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PRACTICAL PROBLEMS IN QUANTIFICATION OF TISSUE VASCULARISATION

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ABSTRACT

The quantitative estimation of tissue vascularisation, especially in neoplasia has raised controversies in literature. Most authors indicate that the numerical density of microvessels may be helpful as independent prognostic factor in various types of cancer. Other authors report contrary results. In this review the methodological differences and details of quantification of the vascular bed especially in neoplasm are traced and the interpretation pitfalls are analysed. Methodological inaccuracies which may influence on irreproducibility and decrease of the diagnostic value of the methods are discussed. The following proposals for methodological improvements are presented: (i) the characterization of the vascularisation of the neoplasm should be based on measurements done at two different, non parallel planes; (ii) the unbiased measurement frame of similar size of reference area (~0.5 mm^2) should be routinely applied in diagnostic studies on neovascularisation; (iii) the measured features should reflect: angiogenesis (count of endothelial cells, or volume fraction or absolute volume of endothelial cells; when the numerical density is estimated a correction for vessel branches should be introduced); risk for metastasis (size distribution of vessels, at least two size classes: below 30 μm and more than 30 μm in diameter); level of tissue nutrition i.e. distinction between vascularisation and blood supply (calculation of the ratio of vessels with red cells to non-perfused vessels).

Keywords: vascular bed, blood supply, vascularisation score, morphometry, stereology, neoplasm.

INTRODUCTION

The evaluation of the status of microvascular network supplying tissues and organs is potentially an important tasks in assessing the neoplastic lesions. The discovery of angiogenetic factors gave a new impetus to the investigation of this problem. Evaluation of vascularisation is of interest to developmental morphology, inflammatory processes, wound healing, atherosclerosis and related diseases, to endocrinology and many other areas of medicine (Kondering et al., 1992, Krupiński et al., 1994, Kulig et al., 1983, Pawlikowski et al., 1996, Pawlikowski et al., 1997, Stępień et al., 1996, Takeshita et al., 1996, Walter et al., 1996, Zieliński and Koktysz, 1989, Zieliński and Kulig, 1994). However, the investigation of

vascularisation has a particular significance in neoplastic histopathology. In this field, in addition to numerous experimental and research studies (Booth et al., 1997, Brem et al., 1972, Fajardo et al., 1992, Folkman, 1996, Kondering et al., 1992, Lamszus et al., 1997, Liotta et al., 1974, Montesano et al., 1996, Pawlikowski et al., 1997, Takamyia et al., 1993, Winiger et al., 1996, Zieliński et al., 1984 a, Zieliński et al., 1984 b), quantification of vascularisation has been proposed a significant position as a possible prognostic factor in human diagnosis. Some authors find the diagnostic value of vascularisation dubious (Alberts et al., 1995, Costello et al., 1995, Goulding et al., 1995, Page and Jensen, 1995, Siitonen et al., 1995), other as well established (de Jong et al., 1995, Fontanini et al., 1996, Lee and Altman, 1996, Weidner et al., 1992, Vartanianin and Weidner, 1995).

