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The association between tumour progression and vascularity in myxofibrosarcoma and myxoid/round cell liposarcoma

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Abstract Angiogenesis is an important factor in the morphological progression and metastasis of many solid tumours. We studied two homogeneous series of myxofibrosarcoma (MFS) and myxoid/round liposarcoma (MRLS), characterised by distinct vascular patterns and correlated the intratumoral microvessel density (IMD) with morphologic progression in both types of sarcoma. In our study, 43 cases of MFS and 42 cases of MRLS were graded according to established diagnostic criteria. For evaluation of IMD, representative sections were stained immunohistochemically for CD31. After selection of "neovascular hot spots", IMD was calculated by measuring the endothelial surface within twenty 200× fields in relation to the total analysed area. In addition to the correlation of IMD with histological grades of malignancy, a correlation of IMD with the inflammatory infiltrate in MFS was done. To determine whether vascular endothelial growth factor (VEGF) and its receptors, KDR and flt-1, may play a role in the progression of both types of sarcomas, we used mRNA in situ hybridisation (ISH) to study VEGF, KDR and flt-1 expression in selected cases. In addition, the expression of thrombospondin-1, which has been reported to inhibit angiogenesis, and of collagen type I was studied using mRNA ISH. Cases of MFS varied histologically from hypocellular, mainly myxoid, neoplasms (low-grade malignant, 18 cases) to intermediate-grade malignant lesions with increased cellularity and mitotic activity (13 cases), and high-grade malignant cases with marked pleomorphism, high proliferative activity and areas of necrosis in many cases (12 cases). Cases of purely low-grade myxoid liposarcoma (16 cases) were characterised by low-cellularity, mucin pooling and plexiform vasculature. In combined MRLS, these hypocellular areas were admixed with hypercellular, round cell areas (5–80% of the analysed tumour area; 23 cases), and in round cell liposarcoma (three cases) rounded tumour cells predominated (>80% of the analysed tumour area). The average IMD in intermediate and high-grade malignant MFS (4.03 and 4.09, respectively) was significantly higher than in low-grade malignant MFS (2.73). Correlation of vascularity with the inflammatory infiltrate in MFS showed increased IMD only in cases with abundant neutrophils; most of these cases were high-grade malignant neoplasms. In contrast, no statistical correlation between morphological progression and IMD was seen in myxoid liposarcoma (6.08), MRLS (6.57) and round cell liposarcoma (4.07). VEGF mRNA was expressed by tumour cells in all histological grades of MFS and MRLS. VEGF receptor mRNA was weakly expressed by endothelia of newly formed blood vessels in both entities. Interestingly, tumour cells of all analysed cases of MFS strongly expressed collagen type I and thrombospondin-1, while these proteins were not detected in tumour cells of MRLS. In conclusion, morphologic tumour progression in MFS is associated with increased IMD, whereas, in MRLS, no such correlation is seen. Whereas VEGF and VEGF receptor mRNA were expressed in both entities, a characteristic expression profile of collagen type I and thrombospondin-1 in MFS emerged. Further studies are necessary to correlate vascularity and clinical course in MFS and MRLS.

Keywords Angiogenesis · VEGF · Thrombospondin · Sarcoma · Soft tissue tumour · Myxofibrosarcoma · Liposarcoma

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Introduction

Tumour-associated angiogenesis refers to the growth of newly formed vessels towards and within a given neoplasm. It has been shown clearly in recent years that neovascularisation plays a fundamental role in tumour progression, and experimental studies have emphasised the necessity of tumour-associated angiogenesis for tumour growth beyond a few millimetres and for metastasis [18, 24, 32, 36]. Angiogenesis is a complex multistep process controlled by angiogenic factors and inhibitors that regulate endothelial cell proliferation, capillary differentiation and extracellular matrix remodelling [28, 32].

Angiogenic factors, such as the members of the vascular endothelial growth factor (VEGF) family, regulate angiogenesis by increasing microvascular permeability [17] and can be secreted by tumour cells, stromal cells and inflammatory cells [4, 17]. In addition, VEGF has direct effects on vascular endothelial cells and increases intracellular calcium, stimulates inositol triphosphate formation, promotes the expression of von Willebrand factor and is a known vascular endothelial mitogen [16]. The identification of endogenous inhibitors of angiogenesis, such as endostatin [44], angiostatin [43] and thrombospondin [29], has supported the 'balance' hypothesis for the angiogenic switch [23, 32].

