Microscopic Assessment of Angiogenesis in Tumors

Stephen B. Fox

1. Introduction

Although it has been recognized for many centuries that neoplastic tissue is more vascular than its normal counterpart, it is only since Folkman’s hypothesis on antiangiogenesis (1) that a more quantitative method for measuring angiogenesis in tissue sections has been pursued. Folkman and colleagues recognized that quantitation of the tumor vasculature might play an important role in predicting tumor behavior and patient management. They therefore developed a microscopic angiogenesis grading system, designated the “MAGS” score, calculated by measuring vessel number, endothelial cell hyperplasia, and cytology in tinctorially stained tissue sections (2). It was hoped that this would be an objective method for quantifying tumor angiogenesis, one that would yield important information on the relationship to other clinicopathological tumor characteristics and help in the testing of antiangiogenic therapies. However, although it was possible to classify tumors into endothelial “poor” or “rich,” the technical limitations of sample selection, inter- and intra-observer variation, and conceptual biological problems were such that the technique could not be easily applied. Interest in grading tumor angiogenesis was rekindled in the 1980s with the advent of nonspecific endothelial markers (3–5), but only in the last five to ten years, with the advent of more specific endothelial markers, have quantitation studies on tissues have been performed.

Most studies have employed a method based on that developed by Weidner et al. (6), in which blood vessels are immunohistochemically highlighted and the number of microvessels quantified in the most vascular areas (so called “hot spots”) of the tumor. These studies have confirmed that tumors are more
vascular than their normal tissue counterparts and have shown that microvessel
density is a powerful prognostic tool in many human tumor types [reviewed in
(7)]. Nevertheless, due to limitations in capillary identification and
quantitation, not all investigators have been able to confirm a relationship
between tumor vascularity and prognosis (8–15). This chapter will briefly dis-
cuss the considerations in quantifying tumor angiogenesis in tissue sections,
give the current optimal protocol for assessment, and then outline other candi-
date techniques with potential for the future.

Since endothelium is highly heterogeneous (16), the choice of antibody pro-
foundly influences the number of microvessels available for assessment. Many
such as those directed against vimentin (17), lectin (4,18), alkaline phosphatase
(3), and type IV collagen (11,19,20) suffer from low specificity and are present
on many nonendothelial elements. Others including antibodies to factor VIII
related antigen, the marker used in most studies (6,8,9,21–25), which identi-
fies only a proportion of capillaries and also detects lymphatic endothelium.
The most specific and sensitive endothelial marker currently available is CD31,
which is present on most capillaries and is a reliable epitope for immuno-
staining in routinely handled formalin-fixed paraffin-embedded tissues (26); a
good alternative antigen is CD34, although this antigen is also expressed by
some stromal cells (25).

Although most quantitative vessel studies have been performed on tumors,
the technique can also be applied to normal tissues. The hot spot method might
not be as relevant as vascularity will vary between particular tissue compart-
ments (e.g., acini and stroma in breast, mucosa and submucosa in intestine,
etc.). Nevertheless, in tumors, once the vasculature has been immuno-
histochemically highlighted, it is scanned at low magnification (×40–100) to
identify angiogenic hot spots (6,22). The number of vessels is then quantified
at high magnification (×200–400) in these regions. These areas of high vascu-
ularity are chosen on the likelihood that they will be biologically important.
Tumors naturally have a limited number of hot spots and they would be diluted
if too many were counted. Thus, although the number of hot spots assessed
varies from one to five (6,8–10,22,27,28), most studies have examined three
from a single representative tissue block (see Note 1). Nevertheless, both the
magnification used and its corresponding tumor field area will determine the
vessel number derived from each hot spot. A high magnification, which will
identify more microvessels by virtue of increased resolution (31), used over a
too small an area will always give a high vessel index whereas a low magnifi-
cation over too large an area will dilute out the hot spot. It is thus recom-
mended that three regions are examined using a microscope magnification
of between ×200 and ×400 (see Note 2), which corresponds to field areas of 0.74–0.15 mm² depending on the microscope type (32,33).