One of the first attempts of semiquantitative assessment of angiogenetic activity was undertaken by Brem et al. (1972). The procedure proposed by Weidner et al. (1991) for routine surgical specimens of breast tumors with after slight modifications (Alberts et al., 1995, Costello et al., 1995, Goulding et al., 1995, de Jong et al., 1995, Fontanini et al., 1996, Siitonen et al., 1995, Vartanianin and Weidener, 1995) is widely used. The procedure involves the preparation of several blocks from different parts of the tumor, cutting of a few serial sections of each and preliminary staining with H&E. "Hot spots" are looked for in these sections under ca 100 x magnification. Hot spots show the highest numbers of vascular profiles according to subjective evaluation. The positivity of the immunohistochemical reaction for endothelial cells is checked (used antibodies are against CD-31, CD-34 or factor VIII related antigen). Then microvessels profiles are counted under a magnification of 400 x in few most vascularized fields. The highest value of vessel profiles count per area ($N_A$ves) is the result. This method, in which the fields are arbitrarily selected, was compared with a systematic sampling scheme of fields by de Jong et al. (1995) discussed by Lee and Altman (1995). The hot spots selection yielded more reproducible results than systematic field sampling. Microvessel profile is defined as any brown-stained endothelial cell or cell cluster clearly separated from adjacent microvessels, tumor cells and other connective tissue elements (Weidner et al., 1991). Some authors supplement the above methodology with additional statements: "vessel lumens with or without red cells were not necessary for a structure to be defined as a microvessel" (Weidner et al., 1991, Siitonen et al., 1995), "longitudinally cut vessels were counted as one vessel but multiple cross-sections of individual convoluted vessel were separately counted" (Alberts et al., 1995, Goulding et al., 1995). Goulding et al. (1995) introduced a significantly different method. The authors used a digital image analyser which enabled them to calculate microvessels with open lumina and the relative percentage of endothelial cells ($A_A$endth). On counting the numerical density of microvessels the upper limit of diameter of countable vessels is sometimes given, e.g. 30 μm (Winiger et al., 1996), 200 μm (Krupiński et al., 1994), 250 μm (Zieliński and Kulig,1994), 500 μm (Zieliński et al., 1984 b), but most authors use a vague term "microvessel". The significant diagnostic efficiency of the method results mainly from the intergroup variance of numerical density of vessel profiles among neoplasms of different degrees of malignancy (Vartanianin and Weidner, 1995). For example for medullary thyroid carcinoma the mean numbers of vessel profiles in 1 mm^2 range from 35 to 72 (Fontanini et al., 1996), in breast carcinoma from 70 and to 140 (Weidner et al., 1991, 1992). The difference of microvessel density between carcinomatous and benign hyperplasia of prostate is 1.9-fold (means: 73 and 39 respectively) (Vartanianin and Weidner, 1995).

The opinions concerning the value of this method as an independent prognostic test in malignant tumors are divided. Approximately 50% of authors (9/21) deny its prognostic value,

whereas the others point out a significant correlation with prognosis (Alberts et al., 1995, Costello et al., 1995, Goulding et al., 1995, Fontanini et al., 1996, Siitonen et al., 1995).

IDENTIFICATION OF BLOOD VESSELS

On morphological assessment of the vascular network the estimation of **blood supply** i.e. the content and distribution of blood in perfused vessels should be distinguished from the estimation of **vascularisation**, i.e. anatomical extent of the vascular bed, including both the functional component and the non-functional areas of the vascular network. Morphometric approach to microvessel quantification may be based on the following principles:

I. Visual identification of vascular profiles as histological objects.

The traditional method involves the identification of the profiles e.g. after H+E or toluidine blue staining (Booth et al., 1997, Brem et al., 1972, Fajardo et al., 1992, Krupiński et al., 1994, Zieliński et al., 1984 b). An important clue for the identifications of such a profiles is the presence of erythrocytes in the lumen. Erythrocytes scarcely distributed in the capillaries are often poorly visible and may be overlooked by the observer, while the presence of numerous pathological vessels with abnormal morphology makes this method problematic, as extravasated red blood cells may not be distinguished from blood vessels. Additionally, the low colour and densitometric contrast of red blood cells and eosin practically excludes the use of digital image analysers for the automation of the count. Thus, immmunohistochemical methods are recommended to improving the identification of the vascular lumens and walls.

II. Identification of vascular lumina

II. a. Injection

The methods of injection (with polyvinyl chloride, barium, Indian ink or stains) do not guarantee the visualisation of the whole patent vascular bed, and they may induce extravasation, as well as increase the diameter of blood vessels. The method can be applied for experimental purposes or for whole organ specimens (Kondering et al., 1992, Kulig et al., 1983, Zieliński et al., 1984 a). Full vascular profiles are clearly identified with this method, and radiographic methods, transparent thick sections, or corrosion methods allow spatial reconstruction of the vascular network. Because, ca. 25-50% of blood vessels are not perfused (Kondering et al., 1992), this methodology cannot fully recommended for quantitative studies.

