Expression of Vascular Endothelial Growth Factor, Its Receptors (FLT-1, KDR) and TSP-1 Related to Microvessel Density and Patient Outcome in Vertical Growth Phase Melanomas

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Microvessel density (MVD) was estimated in a series of 202 vertical growth phase (VPG) melanomas and 68 corresponding metastases, using a marker for angiogenic endothelial cells (CD105) and Factor-VIII. The expression pattern of vascular endothelial growth factor (VEGF), FLT-1, KDR and thrombospondin-1 (TSP-1) was studied by immunohistochemistry, *in situ* hybridization and reverse-transcriptase polymerase chain reaction. CD105 stained significantly less vessels, but gave only limited additional prognostic information compared with Factor-VIII, and MVD was an independent prognostic factor for both markers. Ninety-eight percent of all cases showed expression of VEGF, and higher expression was found significantly more frequent in thinner and less vascularized tumors. Possible autocrine loops were suggested by co-expression of VEGF and its two receptors in tumor cells, and by a significant correlation between KDR and tumor cell proliferation (Ki-67) in the subgroup of thicker tumors. Staining of VEGF receptors in endothelium was not correlated with MVD. Strong expression of TSP-1 in tumor stroma was found in 43% of the primary tumors, and was significantly correlated with increased thickness, proliferation and MVD, as well as decreased survival. These data suggest that MVD is associated with prognosis in cutaneous melanomas, and that the VEGF system and particularly TSP-1 seem to be involved in the regulation of angiogenesis and progression of these tumors. (Am J Pathol 2001, 159:223–235)

The ability of tumors to stimulate endothelial cell growth and differentiation is widely accepted to be of critical importance for progression and metastasis in most tumor types.¹ Microvessel density (MVD), a commonly applied estimate of tumor angiogenesis, is an established prognostic factor in several malignancies, eg, breast cancer,² endometrial cancer,³ and prostate cancer.⁴ However, the reports published on prognostic significance of MVD in

malignant melanoma are contradictory,^{5–12} although survival studies are few. In a previous study, we found a significant prognostic impact of MVD in univariate survival analysis of vertical growth phase melanomas.¹³

Because tumor angiogenesis is considered to be of clinical and therapeutic importance, much effort has been taken to describe new angiogenic stimulators and inhibitors. Vascular endothelial growth factor (VEGF) might have a fundamental role in tumor vessel formation,¹⁴ and VEGF expression has been associated with increased angiogenesis in both clinical^{15–18} and experimental studies.¹⁹ In addition, recent findings by Detmar and colleagues²⁰ demonstrate that VEGF overexpression might induce tumor invasiveness in addition to promotion of angiogenesis and tumor growth. In melanocytic tumors, VEGF expression was increased with malignant progression,^{11,21,22} although significant associations with increased MVD has not been found.

Alternative exon splicing of the *VEGF* gene results in at least 5 different isoforms, having 121, 145, 165, 189 and 206 amino acids, respectively.²³ These isoforms differ in heparin binding and diffusibility,¹⁴ and recent studies of different tumors report increased angiogenic potential and negative prognostic impact for the partially cell-retained isoforms (VEGF 165 and 189).^{24–27}

The FLT-1 and KDR proteins have been identified as VEGF receptors, and are thought to be restricted largely to the vascular endothelium.^{14,28,29} Quite recently though, these receptors have been found in ovarian carcinoma cells,³⁰ melanoma cells,^{31–33} thyroid tumors,³⁴ and breast carcinomas.³⁵ Although the biological relevance of VEGF receptor expression on tumor cells is not clear, one study found that VEGF increased the proliferation of KDR positive melanoma cells *in vitro*,³⁶ whereas others described an inhibitory effect of VEGF on FLT-1 positive tumor cells.³⁷

Thrombospondin-1 (TSP-1), an extracellular matrix glycoprotein,³⁸ has been associated with both a supportive^{39–41} and inhibitory^{42,43} role in tumor invasiveness and

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Table 1. Immunohistochemical Staining Methods

Antibody	Provider	Epitope retrieval	Dilution	Incubation	Signal amplification	Controls
pAb A-082, Factor VIII	DAKO	Proteinase K	1:800	25 min RT*	No	Pos: Colon cancer Neg: Omission
MoAb Sn6h, CD105	DAKO	No	1:1000	30 min RT	CSA (DAKO)	Pos: Colon cancer Neg: Omission
pAb A-20, VEGF	Santa Cruz	3 × 5 min in citrate buffer (pH = 6) at 500 W	1:50	25 min RT	No	Pos: Endometrial cancer Neg: Omission
pAb C-17, Flt-1	Santa Cruz	6 × 5 min in citrate buffer (pH = 6) at 500 W	1:100	Overnight RT	No	Pos: Umbilical vein Neg: Blocking peptide
MoAb A-3, KDR	Santa Cruz	3 × 5 min in citrate buffer (pH = 6) at 500 W	1:500	25 min RT	No	Pos: Umbilical vein Neg: Irrelevant mouse MoAb
MoAb Ab-7, TSP-1	Neomarkers	3 × 5 min in Tris-HCL (pH = 1.0) at 500 W	1:200	Overnight RT	No	Pos: Colon cancer Neg: Omission

*RT, room temperature.

