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Magdalena Białas, Krzysztof Okoń, Jacek Czopek

Assessing Microvessel Density in Gastric Carcinoma: a Comparison of Three Markers

Chair and Department of Pathomorphology, Collegium Medicum, Jagiellonian University, Kraków

Angiogenesis (AG) is necessary for cancer progression. In some cases the intensity of AG may affect the prognosis. The aim of the study was to compare the results of vessel density assessment using the stereological method and immunohistochemical reactions for three endothelial markers: CD31, CD34 and vWf. The material consisted of 40 gastric carcinoma cases. The specimens were processed and the immunohistochemical reactions performed routinely. To assess the microvascular density the stereological parameter of "length density" and the "hot spots" method were employed. Image acquisition and the measurements were done using the image analysis system AnalySIS 3.0 pro with custom-made applications. It was observed that CD34-stained preparations were the easiest to assess. The number of labeled vessels, and especially microvessels, was also the highest in the case of the above reaction. The results achieved in AG evaluation using various endothelial markers are not directly comparable. The vascular network density was significantly associated with tumor stage. Such an association was most clearly seen in CD34 reactions.

Introduction

Angiogenesis (AG), i.e. the formation of small blood vessels, occurs in the course of normal development, but under normal conditions does not take place in an adult human. On the other hand, AG participates in various pathological processes, such as wound healing or organization of inflammatory exudate. AG provides a necessary condition for the development of malignancies. Only very early tumors, few millimeters in size, may survive without a vascular network produced through angiogenesis. The ability to induce AG, as well as the degree of such an induction, are believed to be of a great importance in the biology of cancer. In the case of some diseases, such as breast carcinoma, the density of the vascular network may be an independent prognostic factor; in other entities, such as renal clear cell carcinoma, no such relation has been observed. Therefore, in recent years, the interest in angiogenesis investigations has increased [11].

To detect microvessels immunohistochemical reactions are employed, using primary antibodies against antigens present on endothelial cells. Many such antibodies are commercially available. The methods for AG assessing are not fully standardized. Planimetric methods are usually employed. It can be expected that the use of stereological methods would be more effective, since they relate the density of the vascular network to the volume of a tridimensional structure, which is formed by the tumor and its surroundings. The aim of the study was to develop the methodology for assessing the density of microvascular network that would be based on stereology, to develop necessary software and subsequently to compare the results achieved using three popular endothelial markers: CD31, CD34 and von Willebrand factor (vWf).

Material and Methods

The material for the study consisted of 40 unselected gastric carcinoma cases from the files of the Chair of Pathomorphology, Collegium Medicum, Jagiellonian University. The tissue was fixed in formalin, routinely processed and embedded in paraffin. From each case, a tissue block containing an extensive cancer infiltration was selected. From these, 4μm sections were prepared.

The immunohistochemistry was performed by the standard method. Briefly, the slides were dewaxed, rehydrated and incubated in 3% peroxide solution for 10 minutes to block the endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (0.2% citric acid titrated to pH 6.0 with 2N NaOH) for 5 minutes at 700W, then for 5 minutes at 600W [12]. The primary antibodies used are listed in Table 1. The ENVISION+System kit (DAKO, Denmark) detection system was used. It consists of several goat anti-mouse antibody particles attached to a dextran backbone coupled with horseradish peroxidase. AEC (DAKO, Denmark) was used as the chromogen. The slides were counterstained with Mayer hematoxylin (DAKO, Denmark).