II. b. Visualisation of blood cells in vascular lumina

The benzidine-nitropruside method, developed in 1934 by Pickwoth, based on high peroxidase activity of red blood cells, increases their visibility in thin histological sections (Krupiński et al., 1994, Thomson, 1966). The modification of Pickwoth's method (Zieliński and Kulig 1981) allowed the spatial observation of microvessel network in thick (several hundred μm) sections prepared from routine, non-injected histopathological samples (Zieliński et al., 1984 a). Brilliantine green and acid fuchsin staining (Poley et al., 1964) was also used for the needs of digital image analysis (Pawlikowski et al., 1996, Pawlikowski et al., 1997, Stêpień et al., 1996, Zieliński and Kulig,1994). This way of staining excellently shows the purple erythrocytes within the blood vessels against the background of deep green surroundings. The great colour contrast in this last method or densitometric contrast in Picworth's staining allows the profiles of red cells to be extracted from microscopic image. In digital image analysis manual operations during binary image segmentation are radically

reduced. The methods of visualising blood cells in the vascular lumen "record" the functional status of circulation at the moment of its arrest. A shortcoming of these methods is the necessity to distinguish extravasated erythrocytes as well as discontinuous filling of the vessels - particularly capillaries. Increasing section thickness, e.g. to 8 μm. facilitates the reconstruction of the course of microvessels. The above methods are able to estimate the true blood supply to the tissue, and only indirectly - its vascularisation.

III. The methods of identification of vessel wall components

III. a. Identification of endothelial cells

Histochemical (e.g. Takeshita et al., 1996, Walter et al., 1996) and immunohistochemical methods (e.g. Alberts et al., 1995, Costello et al., 1995, Goulding et al., 1995, de Jong et al., 1995, Fontanini et al., 1996, Krupiński et al., 1994, Siitonen et al., 1995, Vartanianin and Weidner, 1995, Winiger et al., 1996, Weidner et al., 1991, Weidner et al., 1992) can be distinguished. In paraffin sections, the most frequently used endothelial cell markers are antigens CD-31 (de Jong et al., 1995, Goulding et al., 1995, Kuzu et al., 1992, Siitonen et al., 1995, Vartanianin and Weidner, 1995), CD-34 (7,14,25,43), and factor VIII related antigen (Alberts et al., 1995, Costello et al., 1995, de Jong et al., 1995, Kuzu et al., 1992, Siitonen et al., 1995, Winiger et al., 1996, Weidner et al., 1991, Weidner et al., 1992) as well as CD-106 (Krupiński et al., 1994). The CD-36 antigen may be stained in cryostat sections (Kuzu et al., 1992). Unfortunately, these methods of staining demonstrate considerable variations of expression dependent on the type of vessels and investigated organ (Kuzu et al., 1992). Comparative studies using anti-CD31, anti-CD34 and anti FVIIIRA antibodies, carried out by Siitonen et al. (1995) demonstrated rather poor consistency of vessel numbers in serial paraffin sections from breast carcinomas (correlation coefficients ranging from 0.3 to 0.63). A slightly better consistency was obtained when the sum of vascular profiles area (areal fraction) of vessels identified by these antigens was correlated (correlation coefficient values from 0.5 to 0.64). In their opinion, endothelial cells were identified best by anti FVIIIRA, slightly worse by anti CD-31, and considerably worse by anti-CD34. Similar data were reported by other authors, but due to higher sensitivity they chose CD-31 as the marker (de Jong et al., 1995). Comparative studies of vessels present in various vascular tumors also demonstrated a considerable variation of expression of endothelial antigens (Kuzu et al., 1992). The lowest variability was demonstrated by anti CD-31 (de Jong et al., 1995, Kuzu et al., 1992). In cases of active vascularisation occurring in tumors, the expression in immature endothelium may be variable, especially in minute sprouting vessels. On the other hand, when selecting a panel of antigens to identify endothelial cells in non-neoplastic lesions, the likelihood of cross-reactivity of lymphatic and blood vessel endothelium should considered. The above results suggest that immunohistochemical methods may not be reliable enough as the basis for vessel identification in quantitative studies because their results are largely dependent on the technical quality of immunostaining and reflect the degree of immune maturity of endothelial cells forming the vascular bed rather than its extent. The fact that the results of estimation of relative vessel area ($A_A$ves) are more consistent than the results of estimation of numerical density ($N_A$ves) (Siitonen et al., 1995) may be due to the variable staining of single endothelial cells in estimation of $N_A$ves, whereas $A_A$ves mainly on the staining of endothelium in easily identifiable, larger vessels.

