Pergamon

European Journal of Cancer Vol. 32A, No. 14, pp. 2474–2484, 1996

Printed in Great Britain
0959-8049/96 $15.00 + 0.00

PII: S0959-8049(96)00379-6

Quantification of Angiogenesis in Solid Human Tumours: an International Consensus on the Methodology and Criteria of Evaluation

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INTRODUCTION

TUMOUR ANGIOGENESIS is thought to be initiated by an increase in the level of angiogenic stimuli and a concomitant decrease in the level of angiogenesis inhibitors [1]. The locoregional net effect of positive and negative regulators of angiogenesis is based (i) on the activity of tumour cells, stromal cells, inflammatory cells and extracellular matrix components (paracrine interactions), (ii) on the activity of endothelial cells (autocrine interaction) and (iii) on the influx of circulating factors (endocrine interactions). Tumour growth, invasion and metastasis all require angiogenesis [2]. Prognosis of individual cancer patients is profoundly influenced by the intensity of these pathophysiological processes. This is reflected in the clinical and pathological staging criteria. Various experiments suggest that the degree of angiogenesis is closely related to the capacity of a malignant tumour to expand locally and to give rise to distant spread.

Angiogenesis is a reflection of the characteristics of the host tissue. This is, for instance, elegantly illustrated by the fact that tumours grow more slowly in elderly mice [3]. The field of interest of cancer research is shifting towards the tumour-associated stromal tissue.

Important direct and indirect relationships between tumour cell phenotypes and endothelial cell phenotypes have recently been revealed.

Tumour cell proliferation and angiogenesis. Mutations of the *TP53* tumour suppressor gene and the *RAS* oncogene, both augmenting tumour cell proliferation, are also involved in the downregulation of angiogenesis inhibitors, for example, thrombospondin-1 for mutant p53 [4], and in the upregulation of angiogenic factors, for example vascular endothelial growth factor (VEGF) both for mutant p53 [5] and mutant ras [6]. The hypoxic constitution of malignant tumours, as the result of tumour cell proliferation-related oxygen consumption and elevated tissue pressure, stimulates VEGF [7], basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) production [8]. Reciprocal paracrine interactions between tumour cells and stromal cells, for example endothelial cells, may also provide a direct link between the activity of both cell populations [9]. Several studies have investigated the association between tumour cell proliferation and intratumoral microvessel density (IMD) [10–12].

Tumour cell invasion and angiogenesis. There is evidence that tumour cell invasion and endothelial cell migration are interacting processes. Migration of endothelial cells from the wall of pre-existing vessels towards a chemotactic source, another initial step in new vessel development, not only resembles invasion of tumour cells through the basal membrane, but extracellular matrix digestion by endothelial cells also facilitates local spread of tumour cells. Inhibition of angiogenesis *in vivo* by administration of antibodies directed at the integrin $\alpha_v\beta_3$, highly expressed in angiogenic vessels, was indeed shown not only to reduce tumour growth, but also to affect local invasion of a human breast carcinoma [13].

Haematogenous tumour cell spread and angiogenesis. A quantitative relationship between IMD, the number of intravascular tumour cells and the occurrence of pulmonary metastasis was shown in an animal tumour model more than 20 years ago by Liotta and associates [14]. The association between angiogenesis and metastasis is emphasised by a recently reported clinical study, in which peri- and per-operative shedding of cancer cells were shown to be related to IMD in breast carcinoma [15]. Circulating angiogenesis inhibitors can suppress the subsequent outgrowth of metastasis by inhibiting local angiogenesis and increasing apoptotic activity of the tumour cell population [16].

Given the biological interactions between new vessel development and tumour growth, invasion and metastasis,

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quantification of human tumour angiogenesis might provide pathologists and clinical doctors with an integrative parameter of the three key pathophysiological events in disease progression.

Although angiogenesis quantification has been performed in most tumour types, prospective studies analysing the relationship between prognosis and degree of new vessel development are scarce. For the quantification of angiogenesis to yield a reliable prognosticator, the applied methodology has to be characterised by a low intra- and inter-observer variability. Results obtained at different institutes should be comparable in order to allow for meta-analyses. We would like to initiate the discussion on standardisation of angiogenesis quantification and, therefore, here provide an overview of the current techniques.

INTRATUMORAL MICROVESSEL DENSITY AS A MEASURE OF TUMOUR ANGIOGENESIS

Tumour angiogenesis reflects the integration in time of the expression pattern of factors affecting endothelial cell migration, proliferation and differentiation. Angiogenesis influences tumour growth by the perfusion effect and by the paracrine effect of endothelial cells on tumour cells. Migrating endothelial cells also facilitate local tumour cell invasion by degradation of the extracellular matrix, and the newly formed vasculature provides the possible entrance for tumour cells to the circulation, allowing for distant metastasis. IMD is assumed to reflect the intensity of tumour angiogenesis. Several studies have indeed found a correlation of IMD with (i) angiogenic factor expression, (ii) tumour growth and (iii) the occurrence of distant metastasis, suggesting that IMD, as well as quantifying the vascular morphology, contains important information on the degree and functionality of the tumour vasculature.