Image processing and the measurements were performed with the AnalySIS image analysis system (Soft Imaging System GmbH, Germany). For image acquisition, an Axioscop microscope (Zeiss GmbH, Germany) with 10x (NA 0.17) Plan-NeoFluar lens was used . It was coupled with a color CCD camera ZVS-47DE (Optronics, USA) con-

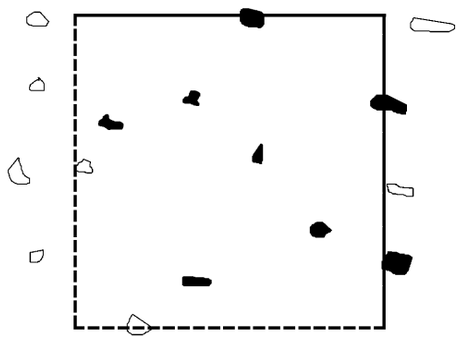

Fig. 1. The idea of unbiased counting frame. The profiles wholly contained inside the frame or touching its two selected sides are counted (filled profiles), the ones outside or touching the two other sides are excluded (empty profiles).

nected by a RGB line to a graBIT PCI frame grabber (Soft Imaging System GmbH, Germany), installed in a standard personal computer. The image analysis software was running under the control of Windows NT 4.0 Workstation operating system (Microsoft Corp., USA). It consisted of a custom-made software developed by one of the authors (K.O.) in the Imaging C (ANSI C) language [1], running in the AnalySIS pro v.3.0 (Soft Imaging System GmbH, Germany) image analysis environment.

The assessment of vessel density was done using the length density stereological parameter. First, the slide was visually scanned and the region containing the visually highest vessel density was chosen. From such a region, five fields were acquired using a 10x lens. The images were entered into the computer system and displayed on its monitor. On the screen, four unbiased frames (Fig. 1) were drawn. The operator pointed to the vessel profiles inside these frames and the system calculated the length density according to the following formula:

$$\hat{L}_V = \frac{\sum_{i=1}^{n} Q_i}{\frac{a}{f}\sum_{i=1}^{n} P_i}$$

where:

Q_i - number of points in field i

P_i - number of frames in field i

a/f - area of frame at the final magnification

In all the assessed cases immunohistochemical staining was performed in identical, serial sections originating from the same paraffin block. A positive reaction was accepted as a cytoplasmatic reaction with orange-brownish granules. In the vWf reaction, apart from morphologically identifiable vessels displaying a lumen, small groups of cells without any lumen, and isolated cells displaying a strong and clear immunohistochemical reaction were also regarded as blood microvessels. In the CD34 reaction, vessels were identified as all the immunopositive, lumen-containing structures, clusters of cells without any lumen, and isolated, scattered cells showing a similar strong reaction. The spindle-like cells showing a clearly weaker reaction were disregarded. In the CD31 reaction, all cell clusters and isolated cells showing a positive reaction were regarded to be vessels.

TABLE 1

Primary antibodies used in the study

Specificity	Source (clone)	Dilution
CD31	DAKO (Qbend10)	1:25
CD34	DAKO (JC/70A)	1:40
vWF	DAKO (F8/86)	1:25

For the comparison of the populations the Kruskall-Wallis ANOVA and Friedmann ANOVA were used. Correlations were tested with the Spearman's rank correlation coefficient. The statistical analysis was done with the Statistica for Windows v.5.5 PL (Statsoft, USA) software. The significance level was set to p=0.05.

Results

The study group consisted of 40 cases, including 30 males and 10 females. The mean patient age was 63 years (range, 35 to 81, SE 0.28). Histologically, 18 cases were Lauren diffuse type, 14 - intestinal type, and 8 cases - mixed type; 16 were Goseki type I, 1 - type II, 10 - type III and 13 - type IV.

The results obtained using the three reactions varied considerably. The CD34 reaction was the strongest and the most evident. The demonstrated vascular structures were quite uniformly distributed within the neoplastic infiltrate. In the majority of cases these were small or very small vascular structures, usually showing a distinct lumen. The assessment of these structures posed no difficulties and a decision whether a given group of cells constituted a vessel was easy. Difficulties in identifying vascular structures emerged infrequently and involved cases, in which neoplastic infiltration was accompanied by abundant inflammatory infiltrate.