Although less subjective than identifying angiogenic hot spots (33), the process of counting vessels has also resulted in significant variation in published series. This has been recently emphasized in the study of Axelsson et al. (15) where the authors, after an initial training period with Weidner, who defined the criteria as to what constituted individual microvessels (see Subheading 3.), did not observe a correlation between microvessel density and patient survival. Even experienced observers occasionally disagree as to what constitutes a microvessel. To overcome these problems, after selection of each hot spot, a 25-dot Chalkley microscope eyepiece graticule (34) has been used to quantify tumor angiogenesis (see Subheading 3.). This method is not only objective, because no decision is required as to whether adjacent stained structures are separate, but rapid (2–3 min per section), reproducible, and gives independent prognostic information in breast (32,35) and bladder (36) cancers. Thus, it is currently the preferred method contained in a recent multicenter discussion paper (33).

The final consideration in quantifying angiogenesis is the differences in the value used for stratification into different study groups. This alone will result in different conclusions being drawn from the same data set. Studies have used the highest, the mean, the median (31), tertiles (32), mean count in node-negative patients with recurrence (23), or variable cut-offs given as a function of tumor area (8,22) or microscope magnification (28). The median and tertile groups do not make assumptions about the relationship between tumor vascularity and other variables, including survival, and is therefore useful clinically. However, there is some loss of information making it optimal to use continuous data where possible. Arbitrary cut-points should be avoided.

2. Materials

1. Silane-coated microscope slides.
2. Dry incubator/oven at 37°C.
3. Citroclear (HD Supplies) or xylene.
4. Graded alcohols (100%, 90%, and 70% ethanol).
5. 5% H₂O₂ in methanol.
6. Phosphate-buffered saline (PBS).
7. Tris-buffered saline (TBS).
8. Protease type XXIV (Sigma).
9. Monoclonal antibody against human PECAM/CD31 (JC70a, Dako, UK).
10. Alkaline phosphatase antialkaline phosphatase (APAAP) kit (Dako, UK).
11. Streptavidin-biotin complex (StreptABC) kit (Dako, UK).
12. Levamisole (Sigma).
13. Aqueous mountant.

3. Methods

3.1. Staining procedure

It should be emphasized that time must be devoted to optimizing the immunohistochemical staining procedure, since quality staining with little background greatly facilitates assessment. Many histopathology laboratories are well versed in immunohistochemistry, necessitating only minor adjustments to the preferred protocol outlined below.

1. Cut 4 µm formalin-fixed paraffin-embedded sections (see Note 3) of the representative tumor block (see Note 4) onto silane-coated slides.
2. Dry at 37°C overnight in an incubator (see Note 5).
3. Dewax using citroclear or xylene for 15 min before passing through graded alcohols into water before placing in PBS for 5 min (block endogenous peroxidase if using StreptABC; see step 8).
4. Pretreat sections with 12.5 mg protease type XXIV/100ml PBS for 20 min at 37°C (see Note 6).
5. Place in TBS (for APAAP) or PBS (for StreptABC) for 5 min, rinse, and apply JC70a (10mg/mL) primary antibody for 30 min.
6. Follow with standard APAAP or StreptABC immunohistochemistry (see Note 7).
7. APAAP (Dako, UK): This method uses a soluble enzyme antienzyme antibody complex (calf intestinal APAAP) to act on new fuschin substrate. The primary and final antibody complex is bridged by excess rabbit antimouse antibody, which binds to the primary mouse antibody with one Fab leaving an Fab site free to bind the tertiary complex. Repeated rounds of secondary and tertiary antibodies amplifies staining intensity. The enzyme hydrolyzes the naphthol esters in the substrate to phenols, which couple to colorless diazonium salts in the chromogen to produce a red color. Endogenous alkaline phosphatase is inhibited by the addition of 5 mM levamisole, which does not inhibit calf intestinal alkaline phosphatase. Incubation steps of 30 min with washing 3× in TBS with two rounds of intensification of 10 min should be used. Rinse in tap water for 2–3 min, tap dry, and mount in aqueous mountant.
8. StreptABC (Dako, UK): This method uses the high affinity of streptavidin for biotin. After application of the primary antibody, a biotinylated goat antimouse antibody at 1/40 is overlayed. The tertiary antibody complex of streptavidin-biotin-horseradish peroxidase (HRP) is then applied. The open sites on the streptavidin complex to the biotin on the secondary antibody. The brown endproduct is formed as 3’3-diamino benzidine HCl (DAB) is oxidized when it donates electrons to activate the HRP/H2O2 reaction. Blocking of endogenous peroxidase in paraffin-embedded tissues is performed by incubation of the sec-
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...tion for 20 min in 0.5% H₂O₂ in methanol. Incubation of antibodies for 30 min with three washing steps of 5 min in PBS should be used. Rinse in tap water for 2–3 min, tap dry and mount in aqueous mountant.