progression. Several experimental and clinical studies have provided evidence for an inhibitory role of TSP-1 on tumor angiogenesis,^{44–49} and TSP-1 expression has been associated with improved survival in studies of colon⁴⁸ and bladder cancer.⁴⁵ Furthermore, recent studies have shown that TSP-1 might modulate angiogenesis in opposite directions, depending on which domain of the molecule is active and/or available, or whether different TSP-1 receptors are present on endothelial cells.^{50,51}

A regulatory role of the *p53* tumor suppressor gene on angiogenesis has been reported to act through VEGF^{18,46,52,53} and/or TSP-1.^{44,45,47} Also, the *CDKN2A/p16* tumor suppressor gene, which is particularly interesting in melanomas,^{54–56} has been associated with regulation of VEGF expression,⁵⁷ and VEGF is reported to down-regulate *p16* and delay senescence in endothelial cells.⁵⁸ On this background, the aim of our study was to examine the expression of VEGF, its receptors and TSP-1 in relation to MVD, *p53* and *p16* protein expression as well as clinicopathologic factors and patient survival in a series of 202 vertical growth phase melanomas.

Materials and Methods

Patients

The patient material of this series is described in detail elsewhere.⁵⁶ Briefly, 202 vertical growth phase melanomas occurring during the years 1981–1997 were included. The presence of a vertical growth phase, and the lack of a radial growth phase, ie, adjacent *in situ* or microinvasive component, were used as inclusion criteria for the present study.⁵⁹ In addition, 68 separate biopsies of local (skin; *n* = 17), regional (lymph nodes; *n* = 44) or distant (*n* = 7) metastases from 58 patients with recurrent disease were available for analyses.

Complete information on patient survival, time and cause of death was available in all 202 cases. Last date of follow-up was December 18th, 1998, and median follow-up time for all survivors was 76 months (range, 13–210). Clinical follow-up (with respect to recurrences) was not carried out in 14 (mostly older) patients, and 21 patients were not treated with complete local excision.

Thus, recurrence-free time could be studied in 167 patients.

Immunohistochemistry (IHC)

The immunohistochemical staining was performed on formalin-fixed and paraffin-embedded archival tissue (5 μ m sections), and the conditions were optimized for each antibody. Some important steps in the respective protocols are summarized in Table 1. The staining procedures and evaluation of *p16*-, *p53*-, and *Ki-67* expression have been described previously.⁵⁶ The results on these biomarkers have also been included in the present study (see Results).

Staining was performed on a DAKO (Copenhagen, Denmark) TechMate 500 slide processing equipment, using the standard avidin-biotin method. Finally, the peroxidase was localized by the 3-amino-9-ethylcarbazole peroxidase reaction with Harris hematoxylin as counterstain.

In situ hybridization

mRNA *in situ* hybridization (ISH) was carried out by using the Super Sensitive ISH Detection kit (Biogenex, San Ramon, CA), optimized for paraffin-embedded archival material, and the Omnislide Thermal Cycler (Hybaid, Ashford, UK) equipment. Twenty-five cases with high VEGF expression (index ≥ 4) and 25 cases with low expression, as well as 5 randomly selected metastases, were selected for *in situ* hybridization on the basis of immunohistochemical staining results. Expression of TSP-1 was studied in 36 randomly selected cases by *in situ* hybridization.

The sections (5 μ m) were dewaxed in xylol. Proteinase K treatment was replaced with microwave treatment in citrate buffer (pH = 6.0) because this method produced more consistent results. After placing the working probe solution (250 ng of probe/ml) on the slides, a 10-minute denaturation step at 95° C on the heating block was performed followed by incubation overnight at 37° C in a

Table 2. Sequences of Primers Used in RT-PCR Analyses for 3 Different Splice Variants of VEGF

Primers	Sequence	Product length
VEGF ₁₂₁ lower	5'-ATC TGG TCC CCG AAA CGC T-3'	173 bp
VEGF ₁₂₁ upper*	5'-TAT GCG GAT CCA ACC TCA CCA A-3'	
VEGF ₁₆₅ lower	5'-ACA TTT ACA CGT CTG CGG ATC T-3'	172 bp
VEGF ₁₆₅ upper*	5'-TAT GCG GAT CCA ACC TCA CCA A-3'	
VEGF ₁₈₉ lower	5'-ACA TTT ACA CGT CTG CGG ATC T-3'	122 bp
VEGF ₁₈₉ upper	5'-AGG GAA AGG GGC AAA AAC G-3'	

*An alternative upper primer, common for VEGF₁₂₁ and VEGF₁₆₅ and corresponding to products of 124 and 123 bp, respectively, was also tested.

humidity chamber. Sections were blocked for endogenous biotin and peroxidase.

The sequence of the biotinylated antisense oligonucleotide probe for VEGF is TGG'TGA'TGT'TGG'ACT'CCT'CAG'TGG'GC. This sequence is previously used by others.^{60,61} Further, a cocktail of two biotinylated antisense probes with the following sequences was used for TSP-1: CAT'GGT'GGA'GCT'GTT'GGT'GCC'CAG'CAG'G and TGG'GGC'AGG'ACA'CCT'TTT'TGC'AGA'TGG'T. The sequences showed 100% homology with the respective genes as determined by a BLAST-search in the NCBI databases (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/) Negative controls were obtained by incubating the control sections with biotinylated sense probes or no probe. Cases showing no reaction when incubated with a polyA probe were regarded to have degradation of mRNA and were excluded from further analysis.

