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Neovascularisation, expression of fibroblast growth factor-2, and mast cells with tryptase activity increase simultaneously with pathological progression in human malignant melanoma

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Abstract

Tissues from 92 proliferative lesions of the melanocytic lineage defining distinct steps in tumour progression were investigated immunohistochemically for changes in angiogenesis, expression of fibroblast growth factor-2 (FGF-2) and density of total mast cells (MCs) and MCs expressing tryptase, an angiogenic-inducing molecule. Although the microvessel number was low in common nevi, it increased significantly in nevi with architectural disorder with varying degrees of melanocytic atypia (termed 'nevi with ADMA'), and these changes persisted during tumour development. Progression of primary melanomas was accompanied by a high microvessel number, and the progression to metastases by another significant increase in the microvessel counts. Expression of FGF-2, evaluated as percentages of positive lesions and positive cells per lesion was upregulated in the course of progression. Changes in expression were associated with nevi with ADMA, tumour changeover, penetration of the tumour into the dermis and metastases. A high correlation was demonstrated in all groups of tissues between the microvessel counts, percentages of FGF-2-positive tumour cells, and both total metachromatic and tryptase-reactive MCs. These results suggest that angiogenesis in human melanoma increases with tumour progression and that FGF-2 secreted by tumour cells and tryptase secreted by host MCs co-operate in its induction.

Keywords: Angiogenesis; Fibroblast growth factor-2; Mast cells; Melanoma; Tryptase

1. Introduction

In solid tumour growth, a specific critical turning point is the transition from the avascular to the vascular phase [1]. Having developed an intrinsic vascular network, the neoplastic mass is able to grow indefinitely both *in situ* and at distant sites (metastasis) in so far as an intrinsic vascular network enables its cells to enter the vascular bed and colonise other organs [2].

Tumour cells are surrounded by an infiltrate of inflammatory cells, such as lymphocytes, neutrophils, macrophages and mast cells (MCs). These cells communicate by a complex network of intercellular signalling pathways mediated by surface adhesion molecules, cytokines and their receptors [3]. The density of MCs is highly correlated with the extent of both normal and pathological angiogenesis, such as that in chronic inflammatory diseases and tumours (for review see Refs. [4,5]. MC-derived heparin and histamine are angiogenic [6,32]. MCs also contain many other angiogenic factors and a variety of cytokines [38], such as transforming growth factor-beta, tumour necrosis factor-α [18], interleukin-8 (IL-8) [8], fibroblast growth factor-2 (FGF-2) [9] and vascular endothelial growth factor (VEGF) [10] implicated in normal, as well as tumour-associated neoangiogenesis. Blair and colleagues [11] have shown that tryptase released by MCs at an angiogenesis site may play an important role in neovascularisation. Direct

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addition of tryptase to microvascular endothelial cells cultured on Matrigel caused a pronounced increase of capillary growth, which was suppressed by specific tryptase inhibitors. Moreover, tryptase directly induced endothelial cell proliferation in a dose-dependent fashion. We have previously demonstrated that in multiple myeloma and B-cell non-Hodgkin's lymphoma there is a striking association between MC and microvessel counts, and both increase with malignancy [12,13]. Moreover, we have further demonstrated that angiogenesis in benign lymphadenopathies and in B-cell non-Hodgkin's lymphoma, measured as microvessel counts, is highly correlated with the total and MC tryptase-positive counts [14].

Human melanoma progresses through different steps: nevocellular nevi, dysplastic nevi, *in situ* melanoma, radial growth phase melanoma (Breslow index $\leqslant 0.75$ mm), vertical growth phase melanoma (Breslow index > 0.75 mm) and metastatic melanoma. In agreement with progression, it acquires a rich vascular network [15,16], whereas an increasing proportion of tumour cells express the laminin receptor, which enables their adhesion to the vascular wall [17]. In the tumour, several angiogenic cytokines are expressed, including (FGF-2) [33,41], IL-3 and IL-8 [33,40] (VEGF) [39].

In this study, we correlate the extent of angiogenesis with the number of tumour cells reactive to FGF-2 and with MCs reactive with tryptase in human malignant melanoma.