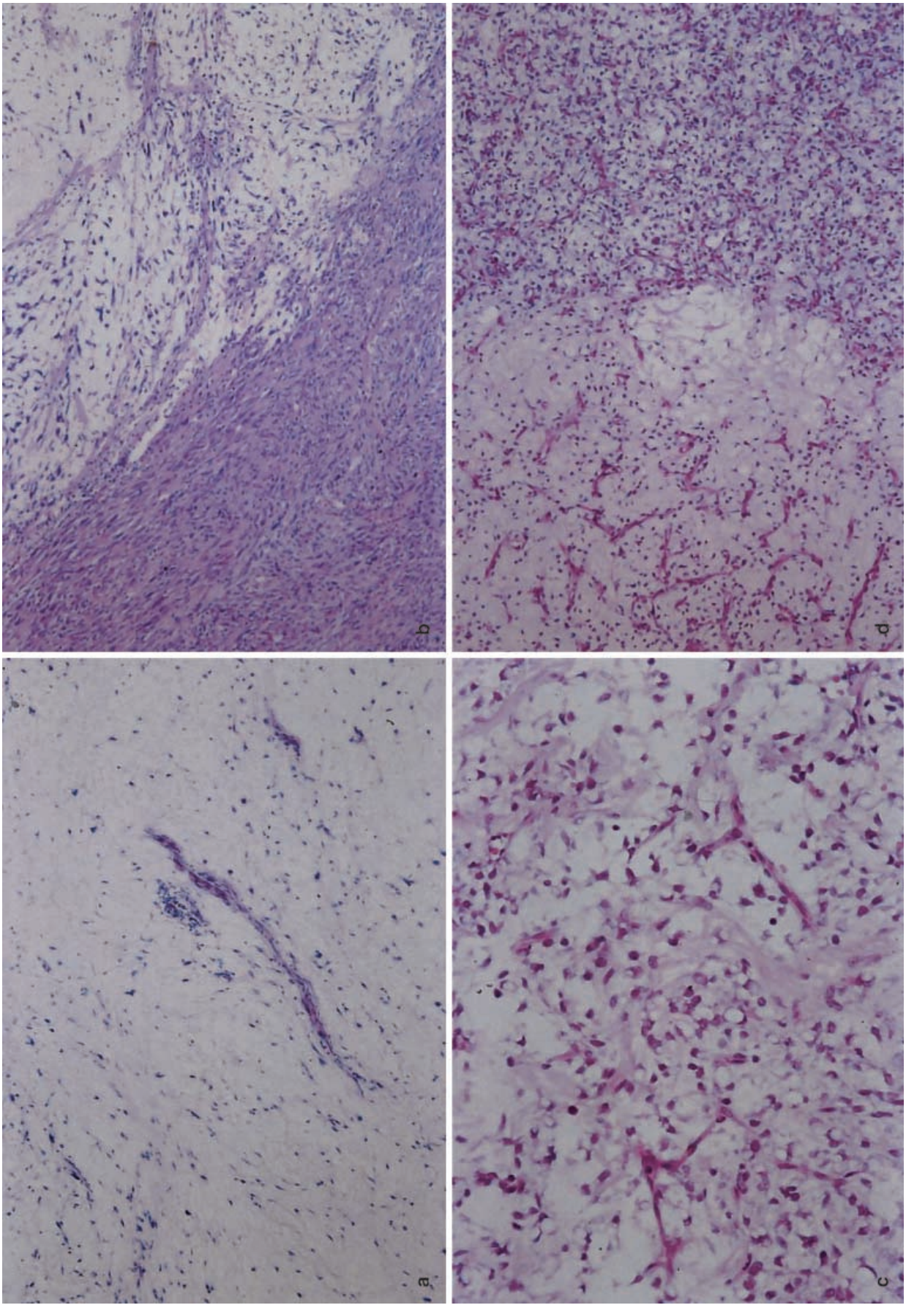
Although it was recognised almost three decades ago that quantification of tumour vasculature might be helpful in patient management [6, 22], most studies correlating vascularity and tumour progression have been done in the last few years with the advent of improved immunohistochemical endothelial markers and computerised image analysis. The quantification of intratumoral microvessel density (IMD) in histological sections is an accurate measure of tumour angiogenesis [57, 58], and a continually increasing number of studies have been reported [26, 55, 56]. Numerous studies have demonstrated a clear correlation between increasing IMD and morphologic tumour progression and a worsening clinical prognosis in various malignant epithelial neoplasms, such as invasive ductal carcinoma of the breast [27, 31, 47, 52, 57], invasive prostate carcinoma [5, 59], carcinomas of the gastrointestinal tract [13, 19, 49, 50] and other entities. However, there have also been studies that were unable to confirm a close relationship between IMD and clinical prognosis [2, 14, 25, 30, 41], and an inverse correlation was found in renal cell carcinoma [33].

