Evaluation of histological techniques for quantifying haemodialysis arteriovenous (AV) graft hyperplasia*

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Abstract

Background. Assessing treatment efficacies for preventing haemodialysis arteriovenous (AV) graft stenosis requires a reproducible method for quantifying intimal hyperplasia. We identified sources of variability in three histological methods for assessing hyperplasia in a porcine AV graft model.

Methods. Carotid-jugular synthetic grafts were placed in pigs. After explantation at 3–6 weeks, the tissue was stained with haematoxylin and eosin (H&E), Masson’s trichrome or elastic tissue Van Gieson (EVG) stains and examined histologically. Hyperplasia at the anastomosis of 14 grafts was quantified using three different methods, each by four blinded observers. These methods were visual scoring, ratio of intima-to-media surface area (I/M ratio), and ratio of intra-graft hyperplasia to graft surface area (H/G ratio) at the graft–vessel interface.

Results. The EVG stain proved superior in delineation of the elastic lamina yet quantification of the intimal and medial layers was still often difficult. This is illustrated by the greater inter-observer median coefficient of variances (CV) found using the I/M ratio method (intimal area CV = 13.7%; medial area CV = 32.7%; I/M ratio CV = 44.0%) than with the H/G method (intra-graft hyperplasia area CV = 7.3%, graft area CV = 5.3%; H/G ratio CV = 6.9%) or by visual scoring (CV = 26.8%). The H/G ratios correlated positively with visual scores (r = 0.941; P = 0.0007; n = 14) and the I/M ratio (r = 0.719; P = 0.0095; n = 14). While hyperplasia was seen in both native vessel and graft lumen, in only one of the 14 anastomoses was the degree of hyperplasia greater in the native vessel than in the graft lumen, suggesting that the degree of hyperplasia occurring within the graft lumen predicted the total hyperplasia around the anastomosis.

Conclusions. The H/G method for assessing hyperplasia is preferred in a porcine model of AV graft because it is quantitative, less variable and does not require the delineation of the elastic lamina, although it infrequently underestimates the total hyperplasia that occurs.

Keywords: arteriovenous PTFE graft; haemodialysis; hyperplasia; intima–media ratio; methodology; porcine model

Introduction

Synthetic haemodialysis arteriovenous (AV) grafts in patients with kidney failure fail at a high rate typically due to underlying neointimal hyperplasia that occurs around the anastomoses between the native vessel and the graft [1]. If the high occurrence of hyperplasia could be prevented in the synthetic grafts, this type of access could be a superior conduit for haemodialysis since they have large luminal diameters and require short periods of time to mature. Thus research is ongoing to develop treatment strategies to inhibit AV graft hyperplasia and prevent graft failure [2–14].

Porcine models of AV graft stenosis have been developed to study anti-hyperplasia treatments [15–17]. The assessment of treatment efficacy, however, requires a reproducible, quantitative method for measuring hyperplasia in such models. The intimal-to-medial ratio (I/M ratio) method is often utilized to evaluate hyperplasia in the coronary arteries [18,19]. Although hyperplasia also occurs at the arterial anastomosis of dialysis vascular accesses in patients [20], it is usually more pronounced at the venous anastomosis. We observed hyperplasia within the arterial anastomosis of the porcine model as well,
but again it is to a lesser degree than what occurs in the venous anastomosis [21]. A disadvantage of the I/M ratio technique is that it requires the delineation of the intimal and medial layers, which are separated by the internal elastic lamina (IEL). The IEL and the external elastic lamina (EEL) are both highly developed and easily discernible in arteries, yet they are typically ill-defined or not discernible in veins in routine histology.

The IEL could potentially be accentuated by special histological stains. Haematoxylin and eosin (H&E) are routine stains for highlighting nuclei and cytoplasm/connective tissue, respectively. Masson’s trichrome stains collagen and muscle fibres. Elastic Van Gieson (EVG) is a stain that specifically highlights elastic fibres. All three stains have previously been used for the evaluation of neointimal hyperplasia at the graft/vessel anastomoses [5,7,13,16]. We examined, in this study, the utility of each stain to facilitate the evaluation of neointimal hyperplasia in our porcine model.

The I/M ratio has also been used to assess hyperplasia in both arterial and venous anastomoses in animal models of vascular access stenosis [4,6,8,13,14,17,22]. However, the appropriateness of this method for assessing hyperplasia in the venous anastomotic tissue has not been thoroughly evaluated. We carried out the following studies to evaluate the I/M ratio and two other methods of assessing venous hyperplasia in a porcine AV graft model and have examined sources of variability with each.

