Morphologic Approaches to the Assessment of Angiogenesis

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INTRODUCTION

Over the past twenty years, the study of organ microcirculation has received more attention than ever before. This is due primarily to the existence of factors which are able to modulate angiogenesis, the outgrowth of new blood vessels from pre-existing ones, which occurs in various organs under physiologic and pathologic conditions. Changing quiescent endothelium into a proliferating phenotype has been one of the most exciting medical discoveries in recent years. Angiogenesis modulation promises to impact the treatment of various pathological processes, including ones in which induction of angiogenesis aids tissue repair (atherosclerosis, myocardial infarction, gastrointestinal ulcers, wounds) or in which it is inhibited, thus delaying disease progression (retinopathies, neoplasia) [1].

ANGIOGENESIS IN TUMORS

The concept of treating solid tumors by inhibiting angiogenesis was first suggested some 30 years ago by Judah Folkman [1]. Although recent discoveries have created intense interest in the use of anti-angiogenic drugs for cancer treatment, significant problems surround its clinical application. Unresolved issues include the identification of reliable indicators of patient need for such treatment, of likely success, and of side effects of anti-angiogenic therapy. Non-invasive assessment of tumor vascularity is possible in vivo by means of Doppler sonography, dynamic contrast-enhanced magnetic resonance imaging (MRI), and postmortem emission tomography (PET). Although these methods may be useful in monitoring the effect of anti-angiogenic therapy, histology remains the ‘gold standard’ for assessing tumor vascularity, predicting disease progression and monitoring the response to treatment.

In tumor pathology, both immunohistochemical and morphometric methods are frequently used in the study of angiogenesis. The potentials and limitations, as well as the challenges posed by each of these methods are also discussed.

KEYWORDS
angiogenesis, cancer, immunohistochemistry, quantification

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involve proliferation of endothelial cells, a cell population usually quiescent, and stable cells in adults. Destruction of the sleeve of basal lamina surrounding the capillary endothelium permits the migration of endothelial cells, a necessary feature of new capillary formation. The latter process is complex and various biomarkers have been proposed to identify what are new blood vessels. Of these, most bind to activated, proliferating endothelium. Cell cycle markers (MIB-1, topoisomerase IIα) or specific antibodies directed against activated endothelial cells (4A11, H4/18, F85, E9) fall into this general group (Fig 3). These are highly specific markers, but they are not often employed because their respective antigens are usually lost during tissue fixation and further processing.

Given rapid progress in the field, the development of new biomarkers of activated and proliferating endothelium continues apace. Among the new markers, integrin αvβ3 deserves special mention, since it is of therapeutic and prognostic significance. Integrin αvβ3 is a cell surface receptor prominently expressed on the surfaces of endothelial cells. Functionally, it links components of the extracellular matrix (vitronectin) with the cytoskeleton, thus generating intracellular signals to regulate various processes, including endothelial cell migration. Accumulating evidence indicates that integrin αvβ3 is at the same time a specific marker for newly formed blood vessels, and a valuable prognostic indicator in different tumor systems. Most promising is the observation that integrin αvβ3 antagonists are anti-angiogenic and substantially reduce tumor growth under different experimental conditions; thus they may have therapeutic potential [2].

Proteolysis of extracellular matrix is required for new blood vessel formation to take place. Thus, it is no surprise that recent studies finding biochemical changes in extracellular matrix composition or increased proteolytic activity in perivascular connective tissue concluded that they were useful markers of neovascularization. For example, it has been shown that such components of vascular basal lamina as tunstastatin (the NCL domain of alpha 3 chain of type IV collagen), endostatin (a fragment of collagen XVIII), and the laminin alpha 4 chain play important roles in regulating angiogenesis. On the other hand, matrix metalloproteinases (MMPs), a family of structurally related proteases that play a role in extracellular matrix degradation and tissue inhibitors of metalloproteinases (TIMPs) have also been included among markers of neovascularization.

Angiogenesis results from the delicate balance between factors that stimulate and inhibit endothelial cell proliferation. In tumors, the process is thought to be initiated by an increase in the level of angiogenic stimuli and a concomitant decrease in the level of angiogenesis inhibitors that results from various factors, including low oxygen concentration. In order to obtain a complete picture of the process, changes in expression of activators and inhibitors of angiogenesis should be evaluated simultaneously. Vascular endothelial growth factor (VEGF) is the most significant growth factor affecting angiogenesis(Fig 4) in that it regulates multiple endothelial cell functions, including mitogenesis [3]. The expression of VEGF and its tyrosine kinase receptors (Flt-1, Flt-4, and KDR/F1k-1), have been shown to be elevated in a variety of tumors. Conversely, decreased expression of thrombospondin 1 and 2, endogenous inhibitors of angiogenesis, may also be of prognostic value in different tumor systems. Recent studies demonstrated that hypoxia-inducible factor 1 (HIF-1) (Fig 5), a transcription factor playing a pivotal role in up-regulation of genes involved in hypoxia adaptive mechanisms, overexpressed in the majority of human cancers [4]. Thus, it too may be a prognostic indicator.

