Computerized Quantification of Tissue Vascularization Using High-resolution Slide Scanning of Whole Tumor Sections

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SUMMARY  Assessment of tissue vascularization using immunohistochemical techniques for microvessel detection has been limited by difficulties in generating reproducible quantitative data. The distinction of individual blood vessels and the selection of microscopic fields to be analyzed remain two factors of subjectivity. In this study, we used imaging analysis software and a high-resolution slide scanner for measurement of CD31-immunostained endothelial area (EA) in whole sections of human neuroblastoma xenograft and murine mammary adenocarcinoma tumors. Imaging analysis software provided objective criteria for analysis of sections of different tumors. The use of the criteria on images of entire tumor section acquired with the slide scanner constituted a rapid method to quantify tumor vascularization. Compared with previously described methods, the “hot spot” and the “random fields” methods, EA measurements obtained with our “whole section scanning” method were more reproducible with 8.6% interobserver disagreement for the “whole section scanning” method vs 42.2% and 39.0% interobserver disagreement for the “hot spot” method and the “random fields,” respectively. Microvessel density was also measured with the whole section scanning method and provided additional data on the distribution and the size of the blood vessels. Therefore, this method constitutes a time efficient and reproducible method for quantification of tumor vascularization. (J Histochem Cytochem 51:151–158, 2003)

Several immunohistochemical (IHC) techniques using anti-CD31 (PECAM-1), anti-CD34, or anti-Factor VIII antibodies have been developed to detect endothelial cells in tumor tissues (Vermeulen et al. 1996). Evaluation of tumor vascularization by these techniques has been hampered by difficulties in obtaining reproducible quantitative data. Tumor vascularization is usually quantified by determining microvessel density (MVD), which consists of a visual count of blood vessels performed under high-magnification light microscopy (Weidner et al. 1991). This method remains variable and poorly reproducible for two reasons (Vermeulen et al. 1996; Fox et al. 2000). First, the counting procedure relies on the subjective distinction of individual vessels by the observer. This distinction may be particularly difficult in areas of tangled capillaries or in areas where a single tortuous vessel can be sectioned several times and, depending on the observer, counted as one or multiple microvessels (Simpson et al. 1996). Different methods based on computerized image analysis have been developed to quantify IHC staining (Lehr et al. 1997) and have therefore been proposed to eliminate the subjective distinction of microvessels. Measurement of endothelial area (EA) corresponding to the surface of immunostained endothelial structure (Simpson et al. 1996; Schoell et al. 1997), microvessel perimeter, and microvessel area, consisting of the EA plus the vessel lumen (Barbareschi et al. 1995), has been used as a more accurate index of tumor vascularization. However, these meth-
methods do not eliminate a second factor of variability, which is the selection of the tissue area to be analyzed. Microvessel quantification is usually performed, either on vascular hot spots corresponding to the most vascularized area (Weidner et al. 1991), or on randomly chosen microscopic fields (Oh et al. 2001). The selection of vascular hot spots is subjective and depends on the experience and the training of the observer (Vermeulen et al. 1997). Vascularization quantification on randomly chosen microscopic fields is dependent on the arbitrary selection of a limited number of fields in a restricted area of a tumor section and does not take into consideration the heterogeneous distribution of microvessels in tumor tissue (Vermeulen et al. 1996). Here we propose to use imaging analysis software and a high-resolution slide scanner to quantify tumor vascularization in whole tumor sections in a time-efficient and reproducible manner.

Materials and Methods

Tumor Tissue Samples

Vascularization was quantified on tissue sections of two types of tumor obtained from animal models. Five suprarenal tumors were collected from a xenograft model of human neuroblastoma previously reported by us, in which a fragment of neuroblastoma tumor is surgically implanted into the adrenal gland of immunocompromised mice (Moats et al. 2001). Five mammary adenocarcinoma tumors were obtained from a transgenic murine model in which overexpression of neuroblastoma tumor is surgically implanted into the adrenal gland of immunocompromised mice (Moats et al. 2001). Five mammary adenocarcinoma tumors were obtained from a transgenic murine model in which overexpression of the Wnt-1 proto-oncogene in the mammary gland induces early duct hyperplasia and transformation into adenocarcinoma (Li et al. 2000). Tumors were carefully dissected, embedded in OCT, snap-frozen in liquid nitrogen, and stored at −70°C.