III. b. Identification of laminar components of vessels wall (including perfusion studies)

In order to trace the course of vascular profiles, antilaminin AB (Booth et al., 1997, Lamszus et al., 1997) and anti-collagen IV antibodies (Booth et al., 1997) are used, among

others. These methods identify the walls of the vessels which have already reached a certain degree of morphological maturity.

Both the methods of endothelium identification and those identifying the laminar vessel wall components determine the anatomical extent of the vascular bed (vascularisation)

Perfusion of experimental animals with the use of dyes such as luconyl blue (e.g. Fajardo et al., 1992), Evans blue and others improve the visualisation of walls of patent vessels under light microscope. This method reflects well the functional status of circulation, without causing artificial changes in the vascular bed due to injection.

FUNCTIONAL AND MORPHOLOGICAL PECULIARITY OF VASCULAR BED IN TUMORS

The vascularisation process, particularly that taking place in malignant tumors, is a complex process involving at least ten proteins initiating the growth of new vessels from the surroundings of the tumor, and at least some proteins inhibiting their development (Booth et al., 1997, Fajardo et al., 1992, Folkman, 1996, Lamszus et al., 1997, Montesano et al., 1996, Mose and Langer, 1991, Takamyia et al., 1993, Walter et al., 1996, Winiger et al., 1996). Some substances regulating the process of angiogenesis demonstrate bidirectional activity, stimulating or inhibitory, dependent on concentration (e.g. TNF α, Fajardo et al., 1992) or degree of degradation (e.g. hyaluronic acid, Montesano et al., 1996)

Thus, the essence of neovascularisation is a local disequilibrium between the inhibitors of angiogenesis and angiogenic factors. It has been demonstrated that intensive angiogenesis (predominance of angiogenic factors) is associated with the growth of more differentiated and clinically aggressive tumors (Booth et al., 1997, Brem et al., 1972, Fontanini et al., 1996, Kondering et al., 1992, Weidner et al., 1992, Zieliński et al., 1984 a, Zieliński et al., 1984 b). From the morphological point of view, the process of neovascularisation of tumors can be divided into several stages (Kondering et al., 1992):

1. formation of endothelial sprouts, particularly in the venular portion of microvascular bed,
2. elongation of endothelial sprouts and formation of vessels lumina (most frequently by vacuolisation of endothelial cells),
3. sprout fusion into arcade loops,
4. formation of a specific network in the tumor and morphological maturation of vessel walls,
5. disorganisation of the tumor network.

I. Anisotropy of vascular network of tumors

Corrosion studies (Kondering et al., 1992), observations of vessel growth in vitro, studies on vascularisation of neoplastic implants in serous membranes (Booth et al., 1997, Lamszus et al., 1997, Montesano et al., 1996, Kondering et al., 1992), as well as observations carried out in thick histological sections (Zieliński et al., 1984 a) indicate that the neovascularisation of the tumor is not completely chaotic as it is suggested by the patterns of vascular profiles in thin histological section. According to the histopathological tumor type, at least some patterns of microvascular network architecture can be distinguished (e.g. sinusoid vessels, spiral vessels, fascicular arrangement, loop-like arrangement, mesh network) (Kondering et al., 1992, Zieliński et al., 1984 a). The organisation and hierarchy of vascularisation is dependent on the region of the tumor. Vascularisation is better visible at the peripheral portion of the tumor and the capsule, where the hierarchical pattern predominates, but may change into loop-like arrangement, sinusoid and corkscrew-like vessels in the more

central parts. In the most central part of the tumor numerous avascular areas are observed, as well as sinusoidal system with numerous blind ends (Kondering et al., 1992). It should be added that the dominating histological structure of the tumor determines the pattern of vascular bed organisation, i.e. the arrangement of vessel is a characteristic feature of the histological type of the tumor (Zieliński et al., 1984 a).