Whereas numerous studies have been performed on epithelial neoplasms, little is known about the association between tumour angiogenesis and morphological progression in malignant mesenchymal lesions, and mostly heterogeneous series were studied [20, 42, 46]. The study presented herein was undertaken to investigate the possible relationship between morphological tumour progression and angiogenesis in two homogeneous series of soft tissue sarcomas. We selected cases of myxofibrosarcoma (MFS) and myxoid/round cell liposarcoma (MRLS), two mesenchymal entities that are characterised by a morphological continuum associated with histological grade in most cases and a prominent and distinctive vascular pattern.

Materials and methods

In order to achieve optimal conditions, only recent cases with known procedures of fixation were included, in which limited or only relatively short clinical follow-up was available. In this study, 43 cases (41 patients) of MFS, and 42 cases (35 patients) of MRLS were analysed. All cases were retrieved from the consultation files of the authors and from the routine surgical files of the Department of Histopathology, St. Thomas's Hospital, London, UK, and from the Departments of Pathology, University of Jena and University Hospital Bergmannsheil, Bochum, Germany. In all cases, tissue was fixed in 10% buffered formalin, routinely processed and embedded in paraffin wax. In addition, snap-frozen material of selected cases of both entities was available for in situ hybridisation (ISH) studies (see below). Sections that were 4-µm thick were stained with haematoxylin and eosin (H&E). Immunohistochemical staining for CD31 was performed using a standardised alkaline phosphatase anti-alkaline phosphatase (APAAP) method. The primary antibody against CD31 (clone JC/70A; diluted 1:30; Dako, Glostrup, Denmark) was incubated for 12 h at 4°C and then routinely processed. In each case, positive and negative controls were employed. The immunoreaction for CD31 was quantified using image analysis (Quantimet 500, software Qwin; Leica, Germany). For this procedure, a "cut-off level" for positive immunohistochemical staining was defined. The counting fields were selected by finding "neovascular hot spots". Areas of necrosis, haemorrhage and tumour degeneration were carefully excluded. In these areas of highest vascularity, a computerised analysis of the CD31 positive, endothelial area was done under the microscope with a magnification of ×200. The structures selected by the computer-aided image-analysis system as microvessels were verified on the computer monitor before they were analysed. The average endothelial area counted in 20 scanned fields per slide (1–2 slides in each case) in relation to the total analysed area (0.062375 mm^2) was designated as the IMD. In addition, snap-frozen material of selected cases (seven cases of MFS and seven cases of MRLS), which had been stored at –70°C, was used for molecular analyses. Frozen sections (6-µm thick) were subjected to mRNA ISH, as described previously [7, 9]. The antisense single-stranded RNA probes for localising VEGF, KDR, flt-1, thrombospondin-1 and collagen type I mRNA and their sense control have also been described previously [7, 8, 11]. Expression of mRNA was rated as strong if silver grains were obvious in numerous tumour cells (>20 grains per cell), moderate (10–20 grains per cell), or low (<10 grains per cell). Statistical analysis was performed using InStat for Macintosh 2.0. Bivariate correlation between different groups was studied using Cox regression.

Fig. 1 **a** Cases of low-grade myxofibrosarcoma are characterised by few, non-cohesive but atypical fibroblastic tumour cells set in a prominent myxoid matrix with curvilinear blood vessels (haematoxylin and eosin, H&E). **b** Transition of lower-grade myofibrosarcoma (*right*) to high-grade myxofibrosarcoma with increased cellularity, cellular pleomorphism and proliferative activity (H&E). **c** In low-grade, purely myxoid liposarcoma, small mesenchymal tumour cells are associated with often univacuolated lipoblasts and plexiform blood vessels (H&E). **d** Combined myxoid/round cell liposarcoma is characterised morphologically by progression of low-grade myxoid liposarcoma areas (*left*) to areas with increased cellularity (H&E) ▶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Results

Clinicopathological findings

Clinical data are summarised as follows: the 41 patients (11 female, 26 male and gender unknown in four cases) with MFS were aged between 17 years and 86 years (mean 60.76 years; median 61 years). The most common locations were the lower extremities (×20), followed by the upper extremities (×9), the trunk (×6) and the head and neck region (×2). In the four unknown gender cases, the exact anatomic locations were also unknown. The tumours excised (41 primary and two recurrent lesions) were graded according to established criteria [37, 38]. Cases of low-grade MFS (×18) were characterised by a prominent nodular growth pattern and were hypocellular, composed of only few non-cohesive plump spindled or stellate tumour cells with atypical enlarged and hyperchromatic nuclei. Tumour cells were set in a prominent myxoid matrix containing elongated and somewhat curvilinear capillaries (Fig. 1a). By contrast, 12 cases showed high-grade features, being composed of more solid or fascicular-arranged, pleomorphic tumour cells with numerous, often atypical mitotic figures and often areas of haemorrhage and necrosis. However, these neoplasms contained, by definition [37], at least 10% of lower-grade myxoid areas morphologically similar to purely low-grade lesions (Fig. 1b). Lesions in the third group (×13), designated intermediate-grade MFS, were more cellular and pleomorphic relative to purely low-grade cases but lacked extensive solid areas or areas of necrosis and pronounced cytologic pleomorphism.