**Subjects and methods**

**Porcine model**

Tissue sections were obtained from a series of seven animals without prior knowledge of the results of the comparisons described subsequently. Anaesthetized Yorkshire cross-domestic pigs, weighing ~30 kg, were used for graft implantation. Under sterile conditions, 7 cm long, 6 mm internal diameter, spiral-reinforced expanded polytetrafluoroethylene (PTFE) grafts were placed between the common carotid artery and the ipsilateral external jugular vein. The former was similarly attached such that the graft loop lay in the cranial direction. The clamps were removed and the anastomoses were inspected for patency and haemostasis. The procedure was repeated on the contralateral side. A total of 200–150 units/kg sodium heparin was given during the surgery. Aspirin EC (81 mg/day) (Pharmaceutical Formulations, Edison, NJ), clopidogrel (225 mg/day) (Bristol-Myers Squibb, New York, NY) and enrofloxacin (5 mg/kg) (Baytril, Bayer, Pittsburgh, PA) were administrated peri-operatively. All animal work was performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Utah and Veterans Affairs Salt Lake City Healthcare System, and conformed to the guidelines established by the *Guidelines for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Grafts remained patent until euthanasia in all animals used as determined by weekly Doppler ultrasound and confirmed by histology after euthanasia.

**Tissue preparation and histological analysis**

At 3–6 weeks, the animals were euthanized by pentobarbital sodium (80–100 mg/kg) injected intravenously. The lumen of the grafts and adjoining arteries and veins were rinsed with saline followed by 10% zinc formalin perfusion in situ, and the native vessels were then severed at ~3 cm on either side of the graft anastomoses. The artery, vein and graft were explanted en bloc and then fixed in 10% zinc formalin overnight. The graft and vessels were cut into five 5 mm blocks in the longitudinal direction to yield lumen cross-sections with one block containing the graft/vessel anastomosis, two blocks containing the consecutive upstream vessel regions and two blocks containing the consecutive downstream vessel regions. These blocks were then embedded in paraffin and 5 μm thick sections were made and stained with H&E, Masson’s trichrome stain or EVG (Richard Allan Scientific, Kalamazoo, MI). The latter was based on Verhoeff’s technique that stains elastic lamina black, collagen red and the remainder yellow. For comparison, carotid arteries and jugular veins from normal animals without graft implantation were also obtained and prepared similarly.

**Morphometric analysis**

Digitized images of the H&E-, trichrome- and EVG-stained histological cross-sections of the graft/vessel anastomoses with various magnifications were obtained with a charge-coupled device (CCD) camera connected to a dissecting light microscope. The images were viewed at the highest magnification that would allow visualization of the entire graft/vessel anastomosis cross-sectional area in a single field. Surface areas of tissue and graft in the cross-sections were acquired using commercial (Bioquant Image Analysis Software, v.6, Bioquant Corporation, Nashville, TN) or non-commercial (ImageJ http://rsb.info.nih.gov/ij/) softwares. Fresh thrombi, when present, were discernible from hyperplasia within the stained tissue sections by their distinctive red colouration and absence of smooth muscle cells; they were not included in the measurement of hyperplasia cross-sectional area.

In each histological section, three methods were used to assess hyperplasia. With the first method, I/M ratio, the intimal tissue was delimited on one side by the IEL, and on the other side by the vessel and graft lumen. The medial tissue was defined as the tissue found between the IEL as inner boundary and the EEL as outer boundary. The observers were also instructed to utilize the appearance of the adventitia to help discern the outer media boundary as the adventitia layer develops a more reddish hue with EVG staining and often contains multiple neovessels.

In the second method, the hyperplasia-to-graft ratio (H/G) was obtained by drawing a straight line across the ‘mouth’ of the graft, then measuring the cross-sectional area of the
hyperplastic tissue delimited by this line and the graft wall (Figure 1). The cross-sectional area of the graft in the same section was measured and used as a normalization factor. The H/G ratio was thus defined in this technique as hyperplasia that developed within the lumen of the graft divided by the graft area.

In the third method, visual scoring was carried out by ranking the amount of hyperplasia formed on a scale of 0–5 (0 = no hyperplasia and 5 = lumen completely occluded by hyperplasia, not fresh thrombosis) with 0.5 increments of scale. Each of the four observers employed each of the three methods to evaluate each histological slide individually and independently from each other.