These observations are of considerable practical importance because they indicate that the vascularisation pattern in tumors is not isotropic. It is a case of spatial (acc. to Weibel, 1979) anisotropy (radial, concentric or spherical) both with respect to the direction of the vessels' course and to their numerical density. Such organisation of vessels suggests two practical conclusions for the evaluation of the above mentioned hot spots of the tumor:

- the highest probability of finding them exists at the peripheral portions of the tumor,
- the area regarded as a hot spot should be checked in at least two cutting planes to avoid incorrect overestimation of the number of vessels, particularly in fascicular system areas.

No macroscopic criteria for hot spots detection in the tumor have been described. In view of the above their macroscopic identification is "blind", and so requires a fixed and uniform methodology for collecting tissue samples from the tumor mass. Some authors slice the tumor in a cruciate manner (e.g. Goulding et al., 1995), others in parallel manner (e.g. de Jong et al., 1995). Taking into consideration the above mentioned spatial structure of the vascular bed of the tumor, the first method seems more correct. The analysis of the discrepancy concerning the estimation of prognostic value of vascularisation in oncopathology, when based on specimens collected by different investigators should take into account the variations associated with macroscopic sampling of the tumor.

II. Biological significance of microvessel quantification

Two-dimensional quantitative morphology of the tissue vascular bed is a particularly difficult area for stereological interpretations. It results from the fact that two-dimensional arrangement of vascular profiles on the plane gives too little information for the reliable reproduction of the vascular network. From model studies (Kamyia et al., 1974, Uylings, 1977) it follows that the essential factor determining the functional efficiency of the vascular network is its spatial topography (network construction) and to a lesser degree the stereological indices such as the number of vascular profiles per reference area (N_A), or area fraction of vessels profiles (A_A). Most stereological analyses are concerned with the estimation of size, shape and distribution of particles or lamellar and fibrillar structures. Blood vessels do not belong to either of these categories, they form a branching structure, the origin of which is practically beyond the reach of histological observation. The most important quantitative data for the assessment of liquid flow conditions in such a system are (Kamyia et al., 1974, Uylings, 1977): the number of ramifications (which determines the length of the vasculature), the hierarchy of particular elements of the system, branching angles and decrease of the vessel's diameter after giving off a ramification, length and width of particular elements, linear character of the vessels' course. This data allow for the qualification of the vascular network in one of four rheological categories: minimum power, minimum drag, minimum resistance, or minimum volume model (Uylings, 1977). Additionally, the area of tissue exchange stereologically estimated by the length of profile circumferences per reference area (L_A) for small vessel diameters is an important element. The combination of these data allows the potential estimation of the nutritional conditions of the tissue, drug availability, sensitivity to radiotherapy etc. A simple example of such analysis - although carried out on the level of macroscopic vessels - was the confirmation of the effect of abnormalities in coronary tissue

geometry on the development of myocardial fibrosis (Zieliński and Koktysz, 1989), giving specific values of ramification angles and heart dimensions with increasing the risk of the disease. The classic (Weibel, 1979) and more recent stereological methods used in biology (Gundersen et al., 1988a, Gundersen et al., 1988b, Weibel, 1989) do not describe the way of estimating of many of the above mentioned characteristics, e.g. ramification angles, the hierarchy of vascular network elements from histological sections. Also the methods of fractal analysis at the present stage do not seem to yield simple and direct solutions of this problem (Losa and Nonnenmacher, 1996, Sanders and Crocker, 1993). It seems that structural syntactic analysis, as well as the methods of three-dimensional reconstruction from serial sections or direct measurement of the third dimension may be helpful in solving this problem (Zieliński et al., 1990). Appropriate statistical analysis of the data on vascular profiles may provide valuable information on the structure of the vascular bed, important for the estimation of nutritional status of the tissues. Apart from the above mentioned parameters such as L_A, A_A, N_A, they include also kurtosis and skewness of the distributive series (Pawlikowski et al., 1996, Pawlikowski et al., 1997, Stępień et al., 1996, Zieliński and Kulig,1994). Markedly right-skewed and platykurtic histograms suggest an excessive development of the capillary portion of the vascular bed and a considerable increase of circulatory resistance with consequent functional inefficiency of the network. Also the comparison of the distribution of vascular profiles containing erythrocytes in their lumen (patent vessels) and those without (non-perfused vessels) may give relevant information.