The age of the patients with MRLS, 19 females and 16 males, ranged from 16 years to 70 years (mean 42.2 years; median 51 years). Most cases of MRLS (35 primary and seven recurrent or metastatic lesions) arose on the thigh (×16), followed by the lower limb girdle (×8), the lower leg and foot (×6), the trunk (×3) and the arm (×1). In one case, location was not stated. In three cases (cases 3, 9, and 30), material from local recurrences and, in case 3, material from metastatic lesions was available in addition to the primary tumours. Sixteen neoplasms showed features of low-grade purely myxoid liposarcoma composed of small, primitive mesenchymal tumour cells associated with mainly monovacuolated lipoblasts and plexiform capillaries (Fig. 1c). Cases containing between 5% and 80% of round cell areas (23 tumours), characterised by increased tumour cells with round and often overlapping nuclei, were designated as combined myxoid/round cell liposarcoma [21] (Fig. 1d). In only three cases, more than 80% of the analysed tumour area was composed of high-grade, round tumour cells with scattered lipoblasts. These cases represented round cell liposarcomas.

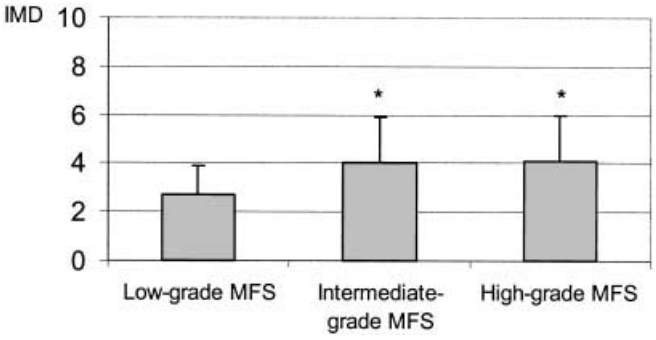

Fig. 2 Intratumoral microvessel density (*IMD*) in myxofibrosarcomas (*MFS*) of varying grades of malignancy [* increased significantly in comparison to low-grade MFS (P<0.035)]

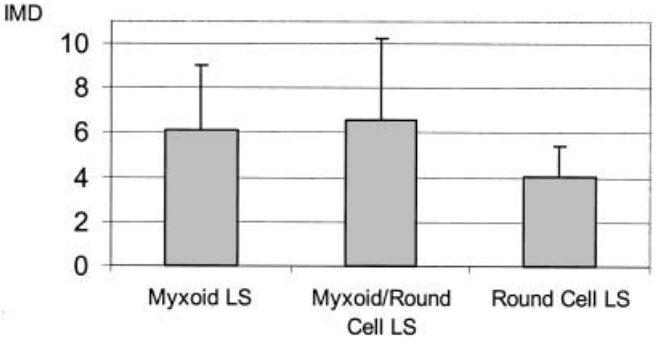

Fig. 3 Intratumoral microvessel density (*IMD*) in myxoid/round cell liposarcomas (*LS*) of varying grades of malignancy

Analysis of IMD

The results of computerised analysis of IMD in MFS and MRLS of varying histological grades of malignancy are summarised in Fig. 2 and Fig. 3. The average IMD in MFS ranged from 2.73 (SD_x=1.13) in low-grade MFS to 4.03 (SD_x=1.85) in intermediate-grade MFS and 4.09 (SD_x=1.85) in high-grade MFS (Fig. 2). Statistical analysis revealed a significant increase in IMD in intermediate- and high-grade MFS relative to cases of low-grade MFS (P=0.0312 and 0.0183, respectively). The IMD of recurrent neoplasms (case 12 and case 17) was comparable to the primary tumours; these recurrent lesions showed features of low-grade MFS. In order to investigate the influence of an inflammatory infiltrate, a common feature in MFS [37], IMD of most cases of MFS was analysed in different groups independent of the histological grade (Table 1). No statistical correlation of the IMD in cases with or without lymphocytes and mast cells was noted. Only cases containing an increased number of neutrophils showed a significantly increased IMD (4.43; SD_x=2.14) relative to cases without neutrophils (2.89; SD_x=1.13). However, only intermediate- and high-grade malignant lesions were seen in this group.