IMD and angiogenic factor expression

Toi and associates have compared IMD with both VEGF and PdECGF expression in 152 invasive ductal breast cancer specimens [17]. The expression of both angiogenic factors was graded according to the staining intensity. IMD was evaluated by counting the number of factor VIII-Related Antigen (FVIII-RAg) positive endothelial cell deposits in the areas considered most active for neovascularisation. An association between VEGF expression (55% of cases) and PdECGF expression (46.7% of cases) was found in approximately 70% of cases. VEGF and PdECGF expression were significantly correlated with the increment of microvessel density.

The same investigators analysed the prognostic value of tumour angiogenesis in a retrospective study including 328 early stage breast cancer patients [18]. Using the same grading criteria for VEGF expression, 50% of the tumours were determined as VEGF positive. The VEGF positive rate increased linearly with the increment of IMD. Multivariate analysis confirmed that IMD had an independent predictive value for relapse-free survival (RFS), with an equal magnitude as the number of nodal metastasis. VEGF status only had a statistical prognostic value in univariate analysis, but in a previous study with a smaller number of patients [19], Toi and colleagues reported the significance of VEGF status in a multivariate analysis. Intratumoral VEGF concentrations, measured by an immunoenzymatic method in 135 breast cancer tissue homogenates, were significantly higher in vascularly-rich tumours than in vascularly-poor tumours [20]. No association was found between VEGF concentrations and bFGF or hepatocyte growth factor (HGF) concentrations.

VEGF mRNA expression, as opposed to bFGF, transforming growth factor (TGF) β and TGFα mRNA levels, was associated with vascular counts in 17 gliomas and 16 meningiomas [21]. VEGF concentrations measured by ELISA in the tumour tissue from 19 brain tumour patients was significantly correlated with vascular density [22]. Almost all tumour cells in the peripheral area of brain tumours that contained a high amount of VEGF protein were associated with increased IMD on immunohistochemical staining. Determination of bFGF levels in the cerebrospinal fluid from children with brain tumours correlated with microvessel counts, which were of prognostic value [23]. The activity of bFGF was illustrated by the stimulating effect of the CSF on *in vitro* endothelial cell proliferation [23].

Immunostaining for VEGF in 129 gastric carcinoma specimens was considered positive by Maeda and associates if more than 5% of the tumour cells had membrane or cytoplasm staining [24]. IMD assessed by anti-FVIII-RAg staining was higher in VEGF positive gastric carcinomas in this study. In cervical neoplasia, increased VEGF mRNA expression, revealed by *in situ* hybridisation, was also associated with high IMD [25]. Vessel density analysis in human renal cell carcinomas xenografted in mice revealed a clear correlation of VEGF mRNA expression with IMD [26].

These studies have all correlated overall expression of angiogenic factors with a locoregional reflection of the angiogenic activity, as microvessels were always counted in vascular hot spots. Studies correlating IMD with the tumour tissue levels of endogenous angiogenesis inhibitors, for example, platelet factor 4, TIMP-1 (tissue inhibitors of metalloproteases) and -2, thrombospondin, cartilage-derived inhibitor and interferon (IFN) α and β, are not available yet.

IMD and uPA/PAI-1 levels

IMD has also been related topographically to factors with a proven influence on angiogenesis. Urokinase plasminogen activator (uPA), an extracellular proteolytic enzyme, is produced by tumour cells, endothelial cells and inflammatory cells such as macrophages. The levels of uPA and its inhibitor plasminogen activator inhibitor-1 (PAI-1) are known prognostic factors in breast cancer [27]. Extracellular matrix degradation and vessel wall destruction by proteolytic enzymes, such as metalloproteases, are a stimulus for endothelial cell migration and proliferation [28]. Hildebrand and associates have compared uPA and PAI-1 contents, measured by ELISA in tissue extracts, in peripheral and central tumour tissue as well as node-negative and node-positive breast cancer patients [29]. uPA/PAI-1 levels were higher in the peripheral breast tumour regions. This was particularly obvious in the node-positive patients. IMD, measured by CD31 staining, correlated linearly with uPA/PAI-1 levels. Vascular hot spots were predominantly found in peripheral tumour regions.

uPA proteolytic enzyme activity may contribute to tumour cell invasion and metastatic dissemination to distant

organs. Since activated endothelial cells produce uPA, this represents part of the reciprocal paracrine interaction between tumour and host cells. In this regard, a higher IMD might indicate a more extensive intercellular interaction at the interface between tumour and host.

IMD and tumour cell shedding

The hypothesis that tumours with a high vascular density are more likely to shed cells into the circulation, thereby enhancing the metastatic process, is elegantly highlighted by the observation of McCulloch and associates [15]. This study indicates that IMD, assessed in the vascular hot spots of breast cancer, quantitatively reflects a major aspect of the pathophysiological role of tumour angiogenesis. Before primary surgical treatment of patients with operable breast cancer, a central venous catheter was fixed in the ipsilateral proximal subclavian vein. Before, during and one day after surgery, blood samples were withdrawn. Cytokeratin-staining was applied on cytospins of these samples and the number of cytokeratin-positive cells, thus tumour cells, was determined. Microvessels were highlighted with an anti-CD34 antibody in tissue sections of the invasive tumour component. IMD was measured in the vascular hot spots. The majority of patients with a high vascular density had cells detected during operation in contrast to a minority of the patients with a low IMD. These preliminary data suggest that the frequency of tumour cell shedding is related to IMD.