9. Confirm satisfactory staining using normal entrapped vasculature as an internal positive control.

10. An optional parallel negative control section using an IgG₁ isotype antibody can also be run.

3.2. Assessment of Tumor Angiogenesis

Most studies use two observers over a conference microscope. The two investigators identify the hot spots and then independently quantify the vessels. Occasional discrepancies are resolved by consensus. Nevertheless, single observers can also accurately quantify tumor angiogenesis. Most solid tumors such as colorectal and breast cancers elicit a similar pattern of neovascularization and do not pose problems in counting. However, papillary tumors, including those of the bladder and thyroid, are difficult to interpret since each papillary formation, characteristic of its morphology, contains a vascular core. Care should thus be taken when assessing tumors with similar growth pattern.

3.2.1. Identification of Hot Spots

The three hot-spot areas containing the maximum number of discrete microvessels should be identified by scanning the entire tumor at low power (×40 and ×100) (Fig. 1). This is the most subjective step of the procedure, because the experience of the observer determines the success in identifying the relevant hot spots (37). Poor selection will in turn lead to an inability to classify patients into different prognostic groups.

Therefore, it is recommended that inexperienced observers be trained, ideally by comparing their hot spots with those chosen by an experienced investigator, and this performed continually on different series until there is >90% agreement. Training can be completed by assessing sections from a series already known to contain prognostic information (33).

Inexperienced observers tend to be drawn to areas with dilated vascular channels, often within the sclerotic body of the tumor. This is a function of the human eye, which is more sensitive to detecting vascular area than microvessel number. It is important to overcome this natural tendency and identify the maximum number of discrete microvessels. The central areas of the tumor often contain dilated vessels, and these regions, together with necrotic tumor, should be ignored. Vascular lumina or the presence of erythrocytes are not a requirement for a vessel to be considered countable. Indeed, many of the microvessels
have a collapsed configuration. Although the hot spot areas can occur anywhere within the tumor, they are generally at the tumor periphery making it important to include the normal-tumor interface in the representative area to be assessed. Vessels outside the tumor margin by one ×200–250 field diameter and immediately adjacent benign tissue should not be counted. The procedure takes 2–5 min.

3.2.2. Chalkley Counting

Once selected, a 25-point Chalkley point eyepiece graticule (see Note 8) is used to quantitate vascularity (34). The graticule covers only a proportion of the microscope field area, so a magnification of ×200–250 is used (see Note 9). The graticule should then be oriented over each hot spot region so that the maximum number of graticule points are on or within areas of highlighted vessels (Fig. 2). It is worthwhile trying several graticule positions because indices can vary greatly depending on its alignment. Particular care should also be taken in the occasional case (<1% breast cancers) where antiCD31 has stained tumors with an intense plasma cell infiltrate. This can mimic a hot spot and obscure the underlying tumor vasculature. Plasma cells can otherwise be disregarded on morphological grounds (see Note 10). The mean of the three

Fig. 1. The tumor is scanned at low power (×40–100) (center), and the three areas that contain the highest number of discrete microvessels are selected.
Chalkley counts is then generated for each tumor and used for statistical analysis. The procedure takes 2–3 min.

### 3.3.3. Intratumoral Microvessel Density

For this index counting can be performed using a magnification of between ×200–400. Any endothelial cell or EC cluster separate from adjacent microvessels, tumor cells, or matrix elements is considered a countable vessel. Those which appear to be derived from the same vessel should also be counted if distinct. Again, vessel lumens and erythrocytes are not included in the criteria defining a microvessel. There is no cutoff for vessel caliber. The procedure takes 3–6 min.