In contrast, no statistical correlation between morphologic progression and IMD was seen in MRLS (Fig. 3). The average IMD in purely myxoid liposarcoma was

Table 1 Correlation of IMD with the inflammatory infiltrate in 39 cases of myxofibrosarcoma.The *t*-test and Mann-Whitney test were used for statistical correlation. *IMD* intratumoural microvessel density; SD_x standard deviation

Group	*P* value	Case number	IMD	SD_x
I No inflammatory infiltrate		13	2.79	0.92
II Few inflammatory cells, any type[a]		21	3.66	1.77
III Prominent inflammatory infiltrate, any type[a]		5	5.50	2.60
Group I vs group II	*P*=0.1564			
Group II vs group III	*P*=0.0683			
Group I vs group III	*P*=0.0593			
IV No lymphocytes		20	2.95	0.95
V Numerous lymphocytes		19	4.30	2.25
Group IV vs group V	*P*=0.079			
VI No mast cells		24	3.63	1.76
VII Few mast cells		15	3.57	1.98
Group VI vs group VII	*P*=0.30			
VIII No neutrophils		21	2.89	1.13
IX Foci of neutrophils		18	4.43	2.14
Group VIII vs group IX	*P*=0.022			

[a] Includes histiocytes, lymphocytes, neutrophils, mast cells and plasma cells

6.08 (SD_x=2.96), and only a slight and nonsignificant increase in IMD was noted in myxoid/round cell liposarcoma (6.57; SD_x=3.70). The IMD was decreased in cases of round cell liposarcoma (4.07; SD_x=1.36). The two metastases in case 3, showing features of myxoid/round cell liposarcoma, were characterised by an increased IMD (5.42) relative to primary and recurrent lesions with features of purely myxoid liposarcoma (3.38 and 2.61, respectively). Also, the local recurrences in case 9 (4.8) and case 30 (11.39) were characterised by an increased IMD relative to their primary lesions (3.4 and 8.28, respectively). However, the number of cases with recurrent and metastatic disease was too small for meaningful statistical analysis.

Evaluation of expression of VEGF, KDR, flt-1, collagen type I and thrombospondin-1

A total of seven cases of MFS (three low-grade, two intermediate-grade, and two high-grade) and seven MRLS (three myxoid, two myxoid/ round cell, two round cell) were studied using mRNA ISH for the expression of VEGF and the VEGF receptors KDR and flt-1, thrombospondin 1 (TSP-1) and collagen type I. VEGF was strongly expressed by tumour cells in both MFS and MRLS, and no clear difference in expression was seen between the two types of tumour. Focal strong expression of VEGF by individual tumour cells was noted in tumours of low, intermediate and high grade on both entities. Strong expression of the VEGF receptors KDR and flt-1 mRNAs by endothelial cells was also seen focally in tumour blood vessels in both MFS and MRLS, independent of the morphological grade of malignancy (Fig. 4). Significant TSP-1 expression was seen in tumour cells and in the blood vessels of all histological grades of MFS. In contrast, TSP-1 expression in MRLS was only focal and limited to endothelial cells of blood vessels, whereas TSP-1 expression was not seen in tumour cells of all histological grades of MRLS. Diffuse strong expression of collagen type I mRNA was found in tumour cells of all cases of MFS studied, including high-grade, intermediate- and low-grade malignant neoplasms. In marked contrast, collagen type I mRNA was not expressed by tumour cells in any myxoid, myxoid/round cell or round cell liposarcomas. Focal strong expression of collagen type I mRNA was noted in non-neoplastic stromal cells near blood vessels in cases of liposarcoma (Fig. 5).

Discussion

Tumour angiogenesis is an important prognostic factor in a number of malignant epithelial neoplasms according to many authors [5, 13, 19, 27, 31, 47, 49, 50, 52, 57, 59]. However, some studies contradict this conclusion [2, 14, 25, 30, 41] or show an inverse correlation [33, 45]. These different results are probably attributable to methodological differences in the quantification of tumour-associated angiogenesis. Intratumoral microvessels are identified using immunohistochemical methods to stain endothelial cells. It has been shown clearly that anti-CD31 antibodies represent the most sensitive and specific markers for this line of differentiation [15, 39, 54]; however, this antibody was not used in all studies. The next problem concerns the selection of representative tissue blocks and identification of areas of highest vascularisation, so-called vascular hot spots and their quantitative analysis.