IMD and intratumoral tissue plasma volume

Magnetic resonance imaging (MRI), performed after the administration of a macromolecular contrast medium, has been developed to estimate tumour tissue plasma volume [30]. Slow and fast growing variants of a mammary carcinoma implanted in rats were analysed for both IMD (FVIII-RAg staining) and plasma volume. A more densely structured vasculature was found in the fast growing tumours. Plasma volume, estimated from the MR images, increased exponentially as a function of histological capillary density (i.e. IMD.)

IMD is stochastically related to factors inducing angiogenesis *in vitro* and *in vivo*. The quantification of pathophysiological processes in tumour progression dependent on angiogenesis, for example, the number of tumour cells in efferent veins and the rate of tumour volume increase, is also reflected in IMD measurements. Taken together, data from the literature strongly support the idea that IMD is a reliable measure of tumour angiogenesis.

ENDOTHELIAL SPECIFIC ANTIBODIES FOR IMMUNOHISTOCHEMISTRY

Intratumoral microvessels can be identified using immunohistochemical methods to stain endothelial cells. In the wide range of currently available human endothelial specific antibodies, two categories can be defined: the pan-endothelial cell markers and the antibodies which bind to activated/proliferating endothelium. Apart from endothelial cells, other constituents of the vascular wall can theoretically be used to visualise the degree of intratumoral vascularisation. These include both the pericytes and components of the surrounding basal lamina. Although in many tumour types the quantification of blood vessels using an anti-CD31 [31] or an anti-FVIII-RAg antibody has provided prognostic information [32], for the assessment of qualitative changes

Table 1. Endothelial cell-specific antibodies: immunohistochemical characteristics

Antibody	Sensitivity	Specificity	Reactivity		Lymphatic staining
			Frozen	Paraffin	
Anti-CD31 [33]	Small and large vessels—equal intensity in normal and tumour tissue	Non-specific in cryostat sections—occasional plasma cell staining in formalin-fixed tissue	+	+	No
Anti-FVIII-RAg [38, 39]	Large vessels—capillaries = variable and focal—polyclonal Ab = more sensitive	Monoclonal Ab: high—polyclonal Ab: stromal staining	+	+	Proportion
UEA [34]	Small and large vessels—equal intensity in normal and tumour tissue	Low: neoplastic cell staining	+	+	Yes
Anti-CD34 [35, 36]	Small and large vessels—equal intensity in normal and tumour tissue	High: variable perivascular stromal staining	+	+	Proportion
Anti-CD36 [37–39]	Intense staining of small vessels—variable/weak staining of large vessels	Monocyte and platelet staining	+	?/+	?
PAL-E [37–39]	Small and large vessels—equal intensity in normal and tumour tissue	High	+	–	No
BMA120-BW200 [37–39]	Small and large vessels—equal intensity in normal and tumour tissue	High: occasional tumour cell and serosa staining	+	+	?
EN-4 [37–39]	Small and large vessels—restricted distribution pattern	Stromal cell staining—infiltrate cell staining—occasional tumour cell staining	+	–/+ (fixation dependent)	?

in microvessels incorporated into tumour tissue, pan-endothelial markers might be less suitable. Table 1 [33–39] lists some commonly applied endothelial cell specific antibodies and their characteristics related to immunohistochemical applications. From this table, it can be seen that anti-CD31 antibodies are superior on paraffin sections, with the second best options on the basis of sensitivity/specificity being anti-CD34 staining, anti-FVIII-RAg staining or BW200 staining. The detection of von Willebrand factor and the CD34 antigen on microvessels in tumour tissue is hampered by co-staining of a proportion of the lymphatic vessels. Although neoplasms are assumed not to elicit the formation of a new lymphatic drainage system [40], the incorporation of pre-existing lymphatic vessels may induce false-positive microvessel counts if these antigens are highlighted. Recent analyses of IMD in breast cancer suggest that anti-CD34 might be a more reproducible and reliable antibody for routine studies [41]. Until this has been reported for other tumour types, it might be useful to combine anti-CD31 and anti-CD34 antibodies.

Several studies have proven that FVIII-RAg, although highly specific for the vasculature, is absent on part of the capillary endothelium in tumour tissue [42]. The occasional CD31 signal on inflammatory cells can be easily differentiated from endothelial cell positivity on the basis of the morphological differences of both cell types. Another disadvantage of CD31 staining is the frequent antigen loss due to fixatives which contain acetic acid. Microwave antigen retrieval efficiently abolishes this problem, but in prospective studies a careful selection of the most suitable tissue fixation procedure should still be performed.