Reverse-Transcription Polymerase Chain Reaction

Thirty cases were randomly selected for the analysis of VEGF isoforms by reverse-transcription polymerase chain reaction (RT-PCR). After deparaffinization of four 10 μ m sections, the samples were incubated with proteinase K at 55°C overnight on a rotator. RNA were then isolated by the phenol-chloroform extraction method.⁶² RT-PCR were carried out by the Sensiscript kit (Qiagen, Hilden, Germany) according to the recommendations of the provider, and random hexamers were used for first strand cDNA synthesis. VEGF cDNA fragments were amplified by 45 rounds of PCR consisting of 1 minute at 95°C, 1.5 minutes at 53°C and 1.5 minutes at 72°C with Amplitaq Gold (Applied Biosystems, Foster City, CA), and PCR products were run on a 3% agarose gel containing ethidiumbromide and visualized by UV-light.

The quality of mRNA was examined by two primer pairs for β -actin (products of 150 and 265 bp, respectively). Table 2 shows the sequences of the primers used, as well as the expected size of the PCR products. The quality of RNA isolated from the archival material was not optimal, and the critical length for successful amplification was found to be around 200 bp in most cases. Thus, to get short products, we used primer pairs specific for each splice variant of VEGF. Negative results were only considered reliable if the actual sample showed a β -actin product longer than the expected product of the isoform being analyzed. cDNA from a snap frozen Ewing sar-

coma, expressing the VEGF isoforms, was used as a positive control for the PCR step.

Evaluation of MVD

MVD was assessed as described previously.¹³ Briefly, the sections were scanned at low magnifications ($\times 25$ and $\times 100$) to identify the most vascular areas of the tumor (hotspots), according to Weidner and colleagues.² Within these areas, which were almost exclusively localized within and around the invasive front at the tumor base, a maximum of 10 fields at $\times 400$ magnification (HPF, 0.16 mm² per field) were examined, and the mean value of these fields was calculated. Vessels more than one-half HPF ($\times 400$) away from (below) the invasive front, or vessels close (< 1 HPF) to ulcerated areas were not counted. Any highlighted endothelial cell or cell cluster, clearly separate from adjacent microvessels, tumor cells, and connective tissue elements, were regarded as a distinct countable microvessel.²

Using Factor-VIII stained slides, MVD was estimated in the hotspot areas. In addition, specific MVD counts were established for the central tumor areas as well as for the tumor base, and a ratio between the two was also calculated. In parallel, MVD in the hotspots was estimated using the CD105/endoglin antibody, which has been promoted as a marker more specific for tumor associated vessels⁶³⁻⁶⁵ and a proliferation-associated marker on endothelial cells.⁶⁶

Evaluation of Staining Results

A staining index, obtained as a product of staining intensity (0-3) and proportion of immunopositive tumor or endothelial cells ($\leq 10\% = 1$, 10 to 50% = 2, $> 50\% = 3$), was calculated for VEGF staining and its receptors, as well as for *in situ* hybridization results for VEGF and TSP-1 mRNA. FLT-1 staining in tumor associated endothelial cells was evenly weak in most positive cases, and quantification by the staining index was not suitable, therefore the expression was recorded as absent or present. When present, the intensity of the VEGF labeling of inflammatory cells and keratinocytes was recorded as minimal, moderate or strong. The mRNA expressing cell type was also noted after *in situ* hybridization for VEGF and TSP-1 mRNA.

Using similar criteria as for the VEGF staining, a staining index (area $\times$ intensity) was calculated for TSP-1

positivity. TSP-1 expression was graded as absent/low (index, ≤ 2) or moderate/high on the basis of extracellular immunostaining in intratumoral or immediate peritumoral (<1 HPF) areas. Nuclear staining, which was observed occasionally in tumor cells and frequently in inflammatory cells, was considered to be nonspecific.^{45,67}

For statistical purposes, cut points for continuous variables, and the variables evaluated by the staining index, were based on the distribution of these values. The staining indices for VEGF, its receptors and TSP-1 showed bimodal distributions, and the cut points were set between distinct peaks.

Statistics

Analyses were performed using the statistical package SPSS, version 9.0.⁶⁸ Associations between different categorical variables were assessed by Pearson's χ^2 test. Continuous variables not following the normal distribution were compared between two or more groups using the Mann-Whitney *U* or Kruskal-Wallis *H* tests. A Wilcoxon signed ranks test was used to compare related samples. Univariate analyses of time to death due to malignant melanoma or time to recurrence (recurrence-free survival) were performed using the product-limit procedure (Kaplan-Meier method), with date of histological diagnosis as the starting point. Patients who died of other causes were censored at the time of death. Differences between categories were tested by the log-rank test. The influence of covariates on patient survival and recurrence-free survival was analyzed by the proportional hazards method,⁶⁹ including all variables with a *P* value ≤ 0.15 in univariate analyses, and tested by the likelihood ratio test. Model assumptions were tested by log-minus-log plots, and significant variables were tested for interactions. Estimated hazard ratio, 95% CI for hazard ratio and *P* values are given in the tables.

Results

MVD

Using the Factor-VIII antibody (F-VIII), median MVD in the primary tumors (hotspot areas) was 125 microvessels per mm^2 (range, 31–456; mean, 132; SD, 59), compared with a median of 106 microvessels per mm^2 (range, 19–281; mean, 124; SD, 58) in the metastases (Wilcoxon signed ranks test, *P* = 0.037). The most active areas were almost exclusively located at the tumor base.