We used CD31 immunostainings in our study, followed the accepted standards of angiogenesis quantification [57, 58] and performed computerised analysis of the CD31 positive, endothelial area. It has been demonstrated that computerised image analysis allows a more objective and reproducible quantification of IMD than manual counts of microvessels [3] and that measurement of the percentage endothelial area is superior to manual counting of vessels [3, 47, 48].

In contrast to epithelial neoplasms, little is known about the association between tumour angiogenesis and progression and prognosis in mesenchymal lesions. Ewaskow et al. [20] found (in a relatively small number of cases) significantly increased vessel density in malig-

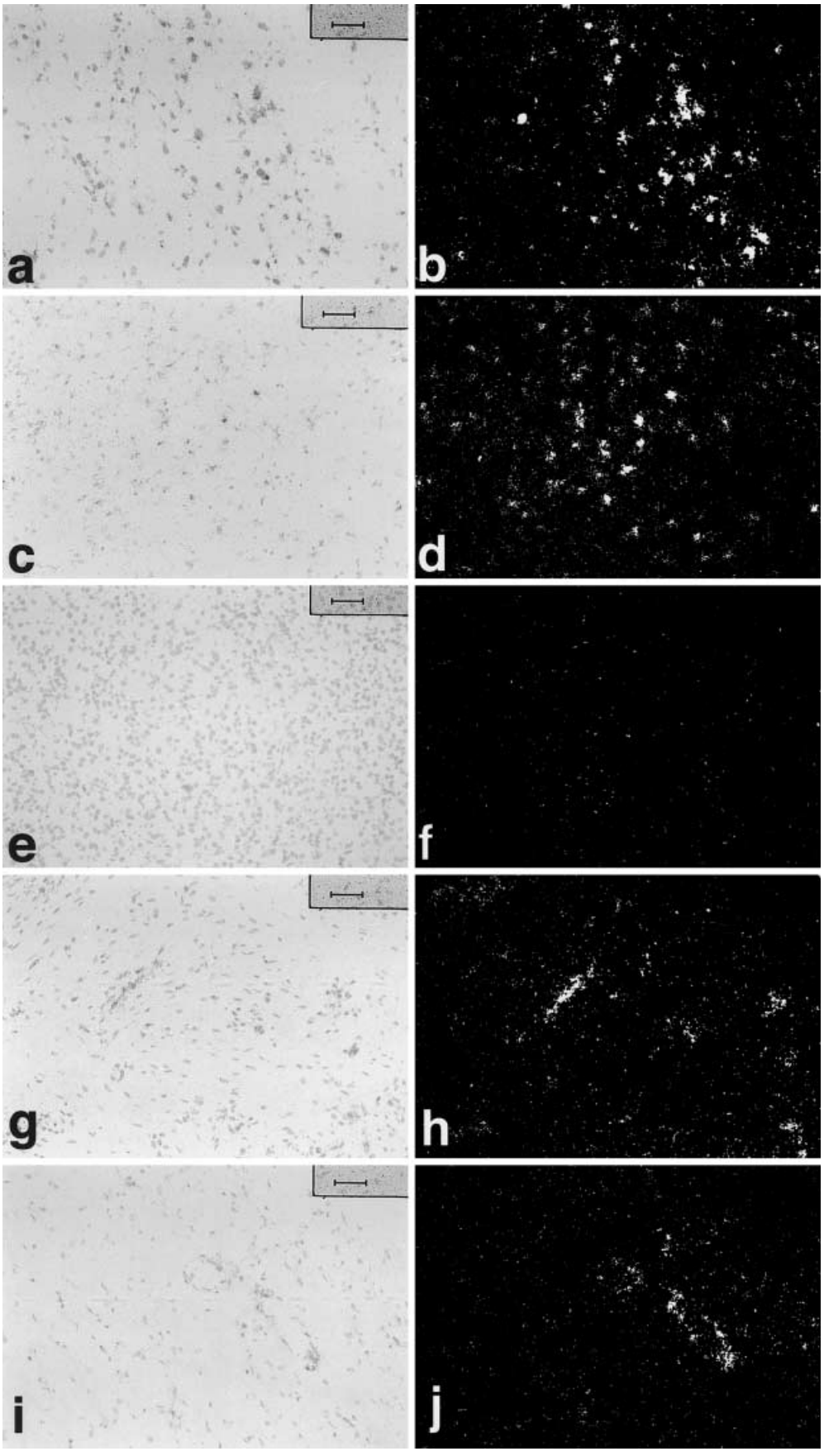

Fig. 4a–j In situ hybridisation studies, bright-field and corresponding dark-field photomicrographs. Strong expression of vascular endothelial growth factor (VEGF) mRNA by tumour cells was seen in both myxofibrosarcomas (**a, b**) and myxoid/round cell liposarcomas (**c, d**). No significant labelling was seen with sense control probes (**e, f**). Strong expression of VEGF receptors was also seen in endothelial cells of small vessels in both myxofibrosarcomas (**g, h**) and myxoid/round cell liposarcomas (**i, j**; 200×)