**Statistics**

The mean, standard deviations and percent coefficient of variances (%CV) of the cross-sectional areas of each parameter (H, G, I, M and visual scores) obtained by the four observers were determined in each of the 14 tissue sections of graft–vein anastomoses. The %CVs of area measurements for each tissue section for each parameter were then combined. The median %CV, 10, 25, 75 and 90th percentiles and minimum and maximum values for area measurements were calculated, as illustrated in Table 1 and displayed in box plots (Figure 2). The correlation of H/G ratios with visual scores, and the correlation of H/G ratios with I/M ratios, were calculated by Spearman’s rank correlation (StatView, v. 4.57, Abacus Concepts, Inc., Berkley, CA). Differences between means of the %CVs of the five parameters (H, G, I, M and visual scores) were tested using a Kruskal–Wallis non-parametric analysis of variance by ranks.

**Results**

Figure 3 shows the typical morphology of a normal porcine carotid artery and jugular vein. The IEL and EEL are clearly visible in the normal artery; the intimal, medial and adventitial layers are therefore distinctly demarcated by these anatomical boundaries. However, in the normal vein, the EEL is not clearly visible and the IEL is much thinner and discontinuous. Thus, even in normal venous tissue, these anatomical boundaries are not as well defined as in arterial tissue.

We next examined the utility of the histological stains H&E, Masson’s trichrome and EVG for the evaluation of hyperplasia in our porcine model.

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**Figure 1.** The H/G method. A tissue section of a graft/venous anastomosis explanted 3 weeks post-graft placement was stained with EVG and digitally photographed at 1× on a dissecting microscope. Using NIH Image J software, a line was drawn across the mouth of the graft, and the area of hyperplastic tissue growing within the lumen of the graft (H1 and H2), and the area of the graft (G) was measured. The intima and media layers within the lumen of the graft are not measured separately with this method. Hyperplastic tissue area (H1 + H2) is divided by graft area to obtain the H/G ratio.

**Table 1.** Evaluation of variability associated with H/G ratio, I/M ratio and visual scoring methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameter Description</th>
<th>Section no. (3 of 14 shown)</th>
<th>Mean ± SD of four observers’ measurements</th>
<th>%CV of four observers’ measurements for each section [(SD/mean) x 100]</th>
<th>Median %CV for all 14 sections</th>
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</thead>
<tbody>
<tr>
<td>H/G</td>
<td>Intra-graft hyperplasia area (H) (mm²)</td>
<td>1</td>
<td>3.19 ± 0.15</td>
<td>4.8</td>
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<td>7</td>
<td>1.80 ± 0.13</td>
<td>7.2</td>
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<td></td>
<td></td>
<td>14</td>
<td>6.93 ± 0.40</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Graft area (G) (mm²)</td>
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<td>9.16 ± 0.56</td>
<td>6.2</td>
<td>5.3</td>
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<td></td>
<td></td>
<td>7</td>
<td>6.59 ± 0.32</td>
<td>4.8</td>
<td></td>
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<td></td>
<td></td>
<td>14</td>
<td>7.11 ± 0.34</td>
<td>4.7</td>
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<tr>
<td>I/M</td>
<td>Intimal area (I) (mm²)</td>
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<td>4.10 ± 0.52</td>
<td>12.7</td>
<td>13.7</td>
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<td></td>
<td>7</td>
<td>1.78 ± 0.29</td>
<td>16.4</td>
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<td></td>
<td></td>
<td>14</td>
<td>6.72 ± 0.34</td>
<td>16.9</td>
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<tr>
<td></td>
<td>Media area (M) (mm²)</td>
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<td>8.79 ± 0.52</td>
<td>5.9</td>
<td>32.7</td>
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<td>7</td>
<td>2.52 ± 1.10</td>
<td>42.5</td>
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<tr>
<td></td>
<td></td>
<td>14</td>
<td>1.64 ± 0.60</td>
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<tr>
<td>Visual</td>
<td>–</td>
<td>1</td>
<td>1.3 ± 0.29</td>
<td>23.1</td>
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<tr>
<td></td>
<td>scoring</td>
<td>7</td>
<td>0.9 ± 0.32</td>
<td>36.5</td>
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<tr>
<td></td>
<td></td>
<td>14</td>
<td>3.4 ± 0.54</td>
<td>15.8</td>
<td></td>
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</tbody>
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*Fourteen graft/venous anastomosis tissue sections were each evaluated by four independent observers using the H/G method, the I/M method and visual scoring. The %CV from the four observers’ measurements of each parameter (intra-graft hyperplasia area, graft area, intimal area and medial area) and visual scoring, for each tissue section, was determined. Only data from three samples, selected by location (1 – beginning, 7 – middle and 14 – end position) in the analysis process, are shown to simplify the presentation.*
In sections of a graft/arterial anastomosis stained with H&E, the adventitial and medial boundaries could be readily discerned (Figure 4A) as the EEL is thick and uninterrupted in arteries. However, the intimal/medial boundaries were much more distinct in a serial section from the same graft/arterial anastomosis stained with EVG (Figure 4B). In contrast to arterial sections, when graft/venous anastomosis tissue sections were stained with H&E, discernment of the intimal/medial boundaries was difficult (Figure 5A), as the IEL was often not visible even at higher power (Figure 5B). When a serial section from the same graft/venous anastomosis was evaluated using trichrome stain, the medial layer stained primarily blue-green, whereas the intimal layer appeared mostly pinkish white, but the borders between the intima and media were still not consistently demarcated (Figure 5C). At higher power, the IEL could faintly be discerned (Figure 5D). In contrast, the lamina becomes highly visible upon EVG staining (Figure 5E and F). Another figure provided as supplementary material (This can be found at NDT online: http://ndt.oxfordjournals.org) further illustrates the superiority of EVG staining over H&E and Masson’s trichrome for the visualization of lamina and the demarcation of intima and media. Although the IEL is not very evident in either the H&E-stained sections or the trichrome-stained sections, it is visible in discrete regions within the vein wall upon EVG staining. Although trichrome staining was occasionally useful in differentiating the intima from the media, EVG staining consistently provided superior visualization of the lamina layers.