The strength of the vWf reaction was somewhat lower. A clear and strong reaction was noted in the endothelium of larger vessels within the specimen, while the number of identified smaller vessels was markedly lower than in the case of the CD34 reaction. In addition, within the endothelium of larger vessels, the reaction lacked continuity or was altogether absent. The strength of the CD31 reaction proved to be the lowest, and the assessment of these specimens was more difficult in comparison to the remaining ones. The reaction was visible in a fairly large number of vessels, although they predominantly constituted major structures, situated at the periphery of the infiltration.

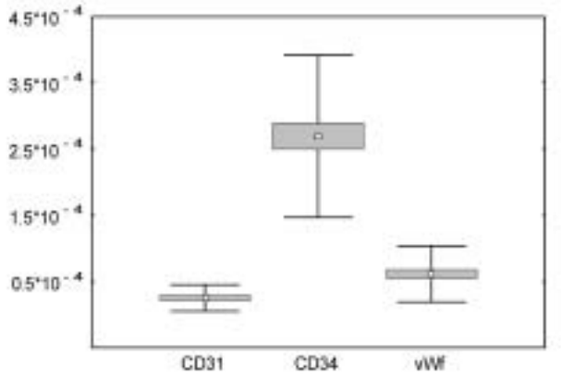

Fig. 2. Distribution of vessel length density. The central point is the arithmetic mean, the box denotes the mean ± standard error, the whisker is the mean ± standard deviation.

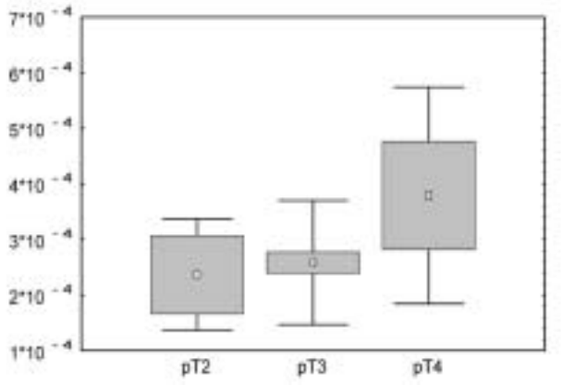

Fig. 3. Vessel length density according to the pT stage. The central point is the arithmetic mean, the box denotes the mean ± standard error, the whisker is the mean ± standard deviation.

Table 2 presents the results of Lv assessment. The highest values of blood vessel density were achieved while detecting the CD34 antigen; the differences between the stains used were significant (Friedman ANOVA $p<0.001$). The results of CD34 stain were also characterized by the highest level of variability (Fig. 2). Although the Lv values obtained by staining for CD31 and vWf demonstrated significant positive correlations ($R=0.35$, $p<0.03$), the value obtained by stain for CD34 was not significantly correlated with the remaining results ($R<0.02$, NS). While analyzing the associations between the density of microvascular network and other clinical-morphological parameters, it was found that the number of vessels was markedly higher in higher stage tumors (pT4) as compared to others (pT2 and pT3) (Fig. 3). In the case of CD34 and CD31, the vessel density was also higher in pT3 tumors than in pT2 carcinomas. The differences were not significant in the investigated material; only in the case of CD34 did they reach the significance level that approximated the assumed value (Kruskall-Wallis ANOVA $p=0.08$). It was interesting that the Lv values were somewhat higher in women (Table 3). Yet the said differences were not significant.

TABLE 2

Differences in vessel length density measured with the 3 stains studied

	Mean	Minimum	Maximum	SD
CD31	2.50×10^{-05}	4.00×10^{-06}	1.00×10^{-04}	2.00×10^{-05}
CD34	26.9×10^{-05}	6.00×10^{-05}	6.59×10^{-04}	12.3×10^{-05}
vWf	6.10×10^{-05}	0.00×10^{-00}	1.66×10^{-04}	4.20×10^{-05}