Despite the fact that quantitative studies of tumor angiogenesis the mechanism relating worse prognosis with the larger number of microvessels in its surroundings is unclear (Page and Jensen,1995). At least three views can be considered.

(i) *The number of endothelial cells is a marker of tumor malignancy*. The observed predominance of the activity of angiogenic factors over that of the inhibitors of angiogenesis in the site the neoplasm is higher the more dedifferentiated the neoplastic cells are. This disequilibrium results in the stimulation of endothelial cells proliferation, which can be considerated a marker of this biochemical abnormality. The direct morphological measure of the magnitude of the disorder is the local increase of the number of endothelial cells, or the total volume of these cells. An increase of the number of vascular profiles is in relation with the number of endothelial cells. The above suggests that for the quantification of the biological phenomenon the number of endothelial cell count in the reference area ($N_A$endth), endothelial cell area fraction ($A_A$endth), and the vessel count per reference area ($N_A$ves) are valuaable, in this order of preference.

(ii) *The increased number of blood vessels in the surroundings of the tumor increases the probability of penetration of the vascular bed by small groups of cells and formation of metastases.* Because the sizes of neoplastic cell agglomerations may be considerable, only the vessels of several tens of μms in diameter may play a role in the transport of these cells. Liotta et al. (1974), demonstrated that the number of circulating neoplastic cells and metastases increase proportionally to the increase of the number of vessels whose diameter exceeded 30 μm in the primary tumor. They did not observe such correlation for the vessels with smaller diameters. It seems that the length of vessel walls per reference area ($L_A$ves), counted for the vessels with diameters exceeding 30 μm may be associated with risk of metastases.

(iii) *The malignancy of the neoplasm is related to the nutritional status.* The summed length of patent vessel walls per reference area ($L_A$ves), or other indices described above,

could describe the nutritional status. This problem is also related to tissue availability for cytostatic agents and its sensitivity to radiotherapy.

III. Ramifications of vascular profiles (Fig.1)

Gundersen et al. (1988a) indicate that the number of vascular profiles in the reference area is a function of their length in space according to the following equation:

$$Lves = 2 * N_Aves * V_Uref$$

where:
Lves = length of structure (vessel)
$N_A$ves = numerical density of profiles in the plane
$V_U$ref = unit of reference volume.

and give an example of simple estimation of vascular loop length in a renal glomerulus. This principle seems correct with regard to such vascular systems as glomerular arteries, which are bundles of several non-ramifying convoluted vessels and when the section thickness is negligible in relation to the average loop length. However, in the case of other vascular networks, especially those occurring in tumors, where numerous ramifications can be observed, the quoted equation is true only if the thickness of the vessels is the same throughout, and the section is infinitely thin (a plane).

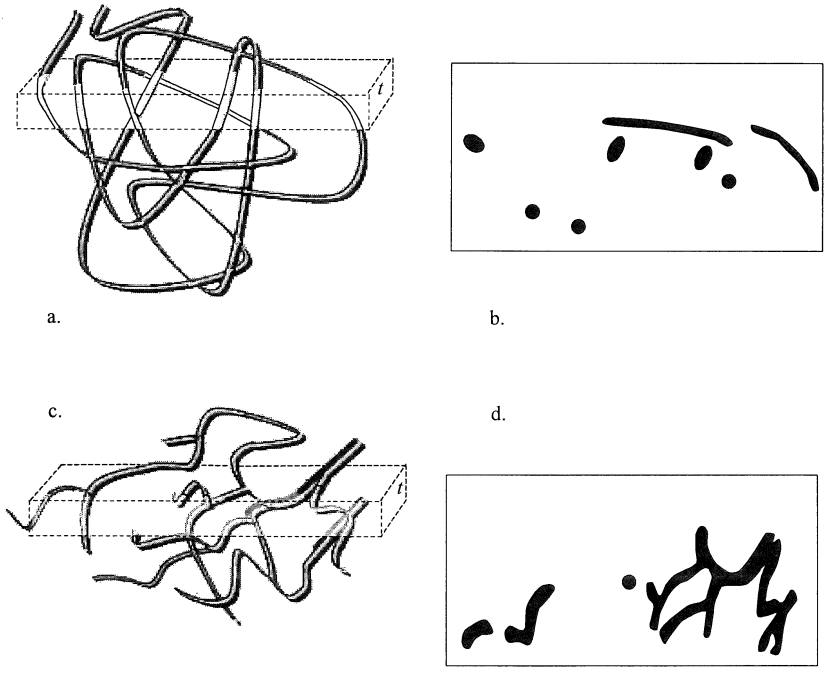

Fig 1. Schematic illustration of differences in plane representation (b, d) of glomerular (a) and tumorous (c) vessels from the block of a thickness t *.*