Although positively associated with the risk of developing metastasis in different tumour types, IMD assessed by using the pan-endothelial cell markers might not accurately represent the dynamic angiogenic capacity of a tumour. In Table 2 [38, 39, 43–46], the antibodies suitable for immunohistochemistry with a variable degree of specificity for activated endothelium are listed. Although the exact nature of this activational state is still under investigation, from the immunising material used to induce antibody production in animals, it can be deduced that inflammation and neoplastic growth induce this phenotype in endothelial cells. The comparable staining patterns of these antibodies in inflammation and tumour progression suggest that the angiogenic processes in both disease conditions might be identical. The highest selectivity for activated endothelium is reported for the antibodies 4A11 and H4/18. Unfortunately, the corresponding antigens of all the listed activation markers are largely lost during tissue fixation and/or paraffin embedding. Information on the success of antigen retrieval techniques is absent in the references mentioned in Table 2. Studies comparing the prognostic value of the antibodies E9 and TEC-11 with that of conventional anti-CD31 staining are ongoing in invasive ductal breast cancer.

CONVENTIONAL METHODS FOR IMD ASSESSMENT

IMD in vascular hot spots according to Weidner and associates

In the first report on the correlation of tumour angiogenesis and metastasis, Weidner and associates [47] described in breast carcinoma the combined method of identifying regions with an elevated vascular density and of subsequently counting the number of microvessel entities within these hot spots [47]. The rationale for assessing microvessel density in selected areas compared to an overall vascular count is manifold. From a practical point of view, given the intratumoral heterogeneity, obtaining a reproducible overall assessment of microvessel density implies the evaluation of large tumour areas. From a pathophysiological point of view, it might be expected that the mechanisms re-

Table 2. Activated endothelial cell-specific antibodies: immunohistochemical characteristics

Antibody	Sensitivity	Specificity	Reactivity		Lymphatic staining
			Frozen	Paraffin	
TEC-11 [43]	>80% of tumour vessels positive	Normal tissue endothelial cells: weakly positive—weak stromal cell staining	+	–	?
E9 [45, 46]	Small intratumoral vessels positive—large vessels negative (fetal—regenerating tissue vessels positive)	Weak normal tissue vessel staining: skin, mucosa, tonsil	+	–	?
EN 7/44 [38, 39, 44]	Small vessels in inflammation, immune reactions and tumours (also Ki67 negative endothelium)	Negative in normal tissue except colon and placenta capillaries	+	?	?
4A11 [38, 39]	Inflammed tissue vessels—tumour tissue vessels	Normal tissue: only vessels in lymph nodes, tonsils and part of vessels in synovial tissue	+	–/+ (fixation dependent)	?
H4/18 [38, 39]	Delayed hypersensitivity—inflammation tissue and Hodgkin's lymphoma lymph node vessels	Negative in normal tissue	+	?	?
FB5 [38, 39]	Large proportion of small tumour vessels—no large vessels	No staining in normal tissue—some malignant cell staining in sarcomas—some fibroblast in tumour stroma	+	?	?

sponsible for the direct and indirect relations between the tumour and the endothelial cell population are particularly active in these highly vascular regions. If vascular hot spots arise due to the existence of angiogenic tumour cell clones, these cells will predominantly enter the circulation and give rise to vascularised metastasis. Although there are convincing arguments that the finding of these regions is critical to assessing accurately the association beween tumour progression and the angiogenic potential, this aspect of the methodology has, since Weidner's paper of 1991 [47], proven to be the most subjective one.

The first problem applies to every pathological evaluation and is related to the selection of a representative tumour block. Weidner and coworkers selected paraffin blocks containing the invasive components of breast cancer specimens by analysing corresponding sections stained with haematoxylin and eosin. In colorectal adenocarcinoma, we have indeed noticed that in the *in situ* growth regions, IMD is approximately half the IMD of the invasive regions [42]. Recently, it has been suggested that multiple blocks per tumour should be assessed for IMD [48]. de Jong and colleagues [49] found a high average coefficient of variation of approximately 24% (range 6–55) if more than one tissue block was analysed as compared to 15% (range 0.5–42) when only counts within one section of one block were made, indicating that careful scanning of all the available tumour material might be necessary to identify the relevant hot spots. The major drawback of this study is the low number, for example 10, of specimens studied and the lack of information on the size of the breast tumours. Martin and associates have determined IMD in sections from three different blocks for 20 breast tumours, one from each end and one from the centre of the tumour [49]. Vessel counts among blocks varied by less than 20% in 14 out of 20 cases (70%). These results are in agreement with Van Hoef and colleagues [50], who also reported a concordance rate of 71–78% between different blocks.

Vascular hot spots are selected by scanning a tumour section on low magnification (10–100×). A low background staining and a highly specific and intense labelling of endothelial cells is required. Using anti-CD31 antibodies, regions with a prominent inflammatory infiltrate might be erroneously taken for a vascular hot spot at low magnification. These CD31 cell infiltrates sometimes obscure microvessels, especially single cells sprouts. The training and experience of the investigator seem to determine the success of finding the relevant hot spot, i.e. with the highest number of vessels at a higher magnification. To assess the degree of subjectivity related to this part of the counting procedure, Barbareschi and associates compared IMD determinations in 91 node-negative, invasive ductal breast carcinomas by light microscopy between two pathologists with different experience [51]. Although their counts were highly correlated ($P = 0.0001$, Spearman's coefficient 0.68), the degree of variability was reflected by a low regression coefficient ($r^2 = 0.4$). Both at univariate and multivariate analysis, only the counts of the experienced pathologist were significantly associated with RFS. In a case–control design, the prognostic value of IMD in a sample set of 38 node-negative breast cancer patients was shown [52]. For three experienced investigators, a classification point could be identified to which a significantly different distribution of IMD values of an unfavourable versus a favourable outcome group was present. Such a classification point could not be observed for the inexperienced investigator. When the counts of two highly experienced investigators were compared according to their median, agreement was reached in 76% of cases. A similar comparison in a study comprising 220 patients resulted in an agreement ratio of 73% [53]. As a preparation of a computer-aided IMD analysis in breast cancer, two investigators independently marked a single area on an individual series of H&E stained slides, identical to the most vascular area on a corresponding series of successive CD34 stained sections [54]. In 7 out of 8 cases the same areas were selected by both investigators. This pilot study suggests that the degree of subjectivity related to the hot spot selection step of vessel enumeration is acceptable.

