SOFT TISSUE SARCOMA

PATHOLOGICAL DEMONSTRATION OF VASCULARIZATION

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Abstract- The investigation and determination of the significance of neovascularization in the progression of soft tissue sarcoma is currently of increasing clinical importance. Microvessel density is the morphologic evidence of neoangiogenesis in the process of malignant progression of sarcomata. This brief review of the markers commonly used in clinical practice was undertaken to determine the validity of the application of these markers in the quantitation of neoangiogenesis in sarcomata. The material selection was based only on primary material from untreated cases that were part of the in-hospital and consultation practices. From this investigation, one can conclude that not all markers have equal value in either the qualitative or quantitative assessment of vascularization in tumors. Laminin was found to have low sensitivity. Factor VIII was found to have extreme sensitivity but lack specificity. A combination of collagen IV, smooth muscle actin, CD34 and CD31 markers were found to represent the most reliable identificators of blood vessels in sarcomata. Morphological identification of vessels may be further supplemented by proliferation markers (e.g. PCNA, Ki67) as indicators for, not only the presence of blood vessels, but also to determine the activity of endothelial cells in blood vessel growth.

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Key words: Neovascularization, immunohistochemical identification, soft tissue sarcomata

INTRODUCTION

Folkman in 1963 introduced the concept that tumor growth and survival depends on angiogenesis (1-8). It was his contention that blood flow plays an important role not only in the physiologic function of normal tissue, but also in the growth, progression, invasion and dissemination of the tumors (2, 4, 5, 9, 10-19). In 1971, he went on to report that tumor cells and blood vessels are part of a highly integrated ecosystem in which endothelial cells could be switched from a resting state to a rapidly growing state by diffuse signals from the tumor cells or from the associated inflammatory infiltration (20-25).This he interpreted as supporting the concept of neo-capillary growth at a rate of 1 mm per day (18). Further, he indicated that microvessel density could be a good predictor of tumor growth and of disease free survival of patients treated by surgery.

The objective of the present investigation was to find a practical and reliable method of microvessel quantitation in soft tissue sarcomas. If this was to be a marker for prognosis and outcome prediction, then the use of specific histomorphologic and immuno-histochemical methods of blood vessel identification needs to be developed in order to see which markers of endothelium, pericyte or basement membrane could be applicable to the assessment of tumor vascularity and the biological significance of these observations.

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To test this thesis we examined a series of diverse sarcomata to see if one could develop a reliable, practical immunohistochemical approach that could be applied to the morphological assessment of microvessel density in soft tissue sarcomas.

MATERIALS AND METHODS

From the files of the senior author, 16 random current cases seen in consultation were retrieved for the study. Cases were chosen from the previous six months on the basis that material to be available for the required histomorphological, immunohisto-chemical and morphometric assessment. No other selection or exclusion criteria were applied.

The material was selected on the basis that it represented the most recent material submitted in consultation to the senior author at Wellesley Central Hospital, Toronto University, Toronto, Canada.

The age range of these patients was from 38 to 86 years (mean age 65.36 years). Seventy-five percent were male and twenty-five percent female. Only after the pathological assessment was completed was the clinical history made available. Since these were current cases, follow-up was limited. However, since the purpose of the study was to determine the validity of various methodologies for the demonstration of vascularization of tumors before undergoing any further study of the significance of vascularization to outcome and prognosis, it was felt that the limited follow-up was not a determining factor. The study was strictly a morphologic one.

Included were five leiomyosarcomas, four gastro-intestinal stromal tumors, two malignant fibrous histiocytomas, one liposarcoma with a mixed histomorphologic pattern, one Kaposi's sarcoma, one spindle celled sarcoma (subclassified as a fibrosarcoma), one aggressive sarcoma (subclassified as neurosarcoma), and one dermatofibrosarcoma protuberans.

The original histopathological slides were retrieved. Blocks were selected for study on the basis of tissue quality and absence of necrosis, hemorrhage or tissue disruption. The areas selected were those in which the tumor was well preserved and intact and in which vascular integrity would be expected to have been preserved. Eventually, this was reduced to a single representative paraffin block from each case. Four to six micron sections were taken from each block. The routine stains were the “WHO” (hematoxylin, phloxine, saffron, alcian green) and H&E stain. Histochemically, only reticulin and elastic stains were carried out. Immunohistochemical studies were performed on formaldehyde fixed paraffin embedded tissue using the avidin-biotin immunoperoxidase complex technique (26).