2. Materials and methods

2.1. Tissues

Tissues were selected from the six clinical steps of melanoma progression described by Clark and colleagues in Ref. [19] (Table 1). Step 1 tissue included 14 common acquired nevi. Steps 2 and 3, which usually coexist, included 11 nevi with architectural disorder with varying degrees of melanocytic atypia (termed 'nevi with ADMA') according to Clark and colleagues [19], as revised by the National Institute of Health Consensus Conference on Early Melanoma [20]. Step 4 included 16 early primary melanomas, i.e. tumours in radial growth phase with limited (0.75 mm or less) Breslow vertical thickness [21]. Step 5 included 30 advanced primary melanomas, i.e. those in vertical growth phase with greater thickness. These tumours were subdivided into three groups according to their thickness ($\leqslant 0.75$, 0.76–1.5, greater than 1.5 mm); these groups define steps in progression in terms of its linear relationship with the competence for and the incidence of metastasis [19,21]. Step 6 included 21 synchronous metastases from regional subcutaneous/skin, regional lymph nodes, and distant visceral (jejunum) sites.

Table 1
Clinical and histological information on patients

Overall		92
	Common nevi	14
	Average age (year)	35
	Men/women	6/8
	Nevi with architectural disorder with varying degrees of melanocytic atypia	11
	Average age (year)	38
	Men/woman	5/6
	Primary skin melanomas[a]	46
	Average age (year)	54
	Men/women	22/24
	Histological type[b]	
	SSM/LMM/NM/ALM	24/7/14/2
	Clark level	
	I/II/III/IV/V	2/19/12/10/03
	Tumour thickness (mm)	
	$\leqslant 0.75/0.76$–$1.5/> 1.5$	16/12/18
	Erosion or ulceration	
	Absent/present	36/10
	Metastatic melanomas	21
	Average age (year)	44
	Men/women	8/13
	Location	
	Subcutaneous/skin	13
	Lymph node	7
	Visceral site	1

SSM, superficial spreading melanoma; LMM, lentigo malignant melanoma; NM, nodular melanoma; ALM, acral lentiginous melanoma.

[a] All primary melanomas were stage I, according to the ITNM classification (International Union Against Cancer (UICC), 1987) [43]. They included 16 early and 30 advanced primary melanomas.

[b] Established according to Clark and colleagues [19].

Tissues samples were fixed in formalin and embedded in paraffin according to standard procedures.

2.2. Immunohistochemistry

Three murine monoclonal antibodies (MAbs) against the endothelial cell marker CD31 (MAb 1A10), FGF-2 (Mab MC-GF1, both from Serotec, Oxford, UK) and tryptase (MAb AA1, Dako, Glostrup, Denmark) were used. Briefly, 4 μm thick sections were collected on 3-amino-propyl-triethoxysilane coated slides, deparaffinised by the xylene–ethanol sequence, rehydrated in a graded ethanol scale and in Tris-buffered saline (TBS, pH 7.6), and incubated overnight at 4 °C with MAbs 1A10 (1:25 in TBS) and AA1 (1:1500 in TBS), after prior antigen retrieval by enzymatic digestion with Ficin (Sigma, St Louis, MO, USA) for 30 min at room temperature for tryptase, and in a pressure cooker for 90 s in ethylene diamine tetra acetic acid (EDTA) buffer, pH 8 for CD31. The immunoreaction was performed with alka-

kine phosphatase anti-alkaline phosphatase (APAAP, Dako) and Fast Red as the chromogen for tryptase, and with the streptavidin–peroxidase complex (LSAB2, Dako) and 3,3′ diaminobenzidine tetrahydrochloride (Dako) 5% as the chromogen for CD31, followed by haematoxylin counterstaining. An unrelated monoclonal IgG1 produced by the P3X63/Ag8 mouse secretory myeloma replacing the MAbs served as negative controls [42].

2.3. Microvessel counts

These were simultaneously assessed without knowledge of the final pathological diagnosis by two investigators using a double-headed light microscope (Axioplan II, Zeiss, Oberkochen, Germany). Four to six 200× fields covering almost the whole of each of four sections per sample were examined with a 144-intersection point square reticulum (0.78 mm^2) inserted in the eyepiece. Care was taken to select microvessels, i.e. capillaries and small venules, from all the CD31-stained vessels. They were identified as transversally sectioned tubes with a single layer of endothelial cells, either without or with a thin basement membrane. Each assessment was agreed upon in turn. Microvessels were counted with a planimetric point-count method with slight modifications [12], according to which only microvessels transversally cut occupying the reticulum points were counted. As the microvessel diameter was smaller than the distance between adjacent points, only one transversally sectioned microvessel could occupy a given point. Microvessels transversally sectioned outside the points and those longitudinally or tangentially sectioned were omitted. Therefore, it was sufficiently certain that a given microvessel was counted only once, even in the presence of several of its section planes. As almost the entire section was analysed per sample, and as transversally sectioned microvessels hit the intersection points randomly, the method allowed objective counts. Means ± 1 standard deviation (S.D.) and medians were determined for each section, sample and group of samples.