Median MVD counted at the tumor base was 125 microvessels per mm^2 , compared with 75 when counted in central areas of the tumor (intratumor MVD; Wilcoxon signed ranks test, *P* < 0.0001). The median intratumor/tumor base ratio for MVD was 0.57, and a higher ratio was significantly associated with Clark's level 5 of invasion, 0.81 versus 0.55 for the others (Mann-Whitney *U* test, *P* = 0.006).

The CD105/endoglin antibody gave a median MVD in the primary tumors (hotspot areas) of 94 microvessels per mm^2 (range, 6–350; mean, 109; SD, 69). Estimates of

Figure 1. Microvessel density (MVD) in vessels/ mm^2 by CD105 antibody plotted against MVD by Factor-VIII antibody in a simple scatterplot. A regression line is shown to illustrate the relationship (see Results).

MVD by F-VIII and CD105 were significantly correlated (Figure 1, linear regression *r* = 0.40, *P* < 0.0001), although MVD counts by CD105 were significantly lower (Wilcoxon signed ranks test, *P* < 0.001). Table 2 shows a comparison between the two vessel markers with respect to associations between MVD and other variables studied, and some differences were present. CD105 was significantly associated with tumor thickness, in contrast to the findings for F-VIII. Also, biomarkers such as p53 and VEGF protein staining were significantly associated with CD105 expression (Table 2).

The median CD105/F-VIII ratio (hotspot areas) was 0.63 (range, 0.03–2.67; mean, 0.69; SD, 0.43). Increased CD105/F-VIII ratio was significantly correlated with increased tumor thickness using median thickness as cut-point (Mann-Whitney *U* test, *P* = 0.018), and presence of p53 staining (Mann-Whitney *U* test, *P* = 0.01).

VEGF Protein Staining (IHC)

Positive staining for VEGF protein in the cytoplasm of tumor cells was present in 98% of the primary tumors, with high expression (staining index ≥ 4) in 68%; 68% of the metastasis also had high VEGF expression. All cases showed positivity in inflammatory cells, and 35% had high VEGF expression in these cells. Seven and 61% of the cases showed strong VEGF expression in the keratinocytes and endothelial cells, respectively (Figure 2c).

As shown in Table 2, MVD, as assessed by CD105/endoglin staining, was lower in cases showing high expression (index ≥ 4) of the VEGF protein (Mann-Whitney *U* test, *P* = 0.04); correspondingly, increased VEGF protein expression was significantly associated with lower tumor thickness (Mann-Whitney *U* test, *P* = 0.04) and lower (≤ 4) Clark's level of invasion (*P* = 0.04). In tumors ≤ 3.55 mm (median), 74% showed strong VEGF expression, compared with 61% in tumors >3.55 mm.

Interestingly, the association between VEGF expression and MVD (CD105) was opposite in thin and thick lesions. As illustrated in Figure 3 a), in tumors ≤ 3.55 mm

Figure 2. MVD at the tumor base as illustrated by CD105- (a) and Factor VIII antibody (b). VEGF protein expression by immunohistochemistry (c) and VEGF mRNA expression by *in situ* hybridization (d). e: Positive staining of Flt-1 receptors. f: KDR receptors in tumor associated endothelium and melanoma cells. (g) Intratumoral stromal tissue expressing the TSP-1 protein. h: TSP-1 mRNA by *in situ* hybridization.

Figure 3. a: Inverse relations between VEGF protein expression and MVD when the cases were grouped in thin and thick lesions. **b:** Opposite associations between expression of TSP-1 protein and MVD depending on p53 expression status (absent or present) of the tumor. * Statistically significant differences, $P < 0.05$ (see Results).

(median), strong VEGF expression was not significantly associated with higher MVD by CD105 (Mann-Whitney U test, $P = 0.6$), whereas in tumors > 3.55 mm, strong VEGF expression was significantly associated with lower MVD (Mann-Whitney U test, $P = 0.001$).

VEGF expression was significantly higher in cases with presence of vascular invasion ($P = 0.04$). There was no association between VEGF expression and MVD by F-VIII in neither primary tumors nor metastases.

VEGF mRNA

As described, 25 cases with high VEGF protein expression (index ≥ 4) were compared with 25 cases with low expression (index, < 4) by *in situ* hybridization for VEGF mRNA. VEGF mRNA expression was found as a granular reactivity in the cytoplasm of tumor cells in positive cases (Figure 2 d). Similar to the immunohistochemical analysis, the expression was diffusely distributed throughout the tumor in most cases. Weak hybridization signals in other cell types, including endothelial cells were detected occasionally (Figure 2). No hybridization was seen when incubating with the sense probe or no probe. Eight cases were negative or very weak (index 0–1), 17 cases were moderate (index 2–3), whereas 25 cases were strong (index ≥ 4). The agreement between the two methods (IHC and ISH) was 74% with a corresponding kappa value $\kappa = 0.63$ (χ^2 , $P < 0.0001$).