The anti-sera used included: anti-Ulex Europaeus agglutinin, anti-human von Willebrand Factor (vWF, also known as Factor VIII related antigen), CD34, CD31, collagen IV, smooth muscle actin and laminin.

Microscopy was carried out with a light microscope (Leitz Orthoplan, at magnification of x 400). Individual microvessels as identified by the individual staining techniques were counted. The typical brown-red stain indicating positive reaction by immunohistochemistry was taken as an indicator of endothelial cell presence, basement membrane presence or perithelial cell presence, depending on the stain.

When a cluster of cells showed positivity for endothelial cell markers, associated with red blood cells in the lumen, identification of vascular channels was most reliable. Ten non-overlapping fields were chosen randomly in each slide avoiding areas of oedema, inflammation, hemorrhage, fibrosis or necrosis. The specific parameter quantitated was the number of vessels as indicated by immuno positivity. A blood vessel was defined as any cell, group of cells or fully developed channel identified by the marker used. This avoided the difficulty of defining a vascular “tube” but reflected presence of endothelial or pericytic cells as well as stromal constituents of a blood vessel wall, *i.e.* basement membrane and supporting connective tissue.

Once the quantitation of the findings in ten fields was obtained, the average of the results were recorded and entered in a personal computer (Excel). The result was expressed as the number of vascular elements showing positivity per field (the field measured approximately 0.180 square millimeters).

Any case in which vascular invasion, thrombosis or blood vessel wall damage or destruction was identified was separately recorded. This usually

applied to the larger vessels. In most cases, the sections did not include the periphery of the tumor and therefore it was not possible to differentiate between the vascularization at the periphery versus the vascularization more centrally in the tumor.

RESULTS

Figure 1 shows the results of different staining methods in different types of tumors. It is immediately apparent that there is no one marker that preferentially or more consistently identifies blood vessels. Laminin, a marker of adhesion molecules to basement membrane of blood vessel wall, was the least sensitive of all. It is obviously not the marker to provide a reliable overview of blood vessel examination in sarcomata.

The other six markers used do not appear to offer a choice in the assessment of the multiple types of the soft tissue sarcoma used in the study. They all seem to provide positivity which within the parameters of the study seem to be relatively comparable. When one looks at individual types of sarcoma examined, one found inconsistency from field to field within each case, variability between cases of similar diagnosis as well as between the various diagnostic groups used in the study (Fig. 1).

In leiomyosarcoma, the blood vessels seem most sensitive to CD34, Ulex Europaeus, CD31 or Factor VIII (Fig. 1, A, B, C, D, E). Smooth muscle actin and collagen IV were less sensitive and laminin was the least sensitive. Because of the variability from field to field, the three top markers in positivity would obviously be needed in each case.

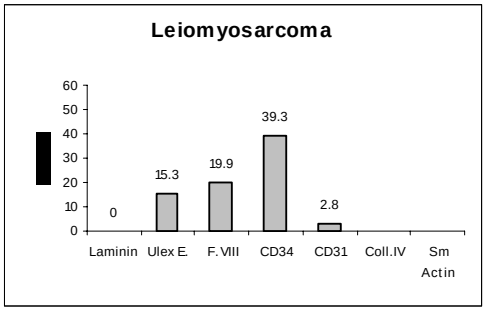
In the assessment of vasculature in the gastro-intestinal stromal tumor, positivity in the tumor cells precluded reliable assessment of positivity in endothelial cells for CD34. The most consistently positive marker in this group was the smooth muscle actin. Factor VIII and CD31 were less strongly positive. Laminin and collagen IV were the least reliable (Fig. 1, F, G, H, I). The vascularity of liposarcoma seems to be sensitive to all markers used quantitatively. In fact, collagen IV and CD34 qualitatively seem the best, easiest and most consistent in the assessment (Fig. 1, J).