TABLE 3

Sex-related differences in vessel length density

	CD31	CD34	vWf
	mean (SD)	mean (SD)	mean (SD)
male	2.20×10^{-05} (1.60×10^{-05})	2.67×10^{-04} (1.06×10^{-04})	5.70×10^{-05} (3.90×10^{-05})
female	3.60×10^{-05} (2.70×10^{-05})	2.78×10^{-04} (1.71×10^{-04})	7.30×10^{-05} (5.10×10^{-05})

Discussion

The formation of blood vessel network is an indispensable step in cancer progression. In the initial phase of their growth, tumors are supplied with oxygen and nutrients through simple diffusion from the surrounding tissues. However, the process is spatially limited and, therefore, tumors that do not possess the capacity of AG induction cannot grow to a diameter bigger than few millimeters. Cancer acquiring the ability to induce angiogenesis is also indispensable for metastasis formation. The formation of vessels within the tumor stroma depends on numerous factors. VEGF is the chief mediator of angiogenesis, while bFGF appears to be less potent. Apart from their paracrine activity, both mediators may be also detected systemically. Serum VEGF has been found to affect the prognosis. VEGF and other angiogenesis-promoting factors may be produced both by tumor cells and by the concomitant inflammatory cells [13, 14, 17].

The assessment of vascular density within tumors is usually performed by the planimetric method, where one calculates the number of vessel sections per a unit of surface area, the latter being expressed as the number of fields of vision. A histological preparation is seen under a microscope as a flat structure; nevertheless, it constitutes a section across a tridimensional structure. Any reference to such a spatial structure may seem difficult, yet stereological methods allow for assessing parameters in a tridimensional space. Moreover, many of these methods are not difficult to employ and their use is reduced to a systematic repetition of simple measurements, such as the

number of elements or the interaction of the measured element and the measurement structure. What is very important is the fact that stereological measurements are unbiased estimators, i.e. they are free from systematic errors and the achieved precision is solely sampling-dependent.

A reliable assessment of microvessel density in a histological preparation requires the vessels to be visualized using an immunohistochemical method. The possibilities for selecting an appropriate marker are wide; here we can refer to EN-4, BMA120-BW200, PAL-E, anti-CD36, Ulex europeus, anti-CD31, anti-vWf and anti-CD34. These markers differ as to their degree of specificity and sensitivity, as well as the power of the reaction and applicability to paraffin-embedded material. The three last antibodies of the listed above are most widely used.

Tanigawa et al. found AG to be a significant prognosticator in esophageal squamous cell carcinoma. The authors compared the results they obtained using antibodies against CD34 and vWf and - similarly as in our material - they observed more numerous microvessels using the first reaction. The prognostic importance of measurements performed using both these reactions was similar [16].

Lenczewski et al. [8] employed the reaction for CD34 and noted that the vascular density affected the prognosis in cervical squamous cell carcinoma. The consensus paper by Vermeulen et al. [18] pointed to CD31 staining as the best marker for AG quantification. However, in their report the authors stated that immunohistochemistry for CD34 might be more reliable, but required more studies before any final recommendation could be made. Chen et al. [2] used CD34 for determining microvessel density in gastric carcinoma and confirmed its prognostic significance.

AG seems to be an independent prognostic factor in gastric carcinoma; it may be associated with the risk of dissemination and relapse [6, 10]. The microvessel density in gastric carcinoma was found to be correlated with VEGF expression [9]. Chen et al. observed that in gastric carcinoma, vessel density assessed in a histological specimen was significantly associated with the color Doppler vascularity index [2]. No such association was found by La Rosa et al. [7] in gastrointestinal endocrine tumors.

Conclusions

- The results of measurements obtained using various vascular markers show a great diversity.
- The results obtained using diverse methodologies may prove impossible to be directly compared.
- The best tool in assessing angiogenesis in gastric carcinoma appears to be CD34 immunohistochemistry.

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Address for correspondence and reprint requests to:
K. Okoń M.D.
Department of Pathomorphology CMUJ
Grzegórzecka 16, 31-531 Kraków