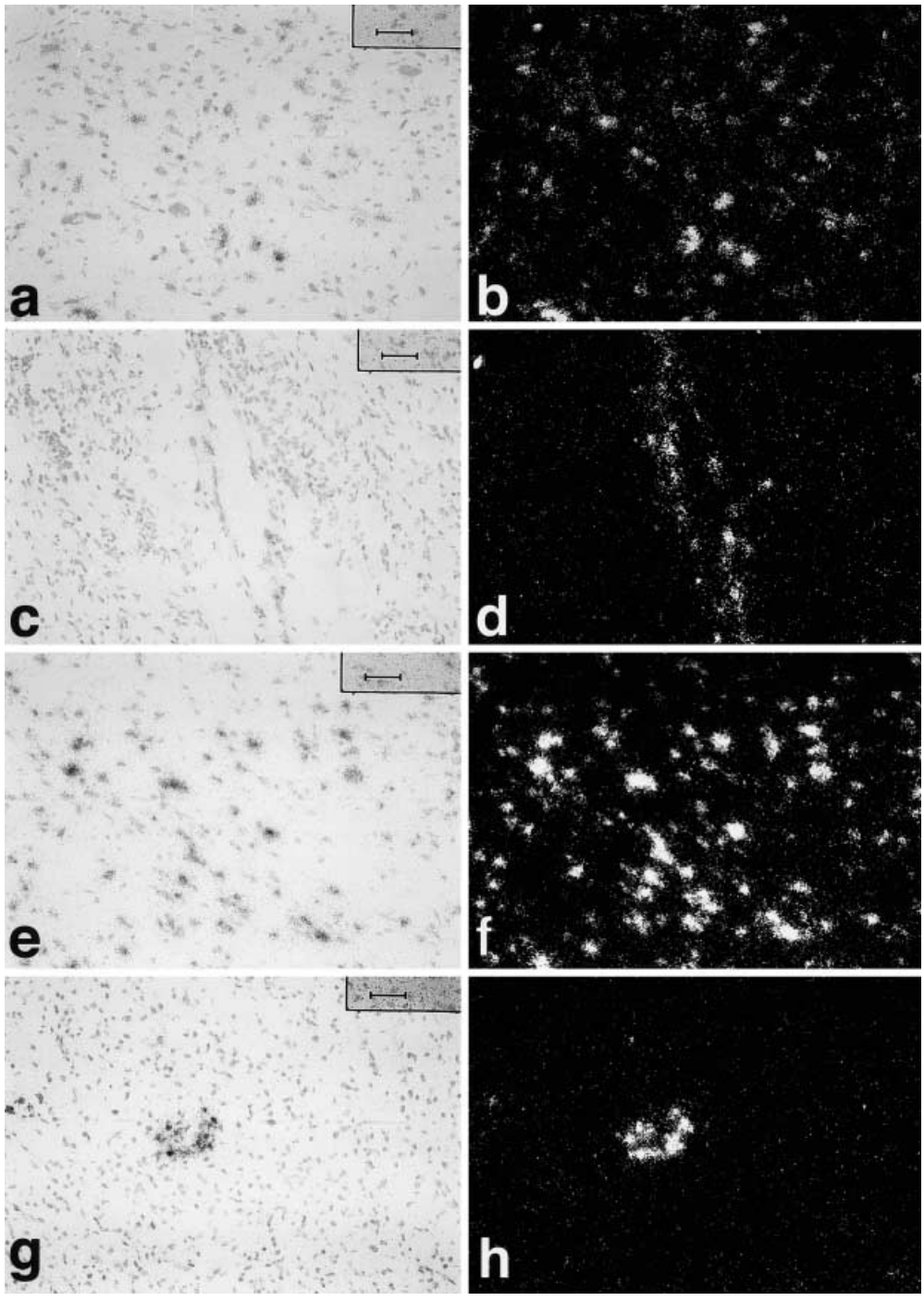

Fig. 5a–h In situ hybridisation studies, bright-field and corresponding dark-field photomicrographs. Strong expression of thrombospondin mRNA was seen in tumour cells and endothelial cells in cases of myxofibrosarcoma (**a, b**). In contrast, thrombospondin mRNA expression was not seen in tumour cells in myxoid/round cell liposarcomas and was limited to endothelial cells of blood vessels (**c, d**). Strong expression of collagen type I mRNA was noted in tumour cells in myxofibrosarcomas (**e, f**). In myxoid/round cell liposarcomas, collagen type I was not seen in tumour cells and was limited to perivascular stromal cells (**g, h**; 200×)