The vasculature of malignant fibrous histiocytoma was demonstrated by all markers except laminin. CD34, and CD31 were the two most sensitive (Fig. 1, K, L). This is not surprising since these are two markers whose specificity is for endothelial cells. Factor VIII did show strong positivity but unfortunately this seemed to be not limited to blood vessel walls but extending into the surrounding tissues indicating vascular injury and possible initiation of the coagulation cascade. Smooth muscle actin seemed to be the most consistent throughout the fields examined. The vasculature in the spindle celled fibrosarcoma demonstrated positivity of most markers used (Fig. 1, M). However, Ulex Europaeus and CD31 were the ones with the strongest, most consistent positivity throughout the examined fields. Factor VIII and CD34 also gave strong positivity.

Kaposi's sarcoma showed its blood vessels to the best identified by smooth muscle actin, Factor VIII or collagen IV (Fig. 1, N). The other markers were inconsistent with marked variability and with weakness of sensitivity. CD34 in Kaposi's sarcoma shows positive staining in the background. With this marker, tumor blood vessels were not obvious for assessment because of the strong background positivity. The aggressive spindle celled sarcoma (neurosarcoma) showed very strong vascular positivity for all markers except laminin (Fig. 1, O).

Dermatofibrosarcoma protuberans showed positivity in the tumor cells for CD34, precluding its usefulness in the assessment of blood vessels (Table 2P). Collagen IV, Factor VIII and Ulex Europaeus were the best in demonstrating the blood vessels. Collagen IV and CD31 were relatively weak and inconsistent. We found out that not all markers have equal value either in qualitative or in quantitative assessment of vascularization in soft tissue tumors, therefore it seemed not necessary to evaluate more patients as a large series, while we reached the goal on a small series of patients.

Samples of different staining methods used in this study are shown in figures 2 to 8.



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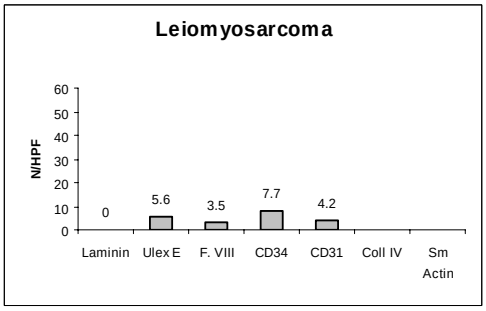

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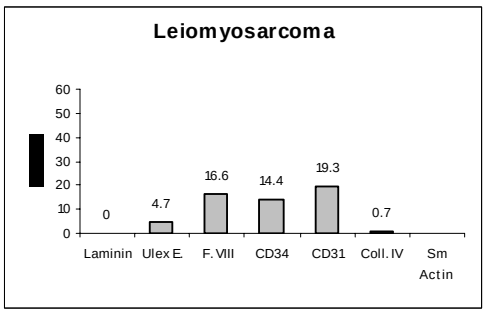

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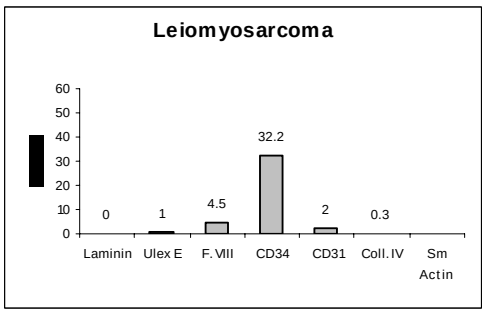

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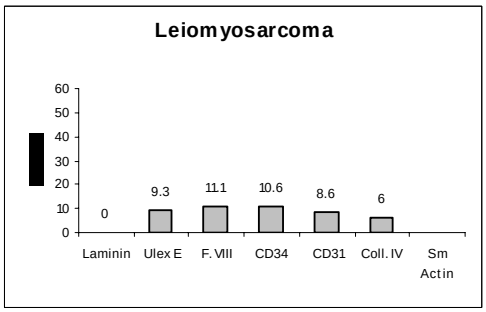