2.4. MC counts

MCs were highlighted in every second section adjacent to that stained for microvessels with 0.5% aqueous solution of toluidine blue (Merck, Darmstadt, Germany), counted in 6–8 250× fields, covering almost the whole section, inside a square reticulum (0.25 mm^2), and calculated as means ± 1 S.D. and median for each group of samples. The MCs were stained with tryptase in every third section and counted as above.

2.5. Tumour cell counts

The percentage of tumour cells reactive with FGF-2 was assessed in every fourth section by counting 6–8 250× fields judged to be representative of the nevus or melanoma section viewed from several 100× fields. A cut-off value of 5% stained cells per section was used to distinguish positive and negative samples. It was based on the finding that up to 5% tumour cells were stained in control sections.

2.6. Statistics

The significance of changes in the counts of microvessels, FGF-2-positive tumour cells and MCs (both total and tryptase-reactive MCs) was assessed with parametric (Fisher's test) and non-parametric (Kruskal–Wallis test) analysis of variance, followed by the Duncan (t), Bonferroni (t) and Wilcoxon tests to compare groups two by two. Correlations between counts were assessed with Pearson's (r) coefficient and simple regression analysis. The Chi-squared test was split into the linear and the residual component, according to Cochran. Data were computed with the Statistical Analysis Software (SAS, SAS Institute, Cary, NC, USA).

Table 2
Tissue density of microvessels and mast cells as a whole and tryptase-positive cell population

Number of	Common nevi ($n=14$)	Nevi with ADMA ($n=11$)	Primary melanomas			Metastatic melanomas ($n=21$)
			⩽0.75 mm ($n=16$)	0.76–1.5 mm ($n=12$)	>1.5 mm ($n=18$)	
Microvessels (per 0.78 mm^2)	3.8±1.5 (4, 2–7)	9±2.8* (8, 6–15)	11.4±3.1 (10.5, 8–17)	10±3.6 (10, 6–16)	10.3±3.4 (10, 6–16)	18.2±8.3** (18, 7–33)
All mast cells (per 0.25 mm^2)	2.9±1.5 (3, 1–6)	8.5±4.7* (8, 3–18)	9.8±4.3 (9.5, 5–20)	12.6±4.9*** (11.5, 5–22)	13.1±5.2 (12, 7–25)	19.7±10.7** (20, 4–35)
Tryptase-positive mast cells (per 0.25 mm^2)	1.6±1.2 (1, 1–4)	4±2.3* (4, 0–8)	5.6±3.1 (5, 0–12)	8.5±4.6*** (6, 4–19)	7.6±3.8 (7, 1–16)	13.4±6.9** (16, 2–23)

Nevi with ADMA = nevi with architectural disorder with varying degrees of melanocytic atypia. Results are expressed as mean 1 standard deviation and (median, interval of variation). *$P<0.001$, **$P<0.01$ and ***$P<0.05$ compared with the preceding group (parametric analysis of variance followed by Duncan t, Bonferroni t and Wilcoxon paired tests).

3. Results

3.1. Angiogenesis and mast cells

Table 2 shows the counts of microvessels and MCs as entire and tryptase-positive cell populations on adjacent tissue sections selected from the six clinical steps of melanocyte tumour progression. The comparison of microvessel counts between groups revealed statistically significant differences (Chi-square = 36.5, degrees of freedom (d.f.) = 2.8, $P < 0.001$, $F = 37.7$, $P < 0.001$). Significantly higher counts were shown in melanoma as a whole (13.0 ± 6.4) compared with nevi (6.1 ± 3.3, $P < 0.001$). Assessment by the progression steps showed a significant increase in the nevi with ADMA group over the common nevi group ($P < 0.001$), and a trend to increase (albeit, non significant) in early melanomas. Progression to advanced melanomas was associated with no significant changes. By contrast, metastases gave significantly higher counts ($P < 0.01$) when compared with advanced melanomas. In parallel, the MC counts varied significantly between groups (Chi-square = 38.8, d.f. = 3.1, $P < 0.001$; $F = 41.2$, $P < 0.001$). The intergroup comparisons showed that the counts of total and tryptase-positive MCs were significantly higher in melanoma as a whole (14.3 ± 6.4 and 9.1 ± 5.8, respectively) compared with nevi (5.4 ± 4.3 and 2.7 ± 2.1, respectively, $P < 0.001$). Both counts also were significantly higher in nevi with ADMA over common nevi ($P < 0.001$), higher still in the primary melanomas ($P < 0.05$), and higher again in the metastatic melanomas ($P < 0.01$).