Vascular hot spots are encountered predominantly at the peripheral tumour margin. Based on the hypothesis that hot spots containing more microvessels provide a more efficient entrance for surrounding tumour cells into the circulation, only hot spots close to tumour cell clusters in viable (i.e. non-necrotic and non-sclerotic) areas have been taken into account in most studies.

Once the region of interest, the vascular hot spot, is defined, a higher magnification is selected in order to be able to count the individual stained microvessels. Although Weidner and colleagues [47] have suggested a field size of 0.74 mm^2, a wide range of magnifications and related field sizes have been applied in breast cancer angiogenesis studies, ranging from 0.12 mm^2 to 1.00 mm^2. A small area, corresponding to a higher magnification, will improve the detail of the image, allowing the identification of more single endothelial cell sprouts. We have noticed a 2-fold higher IMD at magnification 400× than at magnification 200× in CD31 stained breast cancer sections, provided IMD was expressed as microvessels/mm^2 [55]. An area larger or smaller than the hot spot will result in loss of information. Both at magnification 200×, related to a field size of 0.61 mm^2 and at magnification 400×, related to a field size of 0.15 mm^2, the prognostic value of IMD in node-negative breast cancer has been suggested by our group [55]. In order to evaluate strictly the interobserver error of vessel counting, exclusion of the process of the selection of highly vascular areas was performed by taking photographs of individual vascular hot spots. Correlation of the vessel counts done by an inexperienced and an experienced investigator on these photographs was strong, both using a 200× and 400× field size ($r = 0.9$, $P < 0.0001$). This suggests that vessel counting after agreement on the description of a single countable vessel seems to be less dependent on subjective interpretation than the process of hot spot selection.

According to Weidner [47], any highlighted endothelial cell or cell cluster clearly separate from adjacent microvessels, tumour cells and other connective tissue elements, should be regarded as a distinct countable microvessel. This definition has several implications. A lumen is not required, nor is the presence of red blood cells. A cut-off calibre size is not mentioned. Single cell sprouts as well as larger vessels are thus included in the counts. Even if distinct clusters give the impression of being part of one larger vessel transected by the plane of the tissue section more than once, they should be counted as separate microvessels. Strict application of these objective criteria seems to result in low inter-

observer variability when analysing predefined hot spots [55]. CD31 positive inflammatory cells can be differentiated from capillary buds by the pattern of reactivity. New vessel development is associated with a CD31-signal redistribution from a constitutive circumferential membrane pattern to a cell–cell junction pattern [56].

IMD grading

Another technique, in addition to hot spot microvessel density, is semi-quantitative grading. Translation of the continuum of microvessel density values into a categorical type of data will inevitably be associated with a loss of information. Given the highly subjective nature of IMD grading, comparable results will only be obtained by different observers after a long period of training. For multicentre prospective trials involving angiogenesis quantification, this aspect of IMD grading will create a considerable burden. Nevertheless, several studies have reported a positive significant relationship between quantitative and semiquantitative microvessel density scores. In a study in 55 cases of ductal breast carcinoma *in situ*, overall tumour-associated microvessel density was scored on a +1 to +3 scale [57]. Microvessel counts were also performed at 100× magnification in vascular hot spots in the 55 carcinoma specimens. Although lesions with a higher grade of vascularity also had a higher microvessel count, a considerable overlap of quantitative hot spot IMD values between the three categories was found. Overall, grading seems to contain different information compared with hot spot vascularity assessment. Weidner and associates have subjectively graded angiogenesis in vascular hot spots, as well as counting individual vessels in the same fields [58]. Microvessel density values obtained by both methods were a statistically significant predictor of overall survival (OS) and RFS. The obvious advantage of IMD grading is its time-efficiency. By comparing IMD grading with multiparametric computer image analysis of the vasculature in breast carcinoma, a strong correlation was found with the luminal area [59]. The correlation with luminal perimeter and number of microvessels was found to be an order of magnitude lower. This suggests that the investigator when grading is mainly guided by the total area of the tumour vasculature rather than by the number of separate microvessels.

Chalkley counting

In an attempt to facilitate the measurement of tumour angiogenesis, Chalkley point counting has been recently applied by Fox and associates [59]. This method still implies scanning of the tumour section at low magnification to identify the areas giving the impression of containing the maximum number of discrete microvessels. At higher magnification (200–250×), an eyepiece graticule containing 25 randomly positioned dots is rotated so that the maximum number of points are on or within the vessels of the vascular hot spot. Instead of counting the individual microvessels, the overlaying dots are counted.