The I/M method relies on the ability to delineate the intima from the media. Even with the assistance of the EVG staining, however, delineation of the boundary of the medial layer is often difficult in veins. This is due to the inherently ill-defined nature of the lamina in the venous tissue. In addition, the medial layer may undergo remodelling irrespective of changes in the intimal layer, thus altering the I/M ratio. To circumvent these difficulties, a new method, termed the H/G method, was devised in the present
study that measures only the hyperplastic tissue growing within the graft lumen at the anastomosis. In the H/G method, all tissue found within the lumen of the graft, regardless of medial or intimal anatomical derivation, is considered hyperplasia (Figure 1). The hyperplasia cross-sectional area is then normalized by dividing by the graft cross-sectional area.

To determine which technique is most reproducible, the variability that occurred among hyperplasia measurements obtained by four independent observers for each of the three assessment methods was calculated. These three methods were the I/M ratio, the H/G ratio and visual scoring. Representative histological sections used for these studies are shown in Figure 6, demonstrating the variability in the architecture of the vasculature and variability in the conspicuity of the elastic laminae. The variabilities in each parameter (H, G, I, M and visual score) among the four independent observers for selected tissue sections are presented as the %CV (calculated as standard deviation/mean × 100%) (Table 1) and as quartiles and range (Figure 2). Although large variability occurred in the measurement of both the I and M parameters, the greatest %CV was associated with the M parameter. The median CV of the I/M ratios was 44.0%. In contrast, the median CV of the H/G ratios was only 6.9%.

Although the H/G method appears to be less variable, it evaluates only the intra-graft hyperplasia and not the hyperplasia within the native vessel wall. Therefore, this method may considerably underestimate the total hyperplasia. To address this issue, the relative contributions of intra-graft hyperplasia and native vessel hyperplasia to the total hyperplasia

Fig. 4. EVG stain highlights IEL more effectively than H&E. Serial formalin-fixed, paraffin-embedded tissue sections (5 \(\mu\)m thick) from a graft/artery anastomosis explanted at 3 weeks post-graft placement were stained with H&E (A,B) or EVG (B) and digital images were obtained from a CCD camera attached to a dissecting microscope. The external elastic lamina (EEL) is visible with either stain but the IEL is only clearly visible with EVG staining.

Fig. 5. Comparison of stains for highlighting IEL. Serial tissue sections (5 \(\mu\)m) from a graft/venous anastomosis explanted at 3 weeks post-graft placement were stained with H&E (A,B) or Massons trichrome (C,D) or Elastic Van Gieson (EVG) (E,F) and digital images were obtained from a CCD camera attached to either a dissecting microscope at 1× (A,C,E) or a light microscope at 5× (B,D,F) magnification. EVG highlights the IEL more distinctly than H&E stain in the graft/venous anastomosis. A large fresh thrombus is present in the lumen of this tissue section.
surface area were calculated (Figure 7). These data showed that, in nine out of 14 sections, the intra-graft hyperplasia accounted for the majority if not all of the hyperplasia within the section. In four out of 14 sections, the H/G method underestimated the total hyperplasia by 38–42%, and in only one section out of 14 was the hyperplasia within the native vessel greater than that observed within the graft lumen. Thus the H/G method seldom significantly underestimated the amount of hyperplasia that occurred within an anastomosis cross-section. Further, when H/G ratios from the 14 tissue sections were compared with the visual scores (which included the hyperplasia in the native vessels) or the I/M ratios of the same sections, a highly positive correlation was observed in each comparison (Figure 8).

