The lack of staining for this prolif-

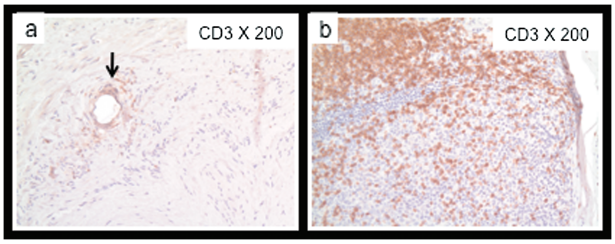

Fig. 7. CD3 stain for lymphocytes. (**a**) Shows minimal staining of a representative vein sample with neointimal hyperplasia for CD3. Note the perivascular pattern of lymphocytic infiltration (arrow). (**b**) Positive control of human tonsil demonstrating significant staining for CD3.

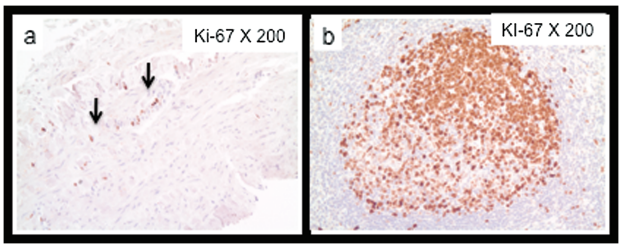

Fig. 8. Ki-67 stain for proliferating cells. (**a**) Shows minimal staining for a representative vein sample with neointimal hyperplasia with only a few proliferating cells present at the intima–media junction. (**b**) Positive control of human tonsil showing significant staining for proliferating cells.

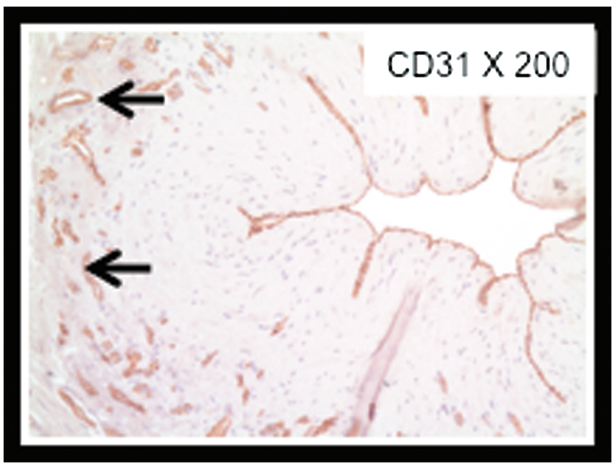

Fig. 9. CD31 stain for endothelial cells. Representative vein sample with severe neointimal hyperplasia showing staining within the endothelium for CD31. Note also the ring of microvessels within the adventitia (arrows). We often used these vessels to determine the outer border of the media.

eration marker may be explained by the fact that our specimens represent a single time point when venous tissue is removed and we may have missed the time frame when maximal proliferation occurs within the cells. This further emphasizes the importance of understanding the sequential mechanisms of intimal hyperplasia formation prior to AV access creation, and uremic animal models where vein samples could be collected at multiple time points would be the best model to evaluate the time frame and sequence of proliferation within vessels.

Our data provide a carefully detailed description and novel insight into the vascular health of patient veins prior to vascular access creation. We have demonstrated that severe neointimal hyperplasia is present in a spectrum of patients that include both pre-dialysis (CKD) and prevalent hemodialysis patients without previous vascular access placement. This may reflect pre-existing changes in veins due to uremia from kidney disease, and in the vessel walls due to age, or other comorbid factors, even before the introduction of blood flow in the arteriovenous anastomosis. The role of uremia and vascular stenosis in AV access dysfunction is relatively unknown. Recent work in animal models has shown that CKD, uremia and oxidative stress accelerate neointimal hyperplasia development in AV fistulas [26,27]. We speculate that the effects of CKD and uremia also play a vital role in the development of pre-existing neointimal hyperplasia and that pre-existing neointimal is associated with impaired AV access maturation, development of early stenosis and poor short- and long-term AV access survival. To date, there are few, if any, truly effective therapies for dialysis access stenosis in AV fistulae or grafts because of the lack of understanding of the cellular and molecular mechanisms that lead to the development of neointimal hyperplasia. Although recent work in the treatment of dialysis access stenosis has focused on local periadventitial drug delivery systems administered at the time of surgery [28–32] and drug therapy after AV access creation [5,7], our work suggests that treatment for dialysis access stenosis may need to begin prior to access placement due to the effects of uremia and other vascular insults on the venous system of patients with CKD/ESRD.

We recognize that our study has several limitations. One limitation is the small sample size and lack of short- and long-term access follow-up. However, this article will be the first of a series of publications that will evaluate, in a larger sample size, the pathophysiology of pre-existing neointimal hyperplasia development and will provide longitudinal data comparing AV access outcomes between patients with pre-existing neointimal hyperplasia and those with normal intima at the time of AV access creation. A second limitation is that we do not have data on previous venipunctures and intravenous line placements prior to access creation but recognize that these factors are potential causes of vessel damage and development of neointimal hyperplasia. However, all patients have pre-operative mapping with either ultrasound or venograms prior to access creation, and vessels with detected stenoses are not used for access creation. A final limitation is that we were not able to perform perfusion–fixation on our vein samples in the operating room. Therefore, our sampled veins may have collapsed down on removal from the patient and this may be a possible explanation for the discrepancy we observed between pre-operative vein mapping data and luminal stenosis.

In conclusion, our results (i) demonstrate a wide spectrum in the magnitude of pre-existing neointimal hyperplasia in advanced CKD and ESRD patients, (ii) highlight the role of the myofibroblast as the predominant cellular phenotype in pre-existing neointimal hyperplasia, (iii) explore the possibility of adventitial migration of fibroblasts to the media and ongoing phenotypic switching within the media and in-

tima, (iv) emphasize the importance of further investigation into the pathogenesis of pre-existing neointimal hyperplasia development and its possible association with short- and long-term AV access outcomes and (v) suggest that novel therapies for dialysis access stenosis may need to begin even prior to vascular access creation.

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