In a pilot series of 30 invasive breast carcinomas [59], a significant correlation was found between microvessel density assessment according to Weidner and Chalkley point counting ($r = 0.71$, $P = 0.00005$). Both in a series of 45 transitional cell carcinomas of the bladder [60] and of 211 invasive breast carcinomas [59], it was then shown that Chalkley point counting gave independent prognostic information. In a recent study, results on the determination of angiogenesis in node-positive breast cancer patients were pooled from two collaborating centres using Chalkley counting [61]. In a multivariate analysis, the Chalkley score was found to be the strongest significant independent predictor of outcome. The study suggests the validity of Chalkley counting for comparing angiogenesis for prognostic purposes between different centres. As the relative area taken by the vasculature is reflected by this new method, it seems to be a suitable alternative for IMD assessment according to Weidner's guidelines. Since no decisions have to be made on whether adjacent stained stuctures are separate microvessels or not, Chalkley point counting should be a more objective approach. The most observer-dependent step though still remains, i.e. the selection of vascular hot spots.

Multiparametric computerised image analysis systems (CIAS)

Semi-automated counting has been suggested as a more objective method of assessing IMD. The main advantage of CIAS is the additional morphometric parameters that can be detected: the number of vessels within a certain dimension range, the vessel lumen area, the vessel lumen perimeter and the percentage of immunostained area per microscopic field. Although the assessment of IMD by CIAS has been reported, other parameters might be more objectively measured, i.e. without the intervention of an investigator. The heterogeneity of microvessel morphology, e.g. size, length and anastomoses, and the locoregional differences in immunostaining intensity particularly hamper a fully automated analysis of tumour tissue sections for IMD.

In a series of 91 node-negative invasive ductal breast carcinomas, both the number of CD31-positive microvessels measured by an experienced pathologist and the microvessel area (MVA), consisting of the endothelial cells plus the vessel lumen and determined by CIAS, were independently associated with RFS [51]. This study provides some indications that the prognostic value of MVA is less dependent on the experience of finding neovascular hot spots. In this way, it may be of help to pathologists unfamiliar with quantifying tumour angiogenesis.

Comparable results have been reported by Fox and associates in 30 invasive breast carcinomas [59]. Manual vessel counts were significantly correlated with MVA, microvessel perimeter (MVP) and microvessel number assessed by CIAS. Charpin and colleagues have determined the total CD31 immunostained area or endothelial area (EA) in 133 breast carcinomas [62]. Automatic screening of the whole tumour section resulted in a positive CD31 surface ranging from 4 to 33% (mean: 15%, S.D.: 5.5%). The CD31 immunoreactivity showed a significant correlation with a prognostic index combining tumour size, node status, number of affected lymph nodes and tumour grade. In another attempt to eliminate the subjectivity related to recognising individual microvessels, Simpson and associates [54] assessed the total anti-CD34 stained area in 178 invasive breast carcinomas. The number of CD34-positive pixels was recorded in five adjacent fields at 20× magnification in the manually identified area of greatest vascular density. Image analysis of EA (number of positive pixels) was significant in multivariate analysis for OS only in lymph

node-negative patients, and for disease-free survival (DFS), only in lymph node-positive patients. Tumour grade, but not manual vessel count, was also significant for OS.

The apparent disadvantage of interactive computer-aided IMD measurements is the time-consuming nature of the method. Automated hot spot selection would solve both this problem and the problem related to the subjectivity associated with manual IMD assessment. Fox and associates were unsuccessful in their attempt to perform completely automatic quantification [59], due to the extremely low level of interference of non-microvessel structures allowed by CIAS. Highly specific endothelial cell markers are needed and variations in staining intensity have to be eliminated or compensated for, for example, by automatic background subtraction. The use of reference slides when staining batches at different times, automated staining and adjustment of fixation techniques in multicentre studies have to be considered.

In conclusion, CIAS seems to provide additional information on the morphology of the tumour vasculature. Most studies seem to indicate that MVA, EA and MVP are a quantitative reflection of the degree of pathophysiological involvement of tumour angiogenesis in tumour progression. Integration of IMD, MVA, EA and MVP with information of the activity status of the tumour-related endothelium might provide a more dynamic picture of the vascular component of a tumour. CIAS might be introduced as a more objective method of microvessel quantification and eventually to perform automated hot spot selection. The high signal-to-noise ratio of the immunostaining required for CIAS IMD assessment might result in a considerable number of tumour sections unsuitable for evaluation. Artificial intelligence-based pattern recognition systems with a learning capability might have to be developed to counter the inherent degree of irreproducibility related to immunohistochemical techniques.

PROPOSED STANDARD METHOD FOR IMD ASSESSMENT

In Table 3, a proposition for the standardisation of the immunohistochemical method for IMD measurement is given. Quality control of (i) the selection of the tissue sample as part of the whole tumour specimen, (ii) tissue processing and immunostaining, (iii) the selection of area(s) for microvessel enumeration and (iv) the technique of IMD evaluation within these areas is required for increased reproducibility and intercentre comparability [63]. For immunohistochemical IMD assessment to become part of the routine pathological protocol in daily cancer medicine, easy, reliable and fast low-cost techniques have to be proposed, based on more elaborate clinical research studies. A systematic scanning of the whole tumour section at low magnification to identify all apparent hot spots and the immediate counting of vessels within these hot spots at higher magnification might be too time-consuming. Martin and associates suggest that the number of hot spots analysed should be at least 10 to reduce the chance of missing the most vascular area in breast cancer sections [41].