### 4. Potential Improvements to Current Methods

#### 4.1. Vascular Grading

Vascular grading facilitates assessment of angiogenesis in tissue sections and is akin to semiquantitative tumor grading. This vascular grading is based
on the subjective appraisal by trained observers over a conference microscope (6,22). Significant correlations between vascular grade and both microvessel density (p = 0.002) and Chalkley count (p = 0.0001) have been demonstrated. The method is reproducible (38), but delineating criteria is difficult owing to the subjective nature of the system, and a considerable investment in time would be required to align the cutoffs required for multicenter studies. However, although there is some loss of power associated with translation of numerical to categorical data, the overall time savings engendered by this technique make it an attractive proposition. Further validation in a large series of randomized patients is warranted, nevertheless, to determine its prognostic utility before being applied in quantitative studies.

4.2. Novel Angiogenic Antigens

Instead of highlighting all the tumor-associated endothelium, an alternative approach would be to identify selectively only the vasculature that is undergoing active neovascularization. This helps to quantify tumor angiogenesis accurately and might also have important implications for antivascular targeting (39). A number of antibodies have been identified that recognize antigens reported to be upregulated in tumor associated endothelium compared to normal tissues. These include EN7/44, endoglin, endosialin, and E-9 (33,40). However, to date no studies assessing their utility have been performed.

4.3. Tumor Vascular Architecture

The vascular morphology of tumors is different within tumors of similar and different histological types (41). Particular vascular patterns might help distinguish benign from malignant lesions (42,43) and be a prognostic marker; in ocular melanomas, a closed back-to-back loop vascular pattern was associated with death from metastasis (44), and in lung carcinomas distinct patterns of neovascularization might potentially respond differently to anticancer treatments (45).

4.4. Automation

Numerous studies have reported automation of the counting procedure by computer image analysis systems (4,17,19,32,37,46–53). These systems have several drawbacks, not including the capital and running costs and those they share with manual methods. An endothelial marker, which gives sensitive and specific capillary staining, is essential for the system to accurately discriminate blood vessels from surrounding tissue elements. A high background signal interferes with this process. Although partially automated systems with area- and shape-filters using defined color tolerances are available, most systems are not fully automated, require a high degree operator interaction, and like manual counting suffer from observer bias. Also, software able to identify
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hot spots is not available but when developed will require motorized stages at additional expense. Thus, computer image analysis systems are currently more costly, time consuming (up to 40 min per case), and no more accurate than a trained observer. They are unsuitable for routine diagnostic practice. Nevertheless, data from these studies have demonstrated that most vascular indices, including microvessel density, vessel perimeter, and vascular area, are significantly correlated, suggesting that they are equivalent indices of angiogenesis (32). Previously it had been hypothesized that microvessel density might not be the most important vascular parameter. A large vascular perimeter or area might be better measures of angiogenesis because these measures may better reflect the functional aspects of endothelial surface and the volume of blood available for interaction with the tumor (32).

5. Potential Novel Techniques

The microvessels highlighted by immunohistochemistry in tissue section are the end result of a dynamic multistep process. The evolving neovasculature is the result of a complex interplay between extracellular matrix remodeling, endothelial cell migration and proliferation, and capillary differentiation and anastomosis (54–56). Although it is not possible to measure these continuous processes, evidence suggests that a number of molecules involved in these events might be surrogate end points of angiogenesis. Thus, partly due to many of the inherent and methodological difficulties of vascular counts, these alternative strategies for quantifying tumor angiogenesis have also been pursued. These have particular drawbacks in that fresh specimens are required and the methodologies have yet to be standardized.

5.1. Angiogenic Factors and Receptors

Angiogenesis is the result of the net change in the balance of angiogenic stimulators and inhibitors (i.e., gain of promoters and/or loss of inhibitors). There are now numerous reports documenting up-regulation of several angiogenic factors and their receptors using a variety of techniques, including immunohistochemistry and in situ hybridization in a range of tumor types (57–72). However, only a few have correlated these data to clinicopathological parameters or survival. Some, such as vascular endothelial growth factor (VEGF) have shown a significant relationship between their tumor levels and microvessel density (in breast [73], brain [74], cervix [70], lung [75], stomach [76], and colon [57] cancers) and expression of KDR, a VEGF receptor, has also been correlated with high vessel counts in advanced stage colon carcinomas (57). Furthermore, in a multivariate analysis VEGF expression levels gave independent prognostic information in breast carcinomas (77). Similarly, thy-
midine phosphorylase in some studies has also been reported to be associated with microvessel density \(^{(78,79)}\) and survival \(^{(79)}\).