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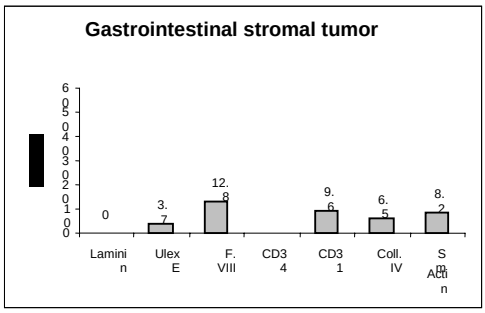

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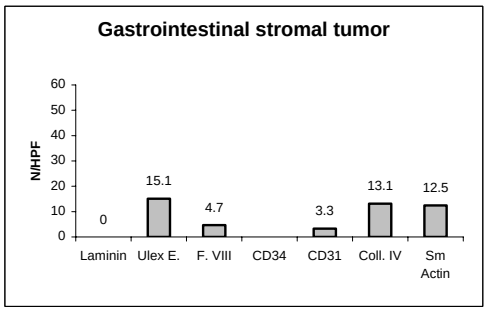

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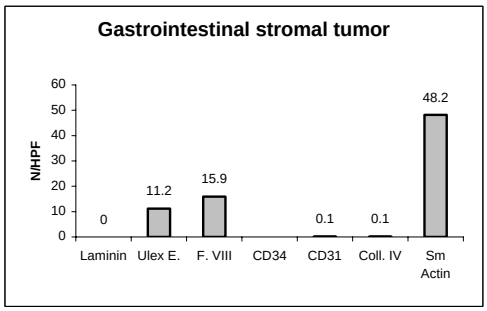
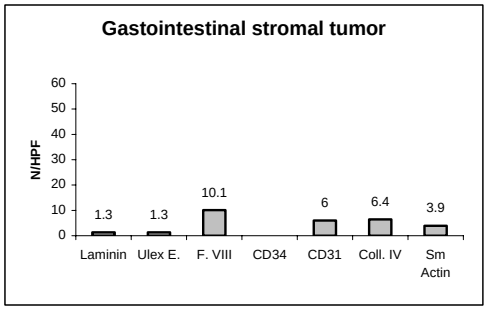


H

Fig. 1. Results of different staining methods in different types of tumors.



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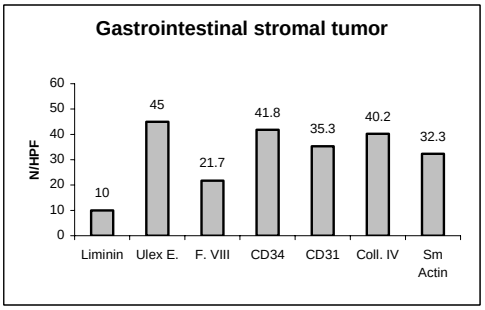

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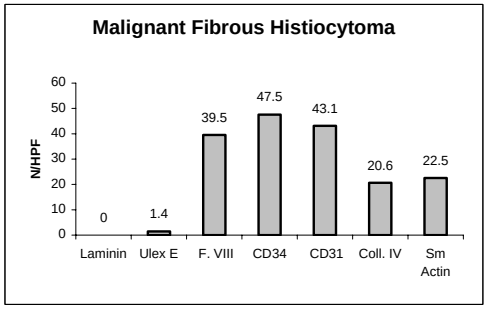

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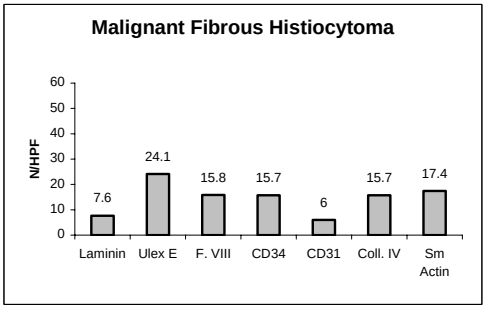

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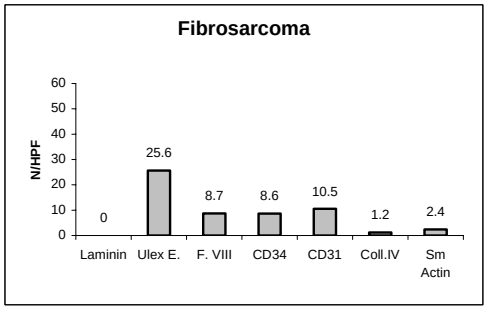