A training programme for the inexperienced pathologist would be of great importance given the subjective methodological step of vascular hot spot selection and, if Chalkley counts are not performed, of the identification of individual microvessels. To deal with the former subjective step, a

Table 3. Proposed standard method for IMD assessment: the unresolved technical aspects require a prospective controlled comparison with the proposed standard

Methodological aspect	Proposed standard	Advantage	Unresolved
1. Tissue processing	Paraffin embedding: non-aggressive fixatives (e.g. neutral buffered formalin) and temperature control	Unsegmented microvessels as opposed to frozen sections	Multiple tissue blocks to be investigated per tumour?
2. Immunostaining	Anti-CD31 monoclonal antibody + microwave antigen retrieval or protease pretreatment	Most sensitive pan-endothelial marker	Activated/proliferating/ intratumoral endothelium specific markers more informative?—parallel anti-CD34 staining?
3. Selection of the quantification field(s)	Manual vascular hot spot selection at low magnification (e.g. 10×)—systematically scan whole section—only in viable tumour tissue or adjacent (e.g. within diameter of one field at 200× magnification) stromal tissue	All highly vascular areas can be detected	Combined with overall IMD assessment?—measurement of the number and the size of the hot spots informative?—selection of the 10 most vascular areas?
4. Quantification of microvessels	Chalkley point graticule method: presence of a vessel lumen not required—adjust field size approximate to the mean size of the vascular hot spots (tumour type dependent)	Exclusion of the subjective step of identifying individual microvessels in an endothelial cell cluster	Total endothelial area determination in hot spots by CIAS more objective?
5. Number of observers	Sequential IMD assessment by two investigators—if difference >10%: third investigator or blind recount	More practical in a clinical setting than co-observation	

comparison of localisation of the highly vascular areas identified by an investigator in training and by an experienced investigator, as performed in the pilot experiment of Simpson and colleagues [54], should be repeated on different and a sufficiently large series of tumour sections, until agreement in more than 90% of cases is reached. As final training, a set of tumour sections belonging to a study in which IMD has already been proven to contain prognostic information, should be re-analysed blind by the inexperienced observer. To reduce the number of patients in such a training set, a case–control setup might be preferable, as used within our group [52]. Such reference sets should be made available by clinical research laboratories with a longstanding experience in IMD measurements and should preferably be related to published data. The set of tissue slides can be used both for training sessions as well as for intercentre standardisation of the applied methodology. A set of unstained slides supplied by reference laboratories could be implemented in the quality control routine of clinical pathology units entering the angiogenesis research field.

Finally, awareness of the potential pitfalls in the processing of the clinical data is required before initiation of the study. Accurate staging and patient follow-up are necessary to detect patients with recurrent tumours. An unbiased case selection is more likely by a prospective study design. Data analysis has to be performed by statistical experts [63].

Categorising tumours in low, intermediate, and high IMD classes discards information. Preferably, a multiparametric formula should be derived in which IMD has to be entered together with the established prognostic factors. This multivariate analysis then yields an individual risk factor on a continuous scale [64]. If a cut-off point is needed, the median value might be used because then groups of comparable size will be obtained.

COMPLEMENTARY METHODS TO ASSESS THE ANGIOGENIC POTENTIAL OF TUMOURS

In this Special Issue of the *European Journal of Cancer*, other authors extensively discuss the techniques for determining the expression level of angiogenic peptides and angiogenesis inhibitors in tumour tissue or body fluids of cancer patients.

Two methods for assessing properties of tumour angiogenesis with a probable future clinical impact are (i) serum and tissue sampling of angiogenic peptides and (ii) contrast-enhanced MRI in cancer patients. These techniques may be complementary to histological assays of angiogenesis quantification and have the apparent advantages of being non-invasive, of analysing the entire tumour and of allowing repetitive sampling pre- and postoperatively and during follow-up/treatment.

We have analysed serum levels of bFGF and VEGF in untreated metastatic breast cancer patients using commercialised ELISA-kits (R&D Systems Inc., Minnesota, U.S.A.). According to clinical criteria, patients were classified as having a fast or slowly progressive disease after a mean follow-up period of approximately 1 year. As cut-off values, the 95th percentile of the serum levels of a control group were taken (7.5 pg/ml for bFGF and 500 pg/ml for VEGF). The first serum level of bFGF and VEGF measured during the follow-up period was taken for further analysis. Around two-thirds of metastatic breast cancer patients ($n = 26$) had elevated serum levels of one or both angiokines. All patients with a fast progression ($n = 14$) had elevated serum levels of at least one angiokine as compared to only 25% of the 12 patients with slowly progressing disease (chi-squared P-value < 0.0001) ([62] and unpublished data). In colorectal cancer, more than 80% of the pre-operative patients were found to have elevated serum levels of bFGF and/or VEGF. This was reduced to 33% after surgery (chi-squared $P < 0.01$). No patient with normal pre-operative angiokine serum levels had elevated levels after surgery. The same positive association of kinetics of tumour progression and serum levels of bFGF and VEGF as found for metastatic breast cancer was valid in metastatic colorectal cancer (unpublished data). Yamamoto and associates recently described significantly higher serum VEGF concentrations in advanced stage primary breast cancer compared with early stage primary breast cancer patients ($n = 137$) [66]. Pre-operative VEGF serum levels were increased in association with an increase in IMD. A decrease in the circulating levels of VEGF after removal of the primary tumour was observed in the majority of patients.