On quantitative analysis of histological preparations originating from hot spots it can be observed that in the thickness of the section a considerable number of vascular ramifications and arising endothelial sprouts has been "captured". The summarical length of vascular sprouts was confirmed by Montesano et al. (1996) as an effective measure of angiogenetic activity in monolayer culture of endothelial cells. However, in the case of histological sections the determination of vascular profile numbers in hot spots by counting only profiles or clusters clearly separated from the adjacent clusters or profiles the number of newly formed vessels will be underestimated. If we assume that is the mass of endothelial cells or local "dissemination" of blood vessels is related to the aggressivity, it would be feasible also to count the ramifications as additional objects (i.e. as a sum vascular profiles, endothelial clusters, and the number of ramifications observed in the inspected field).

From the equation presented above it follows that section thickness is important for the interpretation of the result. Most of the above cited authors of reports on tumor vascularisation do not give this information, even though the thickness variation can be considerable, and increasing the thickness of the section increases the probability of visualisation of ramified vessel profiles.

IV. The measuring frame

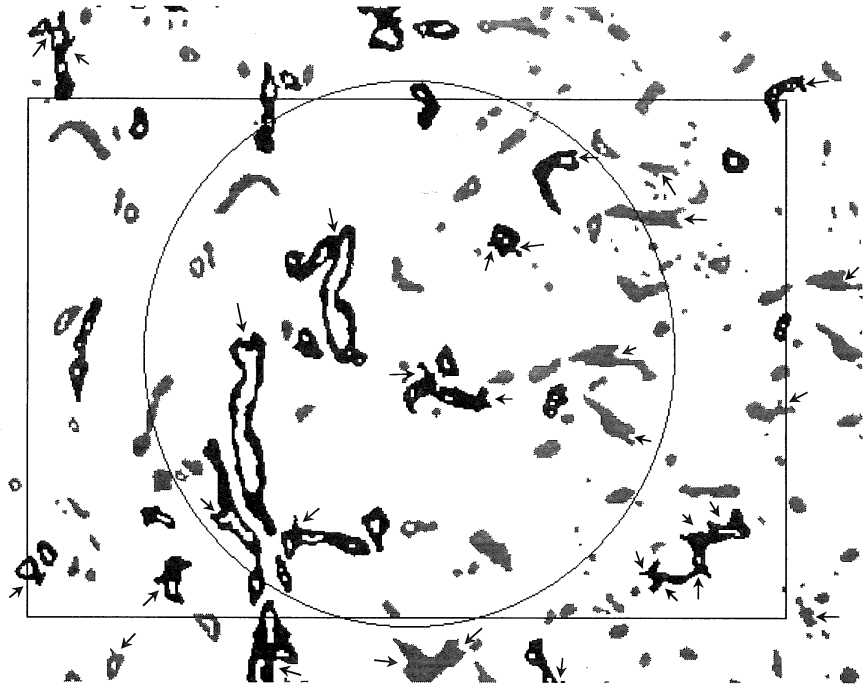

Fig.2. Digitised image from microscopic preparate of hot spot immunostained with anti-FVIIIRA (ductal breast carcinoma). Black profiles – vessels containing red cells, arrows – vascular ramifications.

The presence of numerous irregular profiles of ramified vessels visible in hot spots makes visual estimation of the vessels diameter under the microscope quite difficult. The mean width (diameter) of each profile as the proportion of the profile area to profile circumference $d_{mean} = Aves/Lves$ (Weibel, 1979) should be estimated in this situation. This is not difficult if a digital image analyser is used.

Table 1. The differences of the histometric results from the example field (Fig. 2) of vascular hot spot in dependence on the method of profile counting.