Fig. 1 shows the difference in both microvessel and MCs density between a common nevus (panels a and b) and a nevus with ADMA (panels c and d), and a metastatic melanoma (panels e and f). MCs were generally scattered throughout the neoplastic tissue and within the interstitial stroma where they rested near or around the blood capillaries.

The intragroup comparisons showed that microvessel counts were always significantly correlated with the MCs counts both as the total and tryptase-positive populations (Fig. 2).

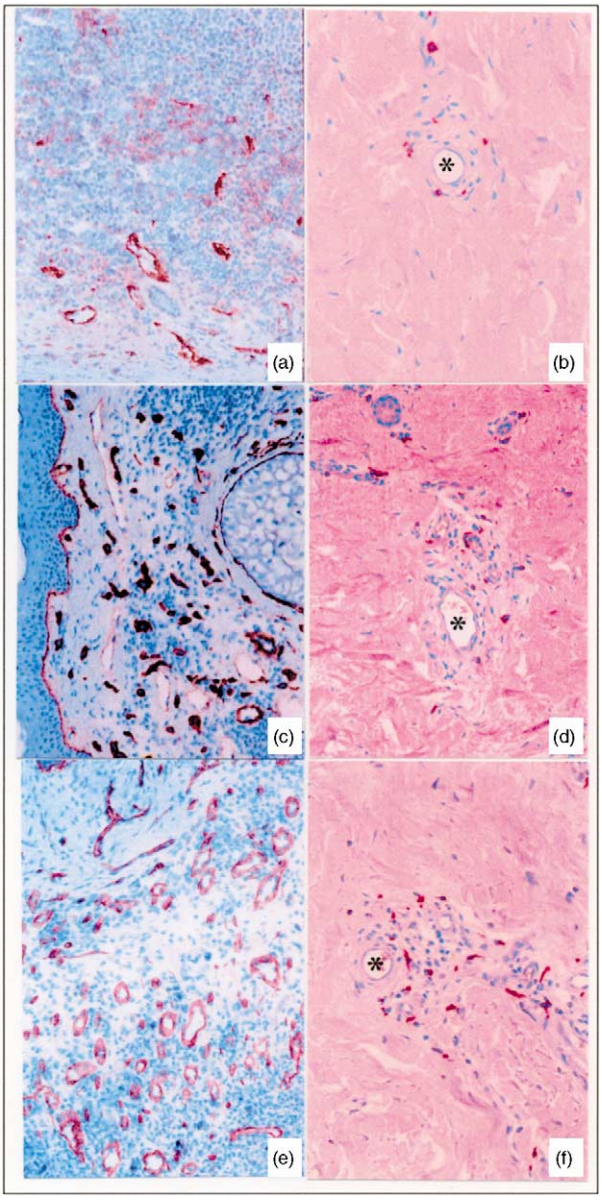

Fig. 1. Adjacent sections of common nevi (a, b), nevi with ADMA (c, d), and metastatic (e, f) melanomas stained with CD31 (a, c, e) for microvessels and with tryptase for mast cells (b, d, f). Note the progressive increase of microvessels and mast cells located around blood capillaries (asterisks) from common nevi to nevi with varying degrees of melanocytic atypia (ADMA) and from latter to metastatic melanomas.

3.2. Tumour immunoreactivity to FGF-2 and correlation with mast cell density

The percentages of lesions reactive with FGF-2 and the fraction of stained tumour cells within these lesions are illustrated in Figs. 3 and 4. The percentages increased in step with tumour progression (Chi-square total = 23.2, $P < 0.001$; Chi-square linear = 10.6, $P < 0.001$). Reactivity of nevi with ADMA was significantly more frequent (82%) than in common nevi (57%, $0.02 > P < 0.05$), and transition between these two steps was marked by increasing stained cell percentages. Progression to the early and advanced melanomas was accompanied by high FGF-2 expression frequency, with approximately 90% of reactive lesions at each step. Progression was associated with an increase in the percentage of stained cells. Immunoreactivity was found in all metastases and occurred significantly more often than in the common nevi ($0.01 > P < 0.05$). Most metastases displayed the highest percentages of stained cells. When percentages of FGF-2-positive tumour cells were compared with the number of total and tryptase-positive MCs significant correlations were found in each step (Fig. 5).