Immunohistochemistry for CD31

Detection of blood vessels was performed by IHC for CD31. Frozen 7-μm sections brought to room temperature (RT) were fixed in acetone and blocked in 5% goat serum supplemented with 2.5% BSA. Sections were incubated overnight at 4°C in the presence of a rat anti-mouse CD31 antibody (dilution 1:50) (product 019151D; Pharmingen, San Diego, CA). A biotinylated goat anti-rat antibody (product 31831; Pierce, Rockford, IL) was used as a secondary antibody (dilution 1:200) for 1 hr at RT. The sections were then processed with an avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), revealed in the presence of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO), and counterstained with methylene/counterstain.(1%).

Digital Image Acquisition

Digital images of microscopic fields of tumor tissue (×5 and ×20 Plan objective) were acquired with a Leica DM RA microscope (Leica Microsystems; Wetzlar, Germany) and an Olympus DP11 color digital CCD camera (Olympus; Melville, NY) with settings of HQ 1712 × 1368 pixels JPEG, ISO 400, 3000 K color temperature (0.05-sec exposure for ×20, 0.04 sec for ×5 objective). Images of whole tumor sections were acquired with a Polaroid SprintScan 4000 35-mm film slide scanner and PolaColor Insight software (Polaroid; Cambridge, MA) connected to a Compaq Professional Workstation SP730 computer (1 GHz Pentium III CPU, 1 Gb RAM) (Compaq; Houston, TX). Microscopic slides were held in the scanner by a PathScan Enabler 4000 microscope slide holder (Meyer Instrument; Houston, TX). Slides were scanned at 4000 dpi resolution with scale 100%, autofocus on for final scan, unsharp mask amount 50, radius 2, threshold 5, dust reduction off, as recommended in the PathScan instructions. In one neuroblastoma tumor, an additional image of the entire tumor section was obtained by digital montage of individual ×5 Plan objective microscopic field pictures assembled using Adobe Photoshop 6.0 (Adobe Systems; San Jose, CA). Montage and scanned images were saved as 24-bit color TIFF format. Before analysis, a shading correction was performed on all the microscopic field images. For each slide, a white reference image corresponding to a blank area was captured using microscope and camera settings described above. The tumor images were corrected using the equation <250 × specimen/white reference> in the <Arithmetic> command of MetaMorph 4.6 imaging software (Universal Imaging; Downingtown, PA). This correction removed any dust shadow and scaled the light to the same intensity range, resulting in consistent thresholding over multiple image acquisition sessions. White balancing for the slide scanner was more constant at acquisition time and scanned images did not require shading correction.

Vascularization Quantification

MetaMorph 4.6 software was used for computerized quantification of immunostained vascular structures. DAB-positive pixels were selectively detected and uniformly displayed with red pixel overlay using the threshold function. Threshold parameters were defined by successively adding regions with heavily DAB staining and deleting regions of counterstaining and background without DAB from the threshold with the command <Threshold Image> from the <Measure> menu (Figure 1A). These last two steps were repeated until all the DAB-positive pixels were selectively thresholded. Independent threshold settings were defined for analysis of the microscope images and the scanned images. The threshold area corresponding to the EA was measured with the <Region Measurement> function. For the whole section images, the EA was selectively measured on the tumor tissue delineated by using the <Trace Region> command of MetaMorph 4.6. Neighboring kidney, peripheral surrounding tissues, and necrotic areas were excluded from the selection (Figure 1B). The EA was expressed as a ratio of DAB-positive thresholded pixels compared to the number of pixels per image (microscopic field images) or per selected region (whole section images). For some tumor sections, the MVD was also quantified with the <Integrated Morphometry Analysis> command of MetaMorph 4.6. Using the total area classifier filter, every separate cluster of thresholded pixels with a minimal size of 5 pixels for the scanned image and a minimal size of 200 pixels for the microscopic field image was defined as one object. The number of objects, the surface, and the total surface (corresponding to the object
Vascularization on Whole Tumor Sections

Statistical Analysis

Variability of the whole section scanning method was compared to the variability of the hot spot method and the random fields method. For each method, a two-way random effect analysis of variance was used to estimate three sources of variability: due to different tissues, due to different observers, and unexplained variability (which was confounded with the tissue × observer interaction effect). The mean sums of squares were used to estimate the variance components. Ratios of the standard deviation and the coefficient of variation (standard deviation divided by the average of all 15 observations for the method and multiplied by 100) were used to summarize the variability associated with each method. A second estimate of interobserver variability was based on the percent disagreement, which was calculated for each tumor and each method by dividing the largest pairwise difference between observers by the average EA for that tumor and method and then multiplying by 100.