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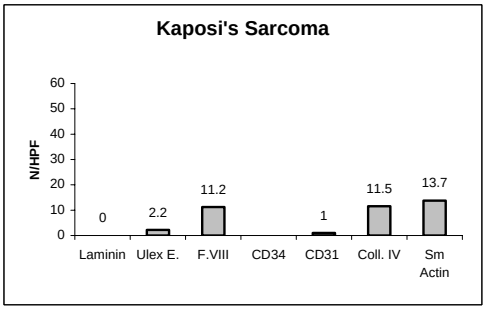

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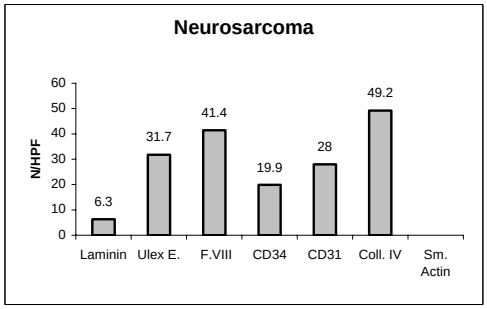

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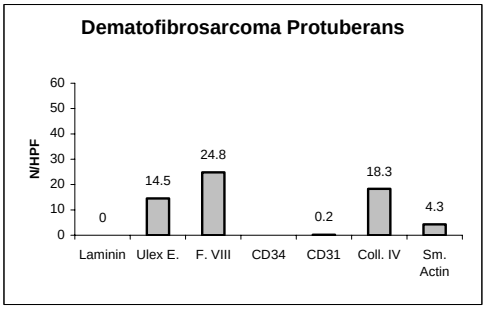

P

Fig. 1. (Continued). Results of different staining methods in different types of tumors.

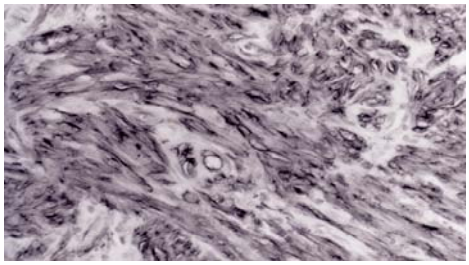

Fig. 2. CD34× 250- DFSP. Strong positivity throughout the lesional tissue precludes identification of vascular channels. Even the cells with the vacuole present in the centre of the field can not be confirmed as a blood vessel.

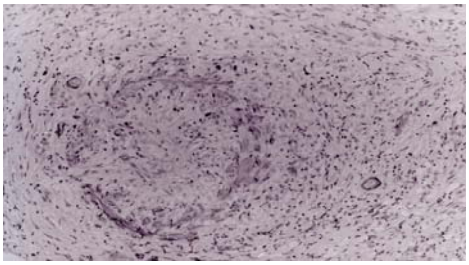

Fig. 3. Laminin × 100. This is a partly disrupted blood vessel in which it is difficult to identify the perimeter of the blood vessel that has been involved by tumor. Even two smaller blood vessels (upper left and lower right have incomplete staining of their wall).

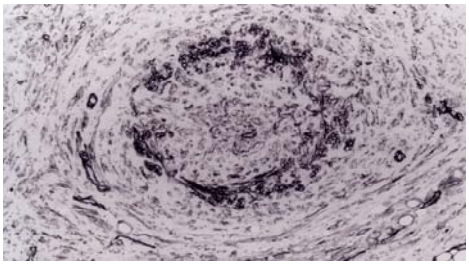

Fig. 4. Collagen IV × 100. Note the disruption of the blood vessel architecture. In spite of the extensive invasion by tumor, the remnants of the blood vessel wall are identified. Several other small vascular channels with complete or partial circlets of positivity are present. This is particularly helpful in identifying damaged blood vessel wall.