Discussion

The purpose of this study was to evaluate three different methods for assessing hyperplasia at the graft/venous anastomoses of a porcine model of AV graft stenosis. A method for the assessment of hyperplasia should be reproducible as well as quantitative. We have previously employed the visual scoring method as an assessment tool for the efficacy of anti-proliferative strategies [2,21]. Visual scoring is, however, only subjective, semi-quantitative and, as shown in the present study, associated with high inter-observer variability (Table 1, Figure 2). The I/M ratio is commonly used for the assessment of arterial hyperplasia in the scientific literature, yet this method had not previously been assessed critically for use in the evaluation of venous anastomotic hyperplasia.

In contrast to arteries, veins have poorly developed IEL and EEL (Figure 3A and B). We found that the choice of histochemical stain can markedly influence the ability to discern between the intimal and medial layers in venous tissues, with the EVG stain yielding the best quality for this purpose. However, even in the presence of EVG stain that optimally highlights these layers, the intima and media of the native venous wall are still often difficult to delineate. Consequently, we found that the I/M ratio suffered from high inter-observer variability, reflecting that subjective observer determination of intimal and medial boundaries significantly influenced the I/M ratios. The variability in M was particularly large (32.7%, Table 1) and usually the more significant contributor to the variability in the I/M ratio. Such variability in the assessment of hyperplasia could significantly influence the accuracy of assessment of treatment strategies. Additionally our laboratory and others have observed that the medial layer can become considerably thickened, regardless of whether intimal hyperplasia has occurred or not. Such remodelling is an adaptive phenomenon that sometimes occurs in response
to increased blood pressure or other mechanical factors [4,23,24]. An anastomosis that shows intimal hyperplasia in the presence of medial thickening would have a lower I/M ratio compared with an anastomosis with the same degree of intimal hyperplasia in the absence of medial thickening. This decreased ratio would erroneously suggest a positive treatment effect on hyperplasia when in fact there is no effect.

For these reasons, we propose a novel parameter for the assessment of hyperplasia, the H/G ratio. In the H/G method, only hyperplasia that grows within the lumen of the graft is measured and it is normalized to the graft area within the same histological section. We, and others, have observed that hyperplasia principally forms around the anastomoses in the porcine model [8,17]. However, since stenosis occurs more often at the venous anastomosis, but far less information is available on the techniques of assessing hyperplasia at this location, the venous anastomosis was the focus of this study. We have found that hyperplasia occurred largely within the graft lumen and never only in the native vessel wall. Thus, intra-graft hyperplasia is largely representative of total anastomosis hyperplasia (Figure 7).

The surface cross-sectional graft area is largely unaffected by physiological forces that can cause distortions to the native vessels or remodelling in medial layers; therefore, the graft area provides a more optimal area for normalization. As expected, graft area and intra-graft hyperplasia measurements showed...
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significantly less inter-observer variability than the intima and media measurements. Accordingly, the H/G ratio showed less variability than the I/M ratio. Rotmans et al. [6] previously reported the separate measurement of the tissue within the lumen of the graft and referred to it as the ‘shoulder region’. In addition, Luo et al. [8] and Cagiannos et al. [7] recently reported using graft area for normalization of intimal area at the venous anastomosis. We propose that the H/G ratio technique be a standard method to measure hyperplasia at the graft/venous anastomosis, but that intima and media areas in the native vessel should also be evaluated with the awareness of the inherent variability associated with each of these methods.

In conclusion, many previous studies have focused on hyperplasia in the artery arising from atherosclerosis or restenosis after angioplasty, and have not focused on hyperplasia in the veins and the grafts. Because of the high prevalence of stenosis at the venous–graft anastomosis, the high variability of the I/M ratio and the presence of the graft that provides an ideal normalization standard, the H/G ratio should be exploited as a standard parameter for the assessment of hyperplasia in animal models of arteriovenous grafts, along with the I/M ratio and visual scores.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data can be found at NDT online.

References


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