Results

Analysis of Different Tumor Tissues with the Same Threshold Setting

First we evaluated whether the threshold setting defined for one tumor section immunostained for CD31 could be used for analysis of sections of other tumors with the same level of sensitivity and specificity. The threshold setting defined on images of \( \times 20 \) objective microscopic fields of a first neuroblastoma tumor (tumor N1) was applied to microscopic field images of sections of four other neuroblastoma tumors similarly processed (tumors N2, N3, N4, N5). Visual examination of images of these five tumors with and without the threshold did not detect any DAB-negative thresholded pixels (false-positive) or DAB-positive unthresholded pixels (false-negative) (Figure 2). In the same manner, the threshold setting defined on an image of a section of neuroblastoma tumor N1 acquired with the slide scanner was applied on similar scanned images of surface plus the lumen) of each object were measured on an entire tumor region delineated as for the EA quantification.

![Figure 1](image)
neuroblastoma tumors N2, N3, N4, and N5. As observed for the microscopic field images, examination of images with and without threshold did not reveal a difference of sensitivity or specificity among the five sections analyzed (data not shown). Therefore, the threshold setting determined in one tissue sample can be used for analysis of other similar tissue samples.

Vascularization Measurement on Whole Tumor Sections

Next we measured the EA on an entire neuroblastoma tumor section and compared the data obtained by scanning the section with the slide scanner to those obtained by assembling serial microscopic field pictures (montage). The data acquisition took 5 min for the scanned image and 2 hr for the montage. The tumor region analyzed contained 0.99 megapixels (39.39 mm$^2$) in the scanned image and 42.2 megapixels (39.37 mm$^2$) in the montage. Despite the difference of magnification between the two images, the EA measurement was similar, with 6.46% of CD31-positive area in the scanned image vs 6.21% of CD31-positive area in the montage. Therefore, the use of the slide scanner consumed less time, generated smaller files, and produced data similar to those obtained on a montage of reassembled individual microscopic fields.

Comparison of Whole Section Scanning Method with Other Methods

We then evaluated the reproducibility of the whole section scanning method and compared it to two previously reported methods, the hot spot method (Weidner et al. 1991) and the random fields method (Oh et al. 2001). Three independent observers (C.F.C., Y.A.D., G.M.) acquired images of tumor sections obtained from five individual neuroblastoma tumors by using the three different methods. EA was measured on these images with common threshold settings (one for the microscope images, one for the scanned images). The measurements and the variability for the three methods are displayed in Figure 3A and Table 1. As expected, EA values measured by the hot spot method were higher than the values obtained by the two other methods. EA values obtained by the random fields method were lower than the EA values obtained by the whole section scanning method. This observation is consistent with the fact that the selection of a limited number of random fields is not representative of the vascularization of the entire tumor section. For all three methods, the interobserver (i.e., observer-to-observer) variability was less than the tumor-to-tumor variability, and there was substantially less interobserver variability with the whole section scanning method. Similarly, the coefficient of variation, using the unexplained or experimental error variation, was less for the whole section scanning method compared to the other two methods. This resulted in a greater ratio of tumor-to-tumor variability over experimental error ($sd_1/sd_2$), suggesting a better ability of the whole section scanning method to detect differences.
among individual tumor samples. Another method used to evaluate the interobserver variability was to calculate the percent disagreement obtained by dividing the largest pairwise difference between observers by the average EA. The whole section scanning method again displayed the least amount of observer-to-observer variability with a percent of disagreement of 8.6% compared to 42.2% for the hot spot method and 39.0% for the random fields method. Because the neuroblastoma tumors examined had few area of necrosis, the delineation procedure was simple and consistent among observers. We therefore asked whether it would result in a higher interobserver variability when tumors with a large necrotic area were analyzed by the whole section scanning method. For this, we used sections of five individual Wnt-1 murine mammary adenocarcinomas, which typically contain highly vascularized areas adjacent to large necrotic areas (Figure 3B). The delineation procedure was standardized by eliminating necrotic areas in contact with the edge of the tumor and internal necrotic areas larger than 25% of the delineated tumor region. Small necrotic areas located inside the tumor tissue were included in the region to be analyzed. The EA measurements obtained on these more heterogeneously vascularized specimens were comparable for the three observers (Figure 3C). The interobserver variability of the EA measurement for the mammary adenocarcinoma, as determined by the percent disagreement, was 6.9%, a value similar to the 8.6% disagreement obtained with the neuroblastoma tumors. Therefore, the whole section scanning method not only constituted a time-efficient method but also was more reproducible than other methods relying on the arbitrary selection of vascular hot spots or on the random assignment of microscopic fields. With specific criteria to exclude large areas of necrosis, this method can be used for analysis of heterogeneously vascularized tumor specimens.