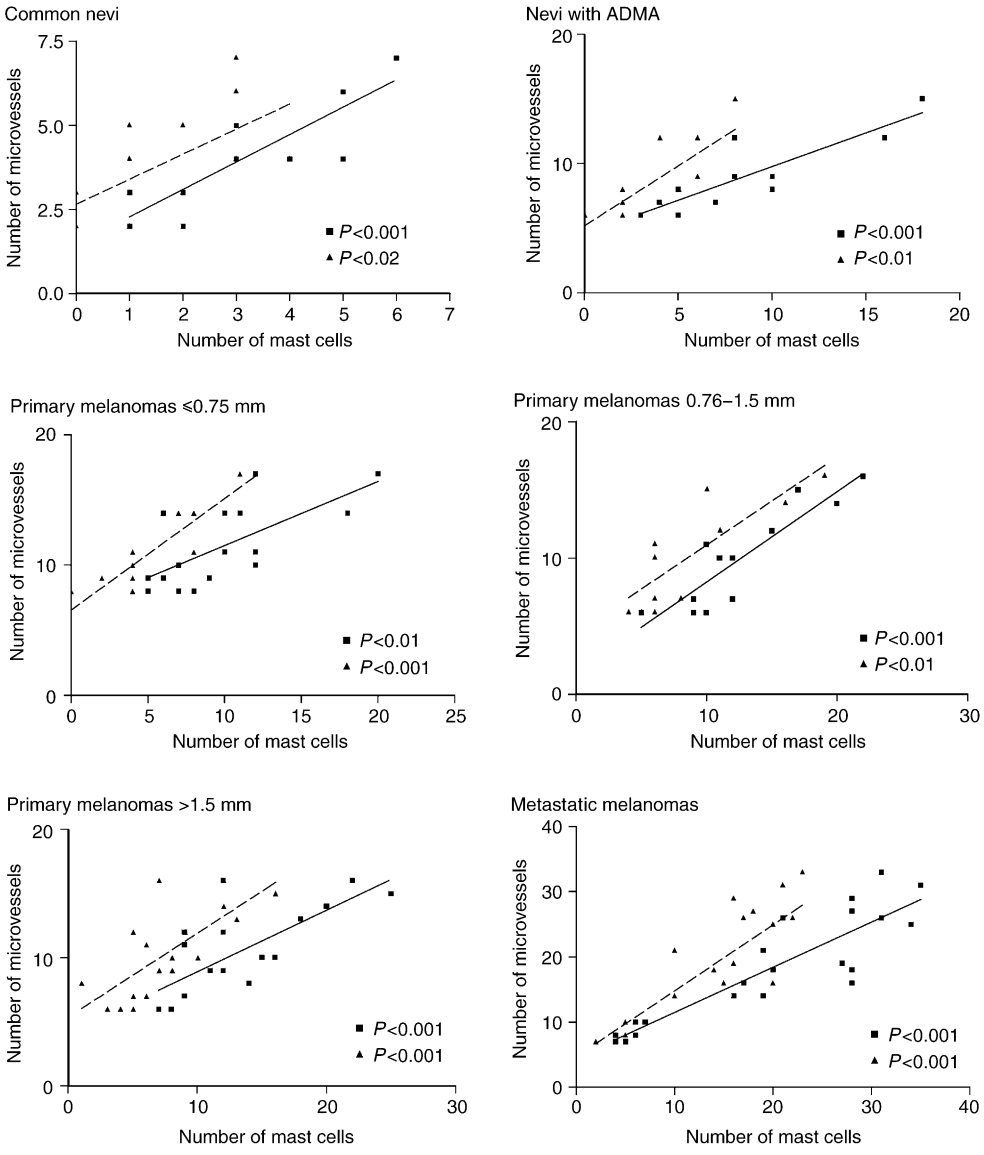

Fig. 2. Microvessel counts in comparison with total (■, continuous line) and tryptase-positive (▲, sketched line) mast cell (MC) counts in tissues. Significance of the regression analysis was calculated using Pearsons' (r) test.