VEGF mRNA Isoforms

Twenty-three of 30 cases included for RT-PCR (77%) produced evaluable bands for β -actin (150 and 265 bp). Six of these cases (26%) were positive for VEGF₁₈₉, and 1 of these 6 was also positive for both VEGF₁₂₁ and VEGF₁₆₅, whereas the remaining 17 cases were negative for all isoforms analyzed. Negative cases that did not show a β -actin band longer than the VEGF isoform product of interest were regarded as not valuable. As illustrated in Figure 4, the amount of amplifiable VEGF mRNA in the positive cases was small in comparison with that of β -actin, as interpreted from the thickness of the bands. The median MVD by CD105 in the 6 cases expressing VEGF₁₈₉ was 31 vessels/mm² compared with 81 in the

Figure 4. RT-PCR analysis of VEGF mRNA isolated from paraffin embedded melanomas. **Lanes 1, 11, 12 and 19:** 100-bp marker. **Lane 2:** positive control for β -actin (150 bp). **Lanes 3–8:** β -actin in melanoma samples (cases 527, 519, 518, 515, 514, and 509). **Lane 9:** VEGF₁₂₁ in case 527. **Lane 10:** Positive control for VEGF₁₂₁. **Lane 13:** VEGF₁₆₅ in case no 527. **Lane 14:** positive control for VEGF₁₆₅. **Lane 15:** positive control for VEGF₁₈₉. **Lanes 16–18:** VEGF₁₈₉ in case 527, 519, and 518.

17 cases positive for β -actin but lacking bands for VEGF₁₈₉ (Mann-Whitney U test, $P = 0.14$).

FLT-1 and KDR Protein Staining

Presence of FLT-1 staining in endothelial cells was recorded in 28% of the cases, and was significantly associated with increased VEGF expression in tumor cells (χ^2 , $P = 0.04$). FLT-1 expression in endothelial cells was further correlated with presence of nuclear p16 staining (χ^2 , $P = 0.008$). No significant association with MVD was found.

FLT-1 staining was present at various levels in tumor cells in 83% of the cases, with high expression (index ≥ 4) in 46%. Strongly positive cases were significantly associated with FLT-1 in endothelial cells (χ^2 , $P < 0.0001$) and presence of nuclear p16 expression (χ^2 , $P = 0.01$).

In tumor-associated endothelial cells, KDR expression was present in 83% of the cases, with high expression (index ≥ 4) in 66%. High expression was significantly associated with absent p53 staining (χ^2 , $P = 0.006$). No significant correlation was found between KDR expression in endothelial cells and clinico-pathological variables or MVD.

Tumor cells showed KDR staining in 89% of the cases, being strong (index ≥ 4) in 72%. High KDR expression was significantly associated with lower MVD (CD105, Mann Whitney U Test, $P = 0.02$), and absence of p53 staining (χ^2 , $P = 0.002$). By comparing the cases by median tumor thickness (3.55 mm), we found that KDR expression in tumor cells was significantly associated with increased proliferative rate by Ki-67 in the thicker cases, with a median proliferative rate of 26% by low KDR expression, as compared to 38% by high KDR expression (Mann Whitney U test, $P = 0.003$). In the same

subgroup of thick tumors, high KDR expression in tumor cells was significantly associated with weak or absent p16 staining (χ^2 , $P = 0.008$).

In cases with nests of infiltrating melanoma cells in the dermis, we often observed a perinodular staining pattern with increased KDR expression in the cells lining the stromal septa compared with cells in the center of the nests. In several cases, we also observed increased expression at the borders of the tumors, although a more homogenous distribution of positive cells was more frequent (Figure 2f).

Significant co-expression in tumor cells was found between FLT-1 and VEGF (χ^2 , $P < 0.0001$). A significant co-expression between KDR and VEGF was also present (χ^2 , $P = 0.02$), and cases that co-expressed KDR and VEGF in tumor cells ($n = 98$) had significantly lower MVD by CD105 (Mann-Whitney U test, $P = 0.01$).

TSP-1 Protein Staining

As illustrated in Figure 2g, the TSP-1 protein was immunohistochemically detected in the tumor stroma, especially near ulcerated areas, in the stromal septa, and at the tumor base. Forty-three percentage of the primary tumors and 41% of metastases showed moderate or high TSP-1 expression (index > 2) by immunohistochemistry. In the primary tumors, moderate or high TSP-1 expression was significantly correlated with increased MVD (both vessel markers), as shown in Table 1. The same trend was found, although not significantly, in the metastases (Mann-Whitney U test, $P = 0.10$). TSP-1 expression was significantly higher in ulcerated tumors (χ^2 , $P < 0.0001$), and when ulcerated and non-ulcerated ($n = 105$) cases were analyzed separately, the relation between TSP-1 and MVD was different in the two subgroups. Increased TSP-1 expression was significantly associated with increased MVD (Mann-Whitney U test, $P = 0.02$) in the non-ulcerated tumors, whereas no association was observed in the ulcerated tumors.

Figure 3b illustrates the relation between TSP-1 expression and MVD depending on the p53 status of the tumor. In p53-positive tumors, moderate or high TSP-1 expression was significantly correlated with increased MVD (Mann-Whitney U test, $P = 0.004$), whereas in p53-negative tumors, the inverse relation was observed (not statistically significant, $P = 0.36$).

High expression of TSP-1 protein was further associated with increased tumor thickness (Mann-Whitney U test, $P < 0.001$), Clark's level 5 versus 2–4 (χ^2 , $P = 0.02$), presence of vascular invasion (χ^2 , $P = 0.04$), increased proliferative rate by Ki-67 (Mann-Whitney U test, $P < 0.001$), presence of p53 expression (χ^2 , $P = 0.014$) and loss of nuclear p16 expression (χ^2 , $P = 0.009$).