**MORPHOMETRIC METHODS OF ANGIOGENESIS ASSESSMENT**

Morphometric quantification of tumoral microvessels is the most frequently used method of estimating tumor angiogenesis. Various methods have been applied to evaluate vascularity; each has its own shortcomings [5]. Since the distribution of vessels within tumors is heterogeneous, different methods have been proposed to adequately evaluate...
representative tumor areas. All are based on the estimation of microvessularity in areas of the tumor showing the greatest vascular density - the so-called ‘hot spot’ (Fig 1). Thus, first at low magnification, tumor areas containing the maximum number of microvessels are identified. The most widely used method of assessing vascular areas involves the determination, at high magnification, of the number of microvessels or the number of randomly positioned dots (vessel cross sections) located within the hot spot. It has been suggested that endothelial cell proliferation is particularly active in these highly vascularized regions and that hot-spot identification may be critical to the accurate assessment of angiogenic potential and thus tumor progression. Unfortunately, all methods of angiogenesis assessment involving hot-spot determination have serious shortcomings. The most striking limitation is high intra- and interobserver variability. Hot-spot determination is highly subjective, and results are difficult to reproduce. It is of note that reproducibility is not necessarily optimized by choosing the same hot spot at a low magnification, in that variation in the selection of a higher power microscopic field often yields different, quite variable counts.

Recently, more objective methods have been suggested to assess tumoral microcircularity. Semi-automated quantification of microvessels within the entire tumor section by randomly distributed field sampling remains a possibility. Using either method, manual or automated, one must select among different morphometric parameters. These include microvessel number, vessel lumen area, and vessel lumen perimeter. Vessel lumen area and vessel lumen perimeter determinations are two different, but frequently assessed stereological parameters. Micrvascular density (MVD) represents the percentage of a tumor occupied by vessels and is determined by measuring their cumulative area within each field. It differs from microvessel surface density (M3D), the percentage of the total vessel circumference in direct contact with tumor cells. Both parameters are closely related and are of functional significance. Since blood flow equals

the volume flow divided by the cross-sectional area of the vascular bed, MVD provides information on the role played by the capillary network in determining blood flow in different portions of the tumor.

Morphometric estimation of microvascularity based on whole tumor section assessment has important advantages over other methods of hot-spot determination. Thus morphometric methods have a high reproducibility with low intra- and interobserver variation. In this regard, the results will be less dependent on the experience of finding hot spots. Stereological studies also take into account the factor of microvessel architectural complexity, defined as degree of vascular branching and tortuosity using fractal analysis. Assessment of microvessel fractal dimension generally shows that tumor microvessels are more tortuous and branching than those of the corresponding normal organ. One recent study reported that microvessel fractal dimension is of prognostic value in several tumor types, including renal cell carcinoma. Although the application of stereological methods will contribute to standardization of angiogenesis quantification, most are time-consuming and difficult to use. The major limitation of stereological methods in the study of angiogenesis relates to heterogeneous microvessel distribution and to the selection of tumor blocks truly representative in large tumors.

TUMOR BEHAVIOR

An important conclusion in diagnostic pathology, particularly in quantification of morphologic features, is the fact that cancer is a very complex process. This must be taken into consideration in studies employing various angiogenic markers.

First, the course of development and progression of tumors differs in individual patients. Indeed, the lesion may be first recognized at any stage in its natural history. For example, Bergers et al. [6] showed that antiangiogenic drugs prove most effective when they are targeted to specific stages of cancer. This observation suggests that microvascularity may play a different role during the course of tumor progression. Secondly, tumors occur in variable sizes. It has been stressed that tumors smaller than 2 to 3 mm in diameter do not require an extended microvascular network in order to grow. Thirdly, it is important to take into consideration that there are more than 300 distinct varieties of tumors, each with its own characteristic biology. Some appear to develop relatively independent of angiogenesis. For example, unlike most tumor systems, our studies of pituitary tumors found them to be less vascularized than the nontumorous adenohypophysis (Fig 2). Thus, it came as no surprise that in pituitary tumors, no significant correlation is seen between vascularity and cell proliferation. It is of note, however, that uniformity of vessel architecture is greater in aggressive pituitary tumors, a finding suggesting a delicate balance between tumor metabolism and blood supply than might be suspected based on low vascular density. It should also be mentioned that the age of patients may affect angiogenesis, tumor growth and progression. Lastly, it has been shown that in aggressive primary and metastatic melanomas, the tumor cells themselves comprise microcirculatory channels. Tumed vasculogenic mimicry, this process is not strictly a vasculogenic event (Fig 6), in that it does not, like angiogenesis, involve the formation of endothelial-lined vessels [7]. Finally, it is noted that some tumors grow under conditions of relatively low oxygen concentration. This is of practical importance in that tumor resistance to radiation therapy is known to be associated with tumor hypoxia.

REFERENCES