4. Discussion

This paper shows that angiogenesis in human malignant melanoma, measured as microvessel counts, is highly correlated with both the percentage of tumour cells reactive to FGF-2 and MC counts (as total cell and tryptase-positive cell counts), and that these parameters increase with tumour progression.

Two distinct types of human MCs have been described based on the protease composition of their secretory granules: MCs containing chymase, carboxypeptidase, cathepsin and tryptase and MCs containing tryptase only. Tryptase, a protease unique to the MC secretory granules, acts as a mitogen for fibroblasts, smooth muscle cells, and epithelial cells [22,23]. Blair and colleagues [11] have shown that MC-released tryptase plays an important role in neovascularisation. Tryptase induces the formation of capillary structures by either directly acting on endothelial cells or by facilitating the early stages of angiogenesis. In fact, tryptase activates latent metalloproteinases and plasminogen activator [7], which degrade the extracellular matrix, a critical step in these stages [25]. MCs are strikingly associated with angiogenesis in tumours, namely haemangioma, carcinomas, lymphoma and multiple myeloma [4,5,9,12–14] where they are preferentially accumulated in the peripheral areas of the tumour, within the surrounding connective tissue, and rest near or around blood or lymphatic vessels [12]. MCs are recruited and activated via several factors secreted by tumour cells: the c-kit receptor [5], FGF-2, VEGF-A and platelet-derived endothelial cell growth factor (PD-ECGF), which are operative at

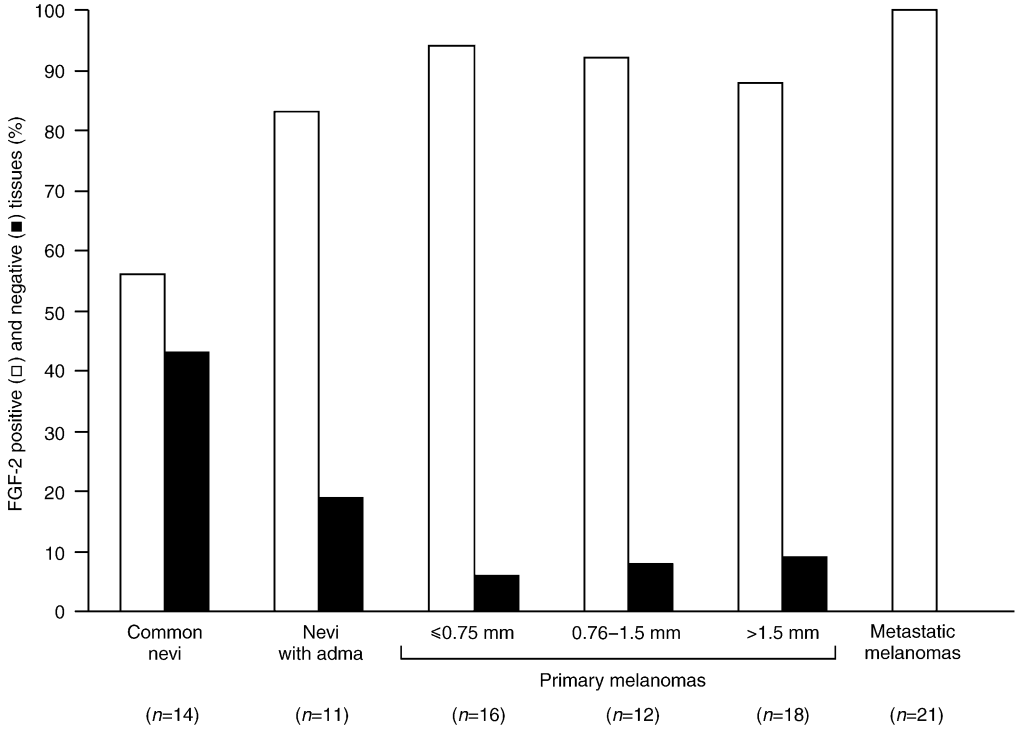

Fig. 3. Percentages of melanocytic lesions positive (open bars) and negative (shaded bars) for the FGF-2 expression at distinct steps in tumour progression. Number of lesions within parentheses.