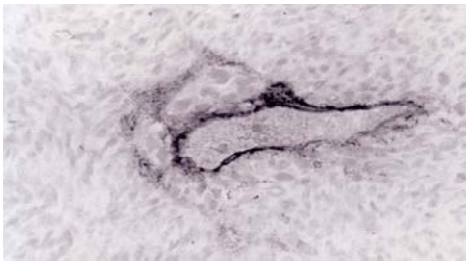

Fig. 5. Factor VIII × 250. Note that in addition to outlining parts of the wall of the blood vessel, There is extensive granular positivity out into the surrounding tumor. This halo of positivity makes it difficult to know if one is indeed seeing a blood vessel or leakage of this factor from the blood vessel into the surrounding tissues.

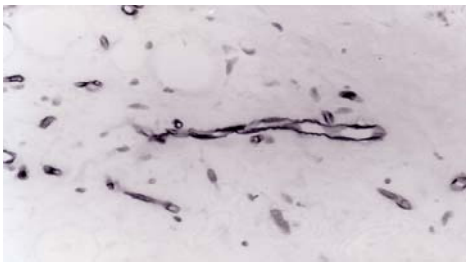

Fig. 6. Ulex Europaeus × 100. Note the crispness of the staining of the blood vessel wall by this marker. In the central larger vessel note the pale grey bulge of endothelial cells into the lumen indicating that the stain is identifying a basement membrane element.

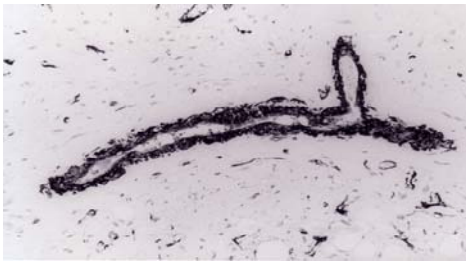

Fig. 7. Smooth Muscle Actin × 100. Note the strong demonstration of blood vessel wall contractile elements. Endothelial cells can barely be seen as a grey shadow along the inner surface of the blood vessel wall. Small vessels are also partly identifiable in the background.

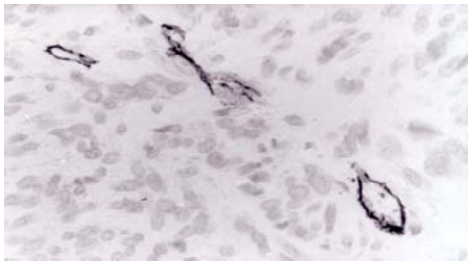

Fig. 8. CD31× 400. CD31 is a marker with high specificity for endothelial cells. In the image, there is a very clear positivity in the capillary-size vessels. Even the central one that does not have a recognizable lumen is identifiable as a tangential cut through a blood vessel.

DISCUSSION

Adequate blood supply is known to be essential to tissue growth and nutrition (1, 2). In tumor development and growth, a small mass less than a few millimeters in diameter (about 106 cells) is known to survive through passive diffusion of nutrients and removal of cell waste (1, 18, 21, 22). As tumors grow larger, the development of a new blood supply (angiogenesis) is essential to tumor growth (1). Antiangiogenic therapies have a number of potential advantages including decrease resistance and fewer side-effects (27). The process of tumor angiogenesis is known to be a multifactorial process which is presumed to be initiated and maintained by growth factors produced directly by tumor cells and indirectly by reactive or inflammatory cells activated by the tumor cells and by the extracellular matrix stores (1, 2, 7, 25). There is a controversy in the literature about the significant correlation between tumor growth or metastasis and microvessel density (5, 14-16, 28). It should be emphasized that tumor angiogenesis alone is not sufficient to cause metastasis (1, 18, 20, 22). Further it is known that there is no direct relationship between microvessel density and disease outcome in patients with soft tissue sarcoma (28). The neovascularization of tumors supports tumor growth but does not necessarily reflect rate of tumor progression or capacity for tumor dissemination. For example, adrenal cortical neoplasia and carcinoid tumors are highly vascularized neoplasms that rarely metastasize to distant sites and are not known for their malignant behavior (23-25).