Kinetic profiles of contrast uptake and washout in contrast enhanced MRI seem to correlate with tumour grade and IMD in breast cancer [67, 68]. MRI derived tumour tissue plasma volume increased exponentially with increasing histological capillary density in a mouse mammary carcinoma model [30].

Parameters measured by both techniques seem to be related to tumour progression or grade rather than simply to tumour volume. This aspect offers promising perspectives for their use in monitoring tumour response during treatment or in predicting tumour relapse. However, it will be necessary to determine the net balance between angiogenic growth factors and natural angiogenesis inhibitors, for example, angiostatin and thrombospondin [1]. Reliable methods to assess the latter are still lacking.

CONCLUSION

Standardisation of angiogenesis quantification is necessary to facilitate confirmation of the suggested prognostic value of IMD in different tumour types in prospective controlled multicentre studies. In addition, IMD assessment might be applied (i) to predict the risk of malignant transformation of premalignant lesions and (ii) to predict response to cancer treatment. The more dynamic serum angiokine sampling and contrast enhanced MRI or computed tomography (CT) techniques might be used (iii) to monitor the response to cancer treatment and (iv) to diagnose preclinical metastasis early. (v) If antibodies directed to activated/proliferating/intratumoral endothelium are compared with pan-endothelial immunostaining, more accurate prognostic information might be obtained after primary tumour resection.

In a retrospective, matched, case–control study comprising 24 case patients who had had a biopsy for a benign breast lesion, IMD was determined on FVIII-RAg stained slides and by using an image analyser [69]. The relative risk of developing breast cancer increased with increasing IMD. These results need to be confirmed in a larger series and the same type of study has to be performed in other tumour types (e.g. preneoplastic colorectal polyps).

In a series of 73 patients with clinical stage II–IV squamous cell invasive carcinoma of the head and neck treated

with concurrent chemoradiation therapy, IMD and performance status retained a significant predictive value for response in a multivariate analysis [70]. Low IMD predicted a good response to therapy. The same group found that angiogenesis quantification adds prognostic information to the oestrogen receptor status in predicting the outcome of breast cancer patients treated with adjuvant tamoxifen [61, 71]. IMD was also reported to predict responsiveness to platinum-based chemotherapy in FIGO stage III–IV ovarian carcinomas [72].

In a trial evaluating the effect of continuous and escalating recombinant leucocyte interferon (IFNα2b) in patients with metastatic carcinoid tumours, contrast enhancement of the tumours on consecutive CT scans was found to be reduced prior to tumour volume reduction. Progression during therapy was preceded by renewed contrast enhancement, prior to tumour enlargement [73]. As well as being indicative of an additional anti-angiogenic effect of interferon in this disease, these results also suggest that changes in the net result of contrast uptake/outflow by the tumour vasculature can reflect response to therapy or progression. Contrast enhancement on CT of lymph node metastasis in head and neck cancer was found to be predictive for response to chemotherapy, suggesting that this enhancement is a measure of vascularity [74].

Future clinical studies exploiting the antitumour effect of biological modifier agents with variable selectivity for elements of the angiogenic process might have to be tailored to the angiogenic profile of individual patients [75–77]. Consecutive serum sampling of angiogenic peptides and their inhibitors might help to design and monitor such a therapy.

Peripheral accentuation of the expression of the adhesion molecules E- and P-selectin on endothelial cells has been reported in breast cancer. The level of expression might reflect the angiogenic drive of the tumour stroma [78]. The role of stromal metalloproteinases in angiogenesis is also under investigation. Zymographical analysis in breast cancer tissue revealed a significant positive association of the expression level of 93 kDa gelatinase with an increase in IMD (M. Toi, Tokyo Metropolitan Komagome Hospital, Japan).

Qualitative changes in the intra- and peritumoral endothelial cells are revealed by immunostaining of tumour sections with markers specific for activated endothelium. E9 and TEC-11, both directed at endoglin (CD105) which resembles the TGFβ type III receptor, and antibodies directed at members of the cell adhesion molecule family of integrins [46], might more specifically recognise the fraction of endothelial cells affected by the angiogenic drive of the tumour/host. A study to verify whether the ratio of activated versus quiescent endothelial cells adds prognostic information to conventionally assessed IMD in breast cancer is ongoing (P.B. Vermeulen, Antwerp University Hospital, Belgium).

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Acknowledgements—P.B. Vermeulen is supported by a grant from the "Vlaamse Kankerliga" (VKL), Brussels. G. Gasparini and G. Viale are supported by grants from the "Associazione Italiana per la Ricerca sul Cancro" (AIRC), Milan.