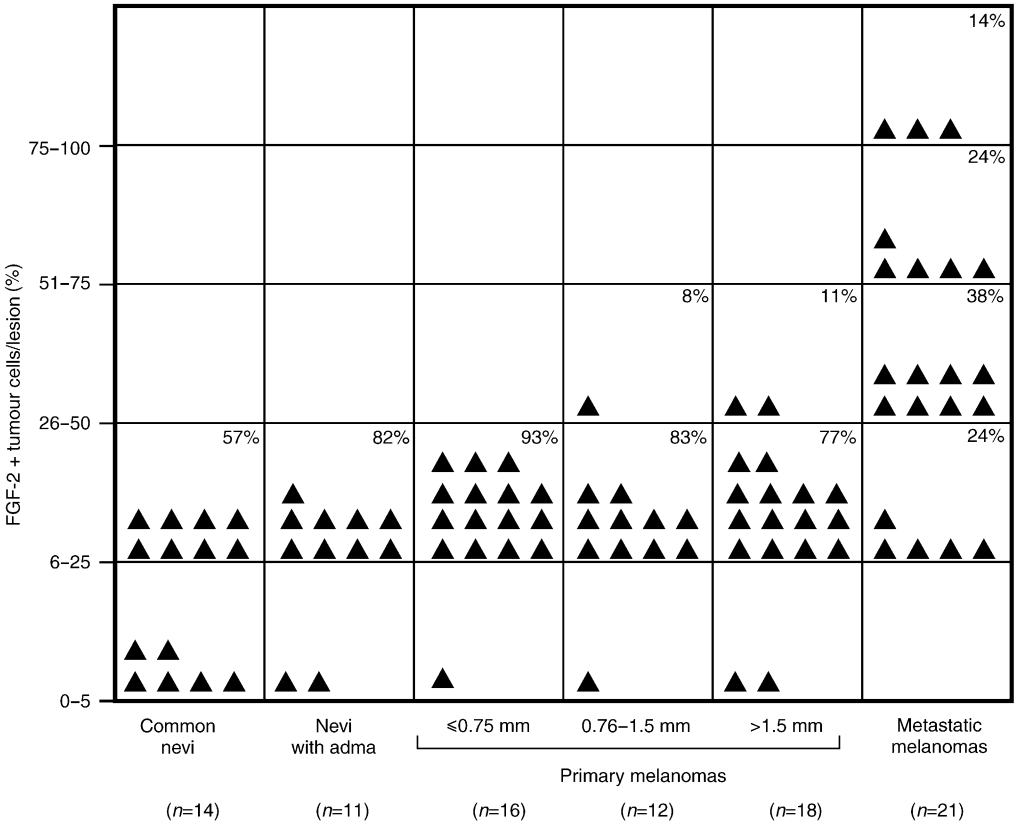

Fig. 4. Percentages of tumour cells expressing FGF-2 in various melanocytic lesions at distinct steps in tumour progression. Each triangle represents a single lesion. The percentages of cells stained per lesion were divided into five intensity groups (0–5, 6–25, 26–50, 51–75 and 76–100%). The percentages of lesions of each intensity group are reported.

picomolar concentrations [26]. The fact that MCs contribute to the induction of tumour angiogenesis stems from studies on MC-deficient mice, which display slow angiogenesis, and its restoration after local reconstitution of MCs [24, 27]. Moreover, in malignant breast lesions the number of MCs with tryptase activity was significantly higher than in benign lesions [28] and MCs derived from human renal tumour tissues contained tryptase [29].

FGF-2 is the most important autocrine growth factor in melanoma [18]. It is expressed by common nevus cells [30] and both primary and metastatic melanomas at the tumour invasion front, while tumour cells adjacent to the epidermis are largely devoid of this factor [31]. Its inhibition by antisense oligodeoxynucleotides leads to inhibition of melanoma proliferation *in vitro* and *in vivo* [17]. Its production by melanoma also promotes angiogenesis via a paracrine mode, through the mitogenic effect on endothelial cells and fibroblasts [34]. In addition, the metastatic potential and invasiveness of human melanoma cells were found to be markedly increased by transduction with the *FGF-2* gene [33]. Ugurel and colleagues [30] found significantly elevated serum levels of FGF-2, angiogenin, VEGF and IL-8 in melanoma patients compared with healthy controls. Blood values of FGF-2, VEGF and IL-8 were positively correlated with the stage of disease and tumour burden and implied a poor overall survival and high probability of progression.