TSP-1 mRNA

TSP-1 mRNA was detected in the nuclei of tumor cells, fibroblasts and inflammatory cells, as well as in endothelial cells in some cases (Figure 2h). The intensity of the chromogenic signal appeared to be comparable in dif-

ferent cell populations within each case, and the staining was relatively homogenous throughout the tumor tissue. In some cases, however, the expression was more marked in the deepest infiltrating part of the tumor. The expression was regarded as high (index ≥ 4) in 12 cases (33%), 18 cases (50%), and 16 cases (44%), for tumor cells, inflammatory cells, and fibroblasts, respectively. There was no statistically significant association between strong protein staining and mRNA expression in either of these cell types, although there was a trend of stronger protein staining in cases with high mRNA expression in the tumor cells (66% with strong TSP-1 protein staining), compared to cases with low mRNA expression (43% of which had strong TSP-1 protein staining; χ^2 , $P = 0.19$).

Presence of ulceration was significantly associated with increased expression of TSP-1 mRNA in tumor cells and fibroblasts (χ^2 , $P = 0.05$ for both). Whereas no association was present between p53 protein staining and TSP-1 mRNA expression in tumor cells, absent p53 staining (tumor cells, index = 0) was significantly correlated to high TSP-1 mRNA expression in inflammatory cells and fibroblasts (χ^2 , $P = 0.02$, and $P = 0.03$, respectively). No significant associations were found with MVD.

Survival Analysis

Table 3 shows the results of univariate survival analysis for the angiogenesis variables. Of these, only MVD and TSP-1 expression were significant and included in multivariate analysis (Figure 5). In addition, the following variables, all significant or of borderline significance in univariate analysis ($P \leq 0.15$), were included: anatomical site, tumor thickness, Clark's level of invasion, vascular invasion, tumor ulceration, p16, p53, and Ki-67 expression.⁵⁶ The MVDs for F-VIII and CD105 were analyzed separately. MVD by CD105 was categorized by the median value, whereas MVD by F-VIII was categorized by the 67th percentile to show a significant difference; using the median value gave no significant difference.¹³ Only cases with complete information on all variables were included in multivariate analysis. Anatomical site, Clark's level of invasion, vascular invasion, p16 expression, p53 expression, Ki-67 expression, and MVD remained as independent prognostic factors in the final multivariate model. The results of multivariate survival analysis including MVD by CD105 ($n = 170$) are shown in Table 4. When MVD with F-VIII was included ($n = 184$), the Hazard Ratio for MVD was 1.9 (1.05–3.4) ($P = 0.03$), with only minor adjustments for the covariables.

Discussion

In the present study we show that angiogenesis, as estimated by MVD, and using two different endothelial cell markers (F-VIII and CD105/endoglin), is an independent prognostic variable in vertical growth phase melanomas. Based on previous reports, the importance of angiogenesis in malignant melanomas has been controversial,^{5–12} although survival studies are few. This might partly be due to differences in patient selection, antibodies used

Table 3. Microvessel Density (Median Vessel Counts/mm²) Related to Standard Clinicopathological Variables

Variable	No. of patients	MVD (F. VIII)	P value*	MVD (CD 105)	P value*
<u>Anatomic site</u>					
Head/neck	47	125	0.75 [†]	100	0.27 [†]
Trunk	49	119		81	
Upper extremity	38	131		91	
Lower extremity	52	113		113	
<u>Clark's level of invasion</u>					
III	21	119	0.77 [†]	84	0.67 [†]
IV	131	125		97	
V	35	131		100	
<u>Tumor thickness</u>					
≤3.6 mm [‡]	92	119	0.05	81	0.001
>3.6 mm	96	131		113	
<u>Tumor ulceration</u>					
Absent	105	112	0.003	91	0.12
Present	79	138		106	
<u>Vascular invasion</u>					
Absent	150	119	0.09	97	0.7
Present	38	131		94	
<u>Ki-67 expression</u>					
≤16% [§]	49	113	0.08	94	0.3
>16%	139	125		100	
<u>p53 staining</u>					
Absent	29	119	0.3	78	0.02
Present	158	125		106	
<u>p16 staining[¶]</u>					
Weak/absent (≤1)	85	125	0.3	94	0.8
Moderate/strong (>1)	102	119		94	
<u>VEGF staining[¶]</u>					
Weak/absent (<4)	60	128	0.4	119	0.015
Moderate/strong (≥4)	127	119		87	
<u>TSP-1 staining[¶]</u>					
Absent/weak (<2)	103	119	0.007	81	0.001
Moderate/strong (≥2)	77	131		113	
<u>Flt-1 staining</u>					
Absent	127	125	0.9	103	0.6
Present	50	128		93	
<u>KDR staining</u>					
Weak/absent (<4)	62	125	0.7	106	0.08
Moderate/strong (≥4)	121	125		88	

Patients Ki-67, p53, and p16 protein expression and angiogenesis regulators in 188 with VGP melanoma, specified for 2 different vessel markers (F-VIII, CD105/endoglin).

*Mann-Whitney *U* test, when otherwise not specified.

[†]Kruskal-Wallis test.

[‡]Median value.

[§]Lower quartile.

[¶]Staining index (see Materials and Methods).

^{||}Staining in tumor-associated endothelium.

and procedures for microvessel quantification. We used two different antibodies, and the hotspot strategy introduced by Weidner and co-workers for microvessel counts. Our findings are in accordance with similar studies on a variety of human cancers,⁷⁰ and support the view of neovascular dependency of tumor growth and progression. However, the prognostic impact was limited, and the statistical significance in multivariate analysis was clearly dependent on sample size, as we, in a previous study of 102 of these cases, reported a borderline significance of MVD.¹³

MVD at the tumor base was significantly higher compared with counts for the central areas of the tumors. It is likely that most tumor-associated vessels in the periphery are recruited from pre-existing vascular networks. The activity of angiogenic factors might be especially elevated at the invasive front, and interactions between tumor cells, stromal cells, and inflammatory cells are prob-

ably important.⁷¹⁻⁷³ In contrast to microvessel counts from areas located at the invasive front, intratumoral MVD was not a prognostic factor in this study, although the ratio between counts from central areas to those at the base increased with tumor thickness.