nant fibrous histiocytomas and malignant peripheral nerve sheath tumours relative to their benign counterparts, whereas low- and high-grade liposarcomas showed no remarkable differences. Similarly, no correlation between vascularity and tumour size, histological subtype, rate of metastasis, depth of the tumour and age and gender was found in a series of malignant fibrous histiocytomas [42]. No statistical difference in microvessel density was reported between benign and malignant smooth muscle tumours of the uterus [12], between different groups of malignant peripheral nerve sheath tumours [40] and in synovial sarcoma [34]. In contrast, a direct correlation between histological grade and the amount of pericartilage vessels was seen in cartilaginous tumours [1]. Whereas newly formed microvessels in carcinomas are predominately found in the area of tumour invasion, capillaries are rather homogeneously distributed in the tumour stroma in sarcomas [53].

Our results indicate that tumour-associated vascularity may be of varying importance in the morphological

progression of different sarcoma types. Whereas intermediate- and high-grade MFS were characterised by significantly increased IMD relative to low-grade MFS, no such correlation was seen in MRLS of varying grades of malignancy. The presence of tumour necrosis in many cases of high-grade MFS is probably an explanation for the lack of difference in IMD between intermediate- and high-grade MFS. The important role of VEGF for tumour growth and prognosis has been reported in different malignancies [8, 9, 10, 35, 51]. In addition, it is known that VEGF is also expressed by normal adult tissues and inflammatory cells, namely macrophages [4]. Because a prominent inflammatory infiltrate is often seen in cases of MFS, we also analysed the IMD for any correlation with an inflammatory infiltrate. An increased IMD was observed only in cases with numerous neutrophils. However, most of these neoplasms were high-grade MFS, and areas of necrosis and neutrophils were seen in these areas. Given the fact that angiogenic factors, such as VEGF, are expressed by macrophages but not neutrophils, it seems that the latter are not of importance in the relationship of tumour-associated angiogenesis and morphologic progression. Purely myxoid liposarcoma is characterised morphologically by prominent plexiform vascularisation. Higher-grade malignant MRLS, in which vascularity often appears less conspicuous upon H&E examination, showed no significant increase in IMD. In contrast, a decreased IMD was noted in the small number of round cell liposarcomas. The number of recurrent and metastasising cases of MRLS that showed slightly increased IMD in comparison to their primary lesions was too small for proper statistical analysis.

Although cases of MFS showed a slightly increased VEGF expression relative to MRLS, no significant differences in the expression of this angiogenic factor and its receptors were noted between the two entities. This strong expression of VEGF by tumour cells and VEGF receptors by endothelial cells suggests that VEGF is playing an important role in the angiogenesis associated with these sarcomas. It is of interest, however, that the patterns of mRNA expression of collagen type I and TSP-1 were remarkably different in analysed cases of MFS and MRLS. The strong expression of collagen type I in MFS is not surprising, because it is commonly accepted that MFS represents a sarcoma of fibroblastic differentiation [37]. Given the fact that TSP-1 has been reported to have antiangiogenic properties [29], it seems somewhat confusing that an increased IMD in higher-grade MFS is associated with strong expression of TSP-1. However, the overall vessel counts in analysed cases of MFS was lower than in MRLS (see Results), emphasising the inhibitory role of TSP-1. Similarly, it has been shown in a recent study of in situ and invasive carcinoma of the breast [11] that expression of TSP-1 may be an attempt to inhibit tumour-associated angiogenesis. However, in the process of tumour progression in cases of MFS, the balance “falls on the side” of angiogenesis.

In summary, our study of homogeneous series of two distinct types of soft tissue sarcoma characterised by gradual morphologic progression and distinct vascular patterns showed that tumour angiogenesis appears to be of differing significance or relevance in the progression of these sarcomas. Whereas an increased IMD was observed in higher-grade MFS, no such correlation was seen in cases of MRLS. Interestingly, MFS and MRLS were characterised by different patterns of the expression of stimulators and inhibitors of angiogenesis. Whereas VEGF and its receptors were expressed in both entities, tumour cells in cases of MFS strongly expressed thrombospondin-1, which was lacking in tumour cells of MRLS. Further studies are necessary to investigate whether tumour angiogenesis is of importance for clinical prognosis in MFS.

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