In line with other reports showing a close relationship between increased number of tryptase-positive MCs and

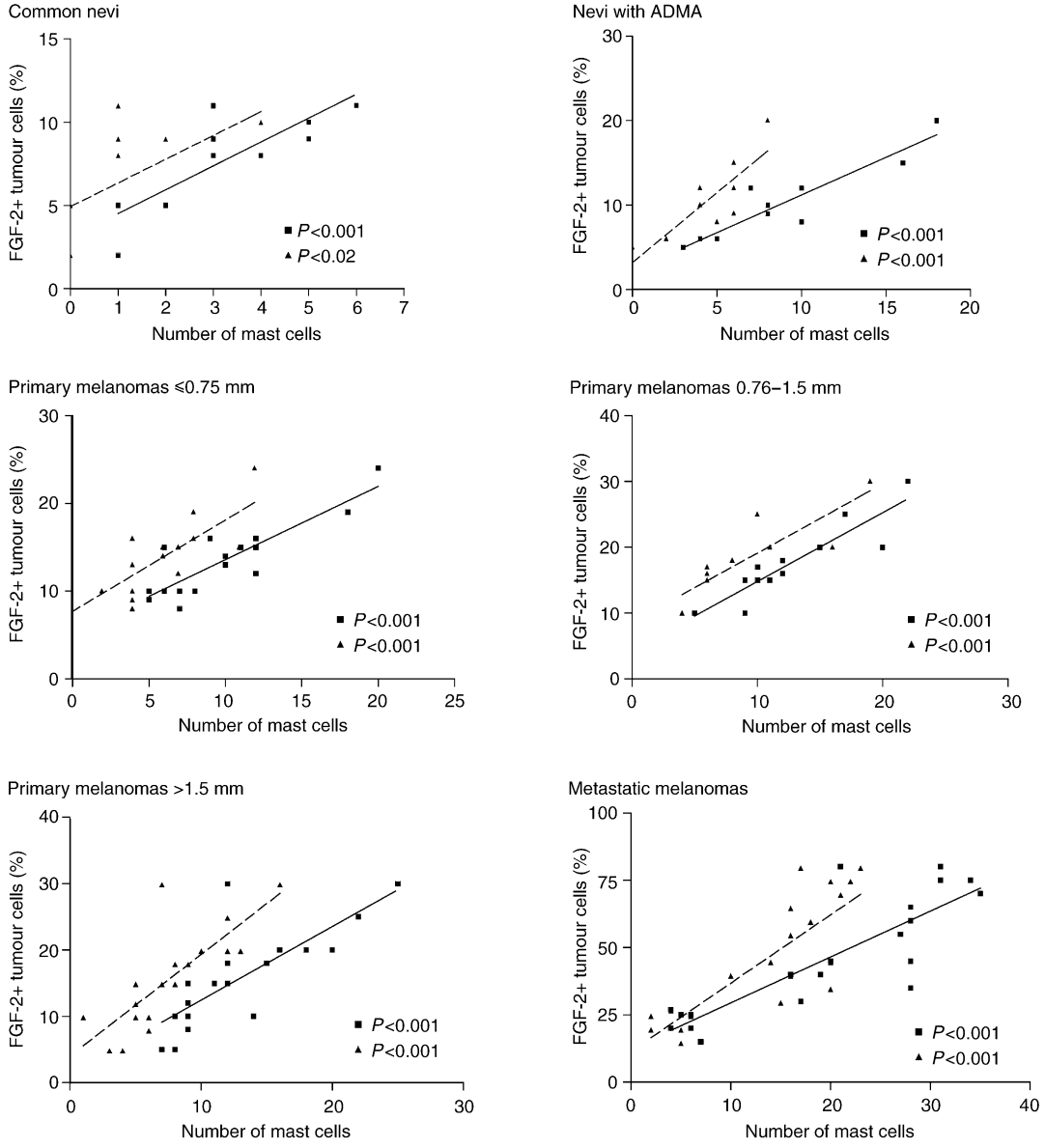

Fig. 5. Percentages of FGF-2-positive tumour cells in comparison with total (▪, continous line) and tryptase-positive (▴, sketched line), mast cell (MC) counts. Significance of the requenion analysis was calculated using Pearson's (r) test.

tumour progression [14,34,35], our data suggest that tryptase-positive MCs may contribute, at least partly, to the melanoma-associated angiogenesis. Furthermore, tumour-derived FGF-2 may have pleiotropic influences, first on tumour invasion, by elevating proteolytic enzymes [36], second on angiogenesis, by paracrine stimulation of endothelial cell growth [31], and third on recruitment and activation of MCs, which express the FGF-2 receptor [37]. MCs, in their turn, secrete FGF-2 stored in their secretory granules [9], which further stimulates endothelial cell growth and amplifies the FGF-2 paracrine stimulatory loop on angiogenesis.

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