Theoretically, the relationship between vessel counts by a marker more selective for activated and proliferating endothelial cells, like CD105,^{63,64} and vessel counts by panendothelial markers, such as F-VIII or CD34, could give additional information on the angiogenic status of a tumor. One study of breast cancer found the CD105 counts to be of stronger prognostic importance than counts by CD34.⁷⁴ We found that an increased CD105/F-VIII ratio was related to increased tumor thickness and presence of p53 staining. One possible explanation is that the relative amount of normal vessels entrapped by the tumor is higher in smaller and p53 negative tumors, whereas the proliferating tumor-associated vessels

Figure 5. Survival curves were estimated according to the Kaplan-Meier method with death due to melanoma as end point. **a:** Survival by MVD using the CD105 antibody. **b:** Survival by TSP-1 expression.

(CD105 positive) are more numerous in larger and p53-positive lesions. However, no significant association was present between patient prognosis and the amount of CD105-positive vessels relative to the amount of F-VIII-positive vessels. Thus, in malignant melanoma, CD105 gives limited additional information to F-VIII.

It has recently been suggested that highly invasive and metastatic melanoma cells are capable of generating vascular channels that might facilitate tumor perfusion, without the involvement of endothelial cells,^{75,76} although the conclusions are disputed by others.⁷⁷ If this property is true for cutaneous melanoma, it might explain the presence of thick, highly proliferative and aggressive cases with low MVD by counting vessels lined with differentiated endothelial cells. Interestingly, our cases showed significantly lower counts of F-VIII positive ves-

sels within the tumor than in the periphery, and still no or minimal necrosis in most cases.

In addition to being a mitogen and permeability factor,¹⁴ VEGF is also a survival factor for endothelial cells,⁷⁸ and the regulation of VEGF expression, as well as the influence of VEGF on tumor vasculature, seems to be complex.^{58,79} In melanocytic lesions, increased expression of VEGF has been associated with malignant progression.^{11,21,22} In accordance with this, we find that practically all vertical growth phase melanomas express VEGF to some extent. However, the level of VEGF expression was significantly, but inversely, related to tumor thickness and MVD. This finding suggests that VEGF might be up-regulated in some smaller tumors, whereas a lower level of expression was found in thicker and more vascularized tumors. In the latter lesions, a lower baseline level of VEGF might be sufficient for the maintenance of an established vascular system, with VEGF acting as a survival factor for newly formed endothelial cells.⁸⁰ One study suggests that the constitutive level of VEGF is more important than the hypoxic up-regulation of VEGF in melanoma angiogenesis.⁸¹ Alternatively, other angiogenic factors, such as bFGF, IL-8, and ephrins, may be more relevant for the vascular phenotype of this subgroup.^{82,83} A recent experimental study indicated that the angiogenesis in poorly angiogenic melanomas was promoted solely by VEGF, whereas multiple angiogenic factors were involved in the angiogenesis of highly angiogenic melanomas.⁸⁴

To be available to endothelial cells, VEGF must be secreted as freely diffusible proteins (VEGF₁₂₁, VEGF₁₆₅), or modified by protease activation and cleavage of the longer isoforms.¹⁴ In a limited number of cases, VEGF₁₈₉-positive tumors tended to have a lower MVD when compared with the others. This might be in accordance with our immunohistochemical findings, although in discordance with some results on other tumor types.^{26,27,85} Our findings should be interpreted with care due to the small number of positive cases, and limited mRNA quality in paraffin embedded tumor material.

Increasing evidence support the expression and functional importance of VEGF receptors in cell types other than endothelial cells^{30,32,33,35,86-88}. We found that VEGF receptors FLT-1 and KDR were present at various levels in tumor cells in most of the cases, and both receptors were significantly co-expressed with VEGF. This might suggest the presence of possible autocrine loops, and we found a significant association between KDR expression and tumor cell proliferation as estimated by Ki-67 staining in the subgroup of thicker tumors (above median). Others have discussed the presence of such autocrine loops in various tumors^{30,32,33,35,88}, and some functional evidence have been published showing increased proliferation of KDR expressing cells,^{36,89} or decreased proliferation of FLT-1 expressing cells^{37,89} in response to VEGF. Further, increased expression of matrix metalloproteinases and increased invasiveness were found after VEGF stimulation of FLT-1 expressing smooth muscle cells.⁸⁷ The VEGF system might therefore be important for various processes involving other cell types, including tumor cells, in addition to its influence on en-

Table 4. Estimated 5- and 10-Year Survival for Patients with VGP Melanoma

Variables	No. of patients	Estimated survival rates, % (SE)		P Value*
		5 years	10 years	
MVD (F-VIII)				
Low [†]	126	72 (4.4)	60 (5.4)	0.06
High	62	54 (7.6)	49 (8.2)	
MVD (CD105)				
Low [†]	86	73 (5.5)	58 (7.6)	0.04
High	84	57 (5.6)	51 (6.0)	
VEGF expression [§]				
Weak/absent (<4)	60	61 (5.6)	58 (7.6)	0.12
Moderate/strong (≥4)	127	70 (5.2)	61 (6.4)	
Flt-1 staining [§]				
Weak/absent (<4)	96	67 (5.1)	58 (7.3)	0.8
Moderate/strong (≥4)	82	63 (6.2)	51 (7.0)	
KDR staining ^{§¶}				
Weak/absent (<4)	51	58 (7.4)	49 (8.0)	0.8
Moderate/strong (≥4)	133	69 (4.5)	58 (5.7)	
TSP-1 expression [§]				
Weak/absent (<2)	103	75 (4.6)	68 (5.8)	0.0001
Moderate/strong (≥2)	77	51 (6.5)	35 (8.4)	

Product-limit method using death from melanoma as end point.