Measured feature	Rectangular frame (area 0.34 mm^2)		Circular frame (area 0.20 mm^2)	
	Value	Difference in %	Value	Difference in %
Unbiased frame counts				
Count of all vessel profiles	121	100.00		
Count of branches	21	100.00		
Sum of all vessel profiles and branches	142	100.00		
Count of vessel profiles $\Phi > 30$ µm	5	100.00		
Count of perfused vessel profiles	22	100.00		
Total area of immunostained objects in %	**10.90**	**95.88**		
Also objects truncated by the frame are counted				
Count of all vessel profiles	125	103.31	63	87.58
Count of branches	23	109.52	13	104.13
Sum of all vessel profiles and branches	148	104.23	76	90.03
Count of vessel profiles $\Phi > 30$ µm	7	140.00	4	134.57
Count of perfused vessel profiles	25	113.64	14	107.04
Total area of immunostained objects in %	**11.37**	**100.00**	**12.56**	**110.46**
Only objects entirely inside the frame are counted				
Count of all vessel profiles	111	91.74	51	70.90
Count of branches	19	90.48	11	88.11
Sum of all vessel profiles and branches	130	91.55	62	73.44
Count of vessel profiles $\Phi > 30$ µm	4	80.00	4	134.57
Count of perfused vessel profiles	19	86.36	11	84.11
Total area of immunostained objects in %	**9.66**	**84.99**	**11.11**	**97.77**

On the evaluation of the number of vascular profiles in hot spots the problem of profiles crossing the circumference of the visual field has been neglected. It should be assumed that each profile, which at least partially is present in the field of measurement, is included in the results. Such an approach leads to overestimation of the number of profiles per reference area (Gundersen et al., 1988a), due to overestimation of the number of larger vessels. On the other hand, an opposite approach, i.e. counting only the whole profiles which are present inside the visual field leads to underestimation of the number of profiles, particularly

large ones. Neglecting this phenomenon may be the source of considerable error, because with numerous vessels and relatively small and differentiated reference area (0.17 – 0.74 mm^2) the measuring frame can fall on as many as 10% of vascular profiles. The error is smaller if the field of measurement is larger and if a circular frame is used instead of a rectangular one (the circumference of a rectangle is 12.9 % longer than the circumference of a circle of the same area (Lrect/Lcirc= 2*sqr (Π)/Π)). The so-called unbiased counting frame (Gundersen et al., 1988a, Weibel, 1979), excludes profiles located under two perpendicular side lines of the frame, and includes those which are located under the other two side lines. To these figures one should add the determination of the caliber of the vessels. This kind of measurement complex could be especially valuable in this field. The problem of frame influence on the results is shown in Fig. 2 and in Table 1. The least biased values are given in bold letters, the percentages of change after recalculation per one square millimetre. The table shows that the numerical density of great vessels profiles is the most variable value and the areal density of immunostained objects (truncated at frame) is the most constant feature.

CONCLUSIONS

The determination of angiogenesis indices in neoplastic hot spots has the potential of becoming a diagnostic method in oncopathology. Combined estimation of the area and number of vascular profiles may have a higher diagnostic value than the estimation of number of vascular profiles only, although the latter method is less tedious. Reproducibility and comparability of results can potentially be improved by following aspects of measurement:

1. the use of the same endothelial cell marker (CD-31 or VIIIRF) throughout one study, and in several studies,
2. the determination of the vascular parameters in two cutting planes of sections collected from the block containing a hot spot,
3. application of an unbiased measurement frame with similar reference area size (~0.5 mm^2),
4. use of uniform section thickness: 4 or 8 μm (with thicker sections used to evaluate blood supply),
5. expressing results by mm^2 or mm^3,
6. the separation of the results into components reflecting:
    - angiogenesis (count of endothelial cell or volume of endothelial cells; the estimation of blood vessel count should be corrected for ramifications)
    - the likelihood of metastasis (division of the vascular profiles into at least two types according to their size: with average diameter up to 30 μm and exceeding 30 μm)
    - the nutritional state of the tumor (comparison of erythrocyte-containing vessels with empty vessels: blood supply vs. vascularisation)

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