Increased numbers of blood vessels do provide enhanced blood flow to a tumor (13). However, tumor blood vessels tend to have incomplete basement membranes and a non-continuous endothelial cell lining. They are said to have a “leaky” nature and more permeable walls than mature, fully developed blood vessels would have (3, 5). It has also been reported that the new endothelial cells can secrete urokinase, collagenase, plasminogen activator and other proteolytic enzymes that enhance the ability of vascular sprouts to extend into the surrounding stroma (5, 29). Obviously, neovasculature in tumors is characterized by capillary size blood vessels which are thin walled and more easily disrupted (4, 16). These features of the new blood vessels may further potentiate the ability of cells of malignant tumors to enter the circulation (17).

The characterization of vasculature in tumors, whether it be in biopsy or definitive resection, would certainly be helpful in assessing the extent and morphologic patterns of the neovascularization. For this, one requires adequate sampling representative of the neoplasm being studied. This is dependent on tissue sampling by the surgeon at biopsy and by the dissector of resected lesions. Fixation and processing must also be of the highest possible standard of quality. In the use of immunohistochemical markers, reliable, well-controlled staining techniques are essential for the accurate identification of the tumor’s blood supply. This requires assessment of presence and activity of endothelial cells present as well as the development of the supporting stroma including basement membrane and perivascular contractile cells. Cellular and extracellular constituents of the “bioconstruct”, that is a blood vessel, must all be assessed individually to fully appreciate the functional completeness of the angiogenesis occurring.

There is ongoing debate about the quantitation of vasculature by manual versus computer assisted semi-automated or fully automated systems (30). In a practical sense, for the laboratory physician asked to assess a tumor, the manual assessment of the tumor and its vascularization remains the principal mode of evaluation. The introduction of immunomarkers that specifically identify cell characteristics has provided a very significant step forward. This study has shown that while a single immunomarker may not be both sensitive and specific enough: a limited but well designed panel of immunomarkers will provide a reliable immunophenotypic assessment of neovascularization and tumor angiogenesis. From the purely scientific point of view, the opportunity for a more comprehensive assessment achievable with computer assisted analytical methodologies would certainly be desirable.

This is a preliminary study of immunohistochemical markers for the qualitative and quantitative assessment of vascularization of tumors. The findings confirm one's suspicion that the use of a single

marker is not reliable in identifying blood vessels in soft tissue neoplasms. No one marker can be relied upon to provide either a qualitative or a quantitative indication of the vascularization within the tumor. This seems to lead to the conclusion that it is essential to have a combination of markers in order to ensure that one has a full reliable, consistent, sensitive evaluation of the blood vessels present within the soft tissue sarcoma.

This study is based on a very small number of cases. It was an attempt to determine the methodologies that would be useful in assessment of vasculature in sarcoma. It has demonstrated that a combination of collagen IV, smooth muscle actin, CD34 and Factor VIII are the very minimum number of markers that will consistently and reliably identify blood vessels in sarcomata, particularly if one is to undertake any quantitative evaluation. This study has certainly confirmed the variability in the distribution and number of blood vessels between tumor types and from field to field in the same tumor. Endothelial cell proliferation (true angiogenesis) proved difficult to assess. From this study, one develope the impression that the combination of basement membrane markers (*e.g*. collagen IV), smooth muscle actin and the more specific endothelial cell markers (CD34 and CD31) would be the most valuable. Laminin was disappointing in its low sensitivity compared to the other markers. Factor VIII (being a coagulation factor) may be helpful in tumors such as Kaposi's sarcoma where one knows that there is blood vessel leakage and the coagulation cascade may be triggered by vascular injury. Aggressive tumors with blood vessel wall injury would, however, be difficult to assess with Factor VIII if one was specifically directing one's attention to the assessment of endothelial cell activity. One concludes from this that in the assessment of angiogenesis, supplementary indicators of endothelial cell activity will be essential in order to assess the proliferative activity present. This may well require the added use of proliferation markers (*e.g*. PCNA, Ki67).

In the every day practice of pathologists, it is extremely difficult to provide objective, quantitative assessment. Very often, one interprets qualitative factors and expresses a subjective opinion. Objective evaluations are extremely difficult. It is therefore imperative that in further studies, in order to assess the validity of histomorphometric assessment of angiogenesis, a battery of markers be used as no single marker is universally conclusive. That is, no one marker can be relied upon to provide complete or consistent information in the evaluation of angiogenesis in soft tissue sarcomata.

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