*Log rank test.

[†]Cut point at the 67th percentile.

[‡]Cut point at the median value.

[§]Staining index.

[¶]In tumor cells.

dothelial cell proliferation, migration, and differentiation.⁹⁰

Staining of thrombospondin-1 protein was mainly found in the tumor stroma, whereas TSP-1 mRNA was detected in the nuclei of both tumor cells, stromal cells and inflammatory cells. The stromal expression of TSP-1 protein was significantly associated with predictors of aggressive tumor behavior, like increased thickness and level of invasion, high proliferative rate (Ki-67), high MVD, tumor ulceration, vascular invasion, altered p53 and p16, as well as decreased survival. Several *in vitro* studies provide evidence that TSP-1 is a suppressor of angiogenesis and tumor progression,⁴³⁻⁴⁹ but the correlation

between *in vitro* experiments and *in vivo* studies of angiogenesis appears to be complex.⁹¹ Recent evidence published by Taraboletti and colleagues show that the 25-kd fragment of TSP-1 potentiates the proangiogenic effect of FGF-2, whereas the 140-kd fragment inhibits FGF-2 induced angiogenesis,⁵¹ further suggesting the importance of environmental settings for the dual role of TSP-1 in angiogenesis. Our results may be in agreement with reports suggesting a proinvasive^{40,41} and prometastatic³⁹ effect of matrix bound TSP-1, possibly through increased attachment to vessel walls,^{39,92} mediated by interactions with different TSP-1 receptors on tumor cells.^{93,94} This mechanism could also explain the asso-

Table 5. Multivariate Survival Analysis for Patients with VPG Melanoma, using Death as End-Point

Variable	Categories	n	HR*	P Value [†]
Anatomic site	Other [‡]	44	1	0.0001
	Trunk	126	3.3	
Level of invasion (Clark)	II, III, IV	141	1	<0.0001
	V	29	5.7	
p16 expression	Absent/minimal [§]	77	3.2	0.0001
	Moderate/strong	93	1	
p53 expression	Absent [¶]	24	1	0.004
	Moderate/strong	146	18.9	
Ki-67 expression	≤16%	43	1	0.02
	>16%	127	2.7	
Vascular invasion	Absent	136	1	0.01
	Present	34	2.2	
MVD (CD105)	Low**	86	1	0.04
	High	84	1.7	

According to the proportional hazards method. Only patients with information on all variables were included (n = 170).

*Hazard ratio.

[†]L ratio test.

[‡]Head/neck/extremities.

[§]Staining index ≤1.

[¶]Staining index = 0.

^{||}Lower quartile.

**Median value.

ciation with vascular invasion. Other studies have shown a proproliferative effect of TSP-1 on tumor cells, in accordance with our findings.^{93,95}

As recently reported, TSP-1 protein, immobilized in the extracellular matrix, might stimulate endothelial cell proliferation through the $\alpha 3 \beta 1$ integrin,⁵⁰ which is considered to be a major TSP-1 binding integrin on human endothelial cells. This may be in accordance with our present findings. Another TSP-1 receptor, of particular interest in melanomas, is the $\alpha v \beta 3$ integrin, and increased tumor cell expression of this multiligand receptor has been associated with progression and metastasis in cutaneous melanomas.^{96–99}

Presence of nuclear p53 staining, as an indication of altered p53 function, was associated with increased MVD, as well as increased expression of TSP-1. We were not able to show any association between lower MVD and increased expression of TSP-1 in tumor cells with normal p53 status (no staining), as found in experimental models and some clinical studies,^{44,45,47} and our findings indicate that other regulators of TSP-1 than p53 might be involved.

The relationship between level of TSP-1 protein expression and MVD was different in ulcerated and non-ulcerated tumors, indicating a possible interaction with angiogenic factors associated with ulceration. Presence of tumor ulceration was related to increased stromal TSP-1 staining and TSP-1 mRNA expression in tumor cells and fibroblasts, suggesting the possibility that TSP-1 might be induced by growth factors involved in wound healing, which might be of further importance for the progression and poor prognosis in this subset of melanomas.

In conclusion, MVD provided independent prognostic information in this series of cutaneous melanomas, without being a very strong prognostic factor. The angiogenic endothelial cell marker CD105 stained significantly fewer vessels than the panendothelial marker Factor-VIII antibody, but gave only limited information in addition to the latter. VEGF and its receptors FLT-1 and KDR were significantly co-expressed at various levels in tumor cells, suggesting possible autocrine or paracrine loops. Increased VEGF expression in tumor cells was significantly more frequent in thinner and less vascularized tumors, whereas the thicker and more vascularized lesions consistently showed a lower baseline level of expression. TSP-1 expression in tumor stroma was significantly associated with several markers of aggressive tumor behavior, reduced patient survival, and increased MVD, suggesting an important role for TSP-1 in melanoma progression and metastasis.

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