A particular use of angiogenic factor measurement in patient sera, urine, or cerebrospinal fluid is the ability to perform serial measurements. Although some of the theses studies have shown a relationship between angiogenic factor expression as a measure of tumor angiogenesis and patient survival \(^{(80–82)}\) none of the current techniques are sensitive or specific enough to use for quantifying tumor angiogenesis \(^{(83,84)}\). Different tumors use different angiogenic factors (breast carcinomas coexpress VEGF and thymidine phosphorylase (TP) \(^{(85)}\), whereas they are reciprocally expressed in bladder cancers \(^{(86,87)}\)), and it is more likely that specific factor profiles for individual tumor types will be a more accurate measure of tumor angiogenesis.

5.2. Cell Adhesion Molecules

Increasing evidence suggests that many of the endothelial cell adhesion molecules of the immunoglobulin, selectin, and integrin superfamilies, which have physiological roles in immune trafficking and tumor metastasis, also play a major role in angiogenesis. Clinical studies showing melanoma patients with upregulated selectins on endothelium and a significantly poorer prognosis are validating the interest in cell adhesion molecules and their cognate ligands in tumor angiogenesis \(^{(88)}\). Indeed soluble cell adhesion molecules are readily identified in sera of cancer-bearing patients although their relationship to tumor angiogenesis is yet unknown \(^{(89,90)}\). Similarly, integrins, including \(\alpha_\beta_3\), have also been shown to have been upregulated in human breast carcinomas compared to normal or benign breast \(^{(91–93)}\) and might also be a potential surrogate marker for angiogenesis.

5.3. Proteolytic Enzymes

Several studies have demonstrated that proteolytic enzymes including the plasminogen activators \(^{(94)}\) and the matrix metalloproteinases \(^{(95)}\) that are important in tumor cell invasion and migration are also important in angiogenesis. Indeed, a positive correlation between microvessel density and both urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI) has been reported \(^{(96)}\). Thus the poor prognosis in tumors \(^{(97–104)}\) associated with elevated levels of the uPA in the system are likely to be partly due to the angiogenic activity of these tumors. Measurement of proteases, particularly components of the urokinase system, might give some indication of the angiogenic activity of a tumor.
6. Summary

Continuing research into angiogenesis using quantitative data will not only broaden our understanding of the angiogenic process but will have several potential clinical applications beyond its use for prognosis. It might help in stratifying patients for cytotoxic therapy (105), aid monitoring and prediction of their response (106), and, with the advent of antiangiogenesis and vascular targeting, treatment could be stratified and altered based on these angiogenic measurements. The next few years will provide the data as to the reliability of quantitation of angiogenesis in tissue sections. During this time it is also probable that basic research will describe several candidate molecules that might become objective, sensitive, and specific enough to supersede the presently used assays.

7. Notes

1. One block is justified by the prevailing evidence, which shows a high concordance in vessel number between different blocks (8, 29, 30).
2. Studies within these microscope magnification/field area ranges derived prognostic information.
3. 8 μm cryostat sections can also be used but the area of tumor assessed is less representative.
4. The tumor block should be selected by examining H&E-stained slides.
5. If sections continually float off after antigen retrieval, drying at 56°C overnight will increase tissue adherence.
6. Alternative antigen retrieval techniques include pressure cooking and microwaving.
7. APAAP is preferred in tissues such as liver and kidney, which contain high endogenous biotin.
8. This can be obtained from Graticules Ltd, Morely Road, Botany Trading Estate, Tonbridge Wells, Kent, TN9 1ZN, UK. NB. Graticule size will depend on the microscope eyepiece diameter.
9. The field area would be too small were a higher magnification to be used. See Subheading 1., paragraph 4.
10. If anti-CD34 is used, positive stromal cells can also interfere with interpretation.

References

prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma. *Am. J. Pathol.* **133**, 419–423.