### Table 1 Summary of variability of the three methods for neuroblastoma tumor area selection

<table>
<thead>
<tr>
<th>Estimate of</th>
<th>Hot spot</th>
<th>Random fields</th>
<th>Whole section scanning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average level of endothelial area (% of tumor area)</td>
<td>EA</td>
<td>13.33</td>
<td>4.43</td>
</tr>
<tr>
<td>Unexplained variability (experimental error)</td>
<td>$s_d^1$</td>
<td>2.62</td>
<td>0.91</td>
</tr>
<tr>
<td>Tumor-to-tumor variability</td>
<td>$s_d^2$</td>
<td>3.86</td>
<td>1.16</td>
</tr>
<tr>
<td>Interobserver variability</td>
<td>$s_d^3$</td>
<td>0.82</td>
<td>0.20</td>
</tr>
<tr>
<td>Coefficient of variation $= 100 \times s_d^1 / \text{EA}$</td>
<td>CV</td>
<td>19.65</td>
<td>20.62</td>
</tr>
<tr>
<td>Ratio of tumor-to-tumor variability to unexplained variability</td>
<td>$s_d^2 / s_d^1$</td>
<td>1.48</td>
<td>1.28</td>
</tr>
<tr>
<td>Percent disagreement</td>
<td>42.2</td>
<td>39.0</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*Sections of five individual neuroblastoma tumors were analyzed by three independent observers. For each tumor, EA was measured with MetaMorph 4.6 software on images of ×20 objective field selected in the highest vascularized area (hot spot) or randomly selected (random fields) and on image of the whole section acquired with the slide scanner (whole section scanning). For each method, the unexplained variability, the tumor-to-tumor variability, and the interobserver variability were calculated. The percent of disagreement was also calculated for each tumor and each method by dividing the largest pairwise difference between observers by the average EA for that tumor and method and then multiplying by 100. Percents of disagreement listed are the average of all five neuroblastoma tumors examined.

**Section-to-section Variability of Tumor Vascularization**

To determine the variability of vascularization quantification between serial sections within the same tumor specimen, we measured the EA by the whole section scanning method in four serial sections of the same neuroblastoma tumor. The average level of EA was 6.48% (SD ± 0.48) of the entire tumor area (data not shown). The coefficient of variation calculated by dividing the standard deviation by the average EA and then multiplying by 100 was 7.46%. Therefore, the method also gave reproducible results in adjacent sections of a tumor specimen.

**MVD Quantification with the Whole Section Scanning Method**

Finally, we examined whether another parameter of vascularization, the microvessel density, could be quantified with the whole section scanning method. Using MetaMorph 4.6, we measured the MVD on images of an entire neuroblastoma tumor section generated by slide scanning and on a montage of serial microscopic field pictures. As for the EA measurement, the difference of magnification between the two images did not modify the MVD quantification, with 45.3 thresholded vessels/mm$^2$ in the scanned image vs 42.3 thresholded vessels/mm$^2$ in the montage (data not shown). Then we compared the EA and MVD values obtained with the whole section scanning method for the five individual neuroblastoma tumor sections. Although there was no significant correlation between the EA and the MVD (correlation coefficient $r^2=0.31$; data not shown), the comparison between these parameters provided valuable information on the size of the microvessels. An example of this comparison for two neuroblastoma tumor samples (N1 and N4) with similar EA values (6.53% and 6.69%, respectively) but different MVD values (47.7 vessels/mm$^2$ and 62.1 vessels/mm$^2$).
The MetaMorph Integrated Morphometry Analysis command as described in Materials and Methods, we measured in both tumors the total surface of each blood vessel (including the lumen) and generated for these two specimens distribution histograms according to vessel size (Figure 4A). We observed a difference in the vessel surface distribution with an increase in the number of blood vessels larger than 90 pixels and a corresponding decrease in the number of blood vessels smaller than 60 pixels in tumor N1 compared to tumor N4 (Figure 4A). Accordingly, the average vessel surface for tumor N1 (34.1 pixels) was greater than the average vessel surface for tumor N4 (25.5 pixels). Microscopic examination of these tumors confirmed the presence of several larger vessels in specimen N1 compared to specimen N4 (Figures 4B and 4C). Therefore, the whole section scanning method not only provides quantitative information on EA but allows us to calculate the MVD and the average vessel surface, thus providing valuable information on the size of individual blood vessels within tumors.
This study describes a novel method for vascularization quantification on entire tumor sections. Charpin et al. (1995) previously reported vascularization quantification on whole tumor sections to avoid the arbitrary selection of particular fields. They measured the mean percentage of CD31 immunostained area of the counterstained area on serial microscopic field pictures obtained by automatic screening of tumor sections. The use of the slide scanner constitutes a more rapid and less expensive method to examine an entire tumor section. It generates a single image of the sample, allowing one to delineate and to easily select the relevant tumor region to be analyzed. Although this delineation remains dependent on the observer, precise instructions regarding the exclusion of necrotic area from the tumor region to be analyzed allows the generation of reproducible measurements by different observers. Consistent with a recent comparison between immunostaining quantification on low- and high-magnification images (Johansson et al. 2001), we showed that vascularization measurement by imaging analysis software does not require an optical magnification at acquisition time.

A potential limitation of the computerized quantification of tumor vascularization resides in the inclusion in the threshold area of nonendothelial structures that are nonspecifically stained by CD31 IHC. Such nonspecifically stained structures could be easily excluded by a trained pathologist. Anti-CD31 antibody has a high sensitivity for endothelial cell recognition but has been reported to occasionally label some plasma cells (Horak et al. 1992). In our frozen tumor sections, the only nonspecific DAB staining observed was due to residual peroxidase activity in highly necrotic areas. Because its intensity was weaker than the intensity of endothelial cell staining and because it was located in necrotic area excluded from the analyzed tumor region, this nonspecific staining was not included in the thresholded area actually quantified. Nevertheless, this limitation may represent a problem in tumor samples heavily infiltrated by CD31-positive nonendothelial cells, such as plasma cells, or in specimens that contain multiple areas of focal necrosis. A careful examination of the sections by a trained observer remains important before scanning and digital analysis.

The whole section scanning method was found to be highly reproducible when serial sections in the same tumor sample were examined. However, in heterogeneous tumors, it is anticipated that the analysis of nonserial sections will generate different values. Although this was not examined in this study, our method, which eliminates the need to examine several adjacent sections, allows examination of a greater number of nonadjacent sections in complex tumors in.
a time-efficient manner, thus increasing the ability to address tumor heterogeneity.

In this study we used the EA to quantify the tumor vascularization. Measurement of this parameter does not require the distinction of individual microvessels and may better reflect the interaction between tumor cells and peripheral blood, as previously suggested (Fox et al. 1995; Simpson et al. 1996). However, we showed that other parameters of vascularization, such as the MVD, can also be quantified by the same method using Metamorph software. Whether EA measurement is superior to MVD measurement to assess tumor vascularization is controversial in the literature (Kohlberger et al. 1996; Simpson et al. 1996, 1997). Consistent with previous reports, we found no significant correlation between the EA and the MVD. This is not unexpected because, for similar EA values, tumor samples with large blood vessels will have a lower MVD value than tumor samples with smaller blood vessels. A major advantage of the method reported here is that it allows one to obtain both EA and MVD values for each section, thus providing additional information about the size of blood vessels in tumor specimens.

In conclusion, imaging analysis software such as MetaMorph provides an objective criterion to detect and quantify immunostained microvessels in tumor tissue sections that could be applied to other immunohistochemical and histological stainings. The use of this criterion for vascularization quantification on scanned images of whole tumor section constitutes a time-efficient and reproducible method. Because it can be easily standardized, this method could also be a valuable tool for large multicenter studies in which the comparison of tumor tissues analyzed by several observers is required.

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Literature Cited


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