Human Melanoma Cells Secrete and Respond to Placenta Growth Factor and Vascular Endothelial Growth Factor

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The vascular endothelial growth factor is produced by a large variety of human tumors, including melanoma, in which it appears to play an important role in the process of tumor-induced angiogenesis. Little information is available on the role of placenta growth factor, a member of the vascular endothelial growth factor family of cytokines, in tumor angiogenesis, even though placenta growth factor/vascular endothelial growth factor heterodimers have been recently isolated from tumor cells. To investigate the role of placenta growth factor and vascular endothelial growth factor homodimers and heterodimers in melanoma angiogenesis and growth, 19 human melanoma cell lines derived from primary or metastatic tumors were characterized for the expression of these cytokines and their receptors. Release of placenta growth factor and vascular endothelial growth factor polypeptides into the supernatant of human melanoma cells was demonstrated. Reverse transcriptase polymerase chain reaction analysis showed the presence of mRNAs encoding at least three different vascular endothelial growth factor isoforms ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$) and transcripts for two placenta growth factor isoforms (PlGF-1 and PlGF-2) in human melanoma cells. In addition, placenta growth factor expression in human melanoma *in vivo* was detected by immunohistochemical staining of tumor specimens. Both primary and metastatic melanoma cells were found to express the mRNAs encoding for vascular endothelial growth factor and placenta growth factor receptors (KDR, Flt-1, neuropilin-1, and neuropilin-2), and exposure of melanoma cells to these cytokines resulted in a specific proliferative response, supporting the hypothesis of a role of these angiogenic factors in melanoma growth. ***Key words: angiogenesis/melanoma progression. J Invest Dermatol 115:1000–1007, 2000***

The growth of solid tumors and the development of metastases are greatly favored by the angiogenic process. Actually, the vascular density of a number of different types of primary tumors is predictive for their metastatic potential (Gasparini, 1997). Several factors produced by cancerous cells influence the angiogenic process (Folkman, 1995). Among them, the vascular endothelial growth factor (VEGF) is produced by a variety of normal and neoplastic cells and acts as an endothelial-cell-specific mitogen and permeability factor (for review see Neufeld *et al*, 1999). At least five VEGF species of 121 ($VEGF_{121}$), 145 ($VEGF_{145}$), 165 ($VEGF_{165}$), 189 ($VEGF_{189}$), and 206 ($VEGF_{206}$) amino acids are generated by alternative mRNA splicing from a single gene (Neufeld *et al*, 1999) and characterized by different biologic properties (Cohen *et al*, 1995; Poltorak *et al*, 1997).

Placenta growth factor (PlGF), a secreted dimeric protein of the VEGF family, shares a 53% sequence identity with the platelet-derived growth factor (PDGF)-like region of VEGF (Maglione *et al*, 1991). Similarly to VEGF, alternative splicing of PlGF mRNA produces at least three polypeptides of 149 (PlGF-1), 170 (PlGF-2), and 221 (PlGF-3) amino acids (Maglione *et al*, 1993; Cao *et al*, 1997). A highly basic 21 amino acid insertion in the carboxi-terminal region of PlGF-2 sequence results in the high heparin-binding affinity of this polypeptide, whereas neither PlGF-1 nor PlGF-3 bind heparin. PlGF has been detected in a limited number of tissues and cell types (Clark *et al*, 1998) and its *in vivo* and *in vitro* angiogenic activity has been demonstrated only recently (Ziche *et al*, 1997). Naturally occurring VEGF/PlGF heterodimers have also been found in the supernatant of some tumor cell lines (DiSalvo *et al*, 1995; Cao *et al*, 1996b). VEGF proangiogenic activity is mediated by two tyrosine kinase receptors present on endothelial cells: the 180 kDa *fms*-like tyrosine kinase (Flt-1) and the 200 kDa kinase-insert-domain-containing receptor (KDR) (Neufeld *et al*, 1999). The binding of VEGF to KDR has been correlated with endothelial cell proliferation (Millauer *et al*, 1993), whereas Flt-1 activation seems to result in the enhancement of cell migration (Waltenberger *et al*, 1994; Barleon *et al*, 1996; Clauss *et al*, 1996). The role of Flt-1 in other biologic activities triggered by VEGF is still controversial. A soluble form of Flt-1 (sFlt-1), lacking part of the extracellular domain, the transmembrane, and intracellular regions (Kendall and Thomas, 1993), has been cloned. sFlt-1 binds VEGF and inhibits its mitogenic activity in vascular endothelial cells by impairing VEGF binding to KDR (Kendall *et al*, 1993). PlGF shares with VEGF the capability to bind with high affinity the Flt-1 receptor (Sawano *et al*, 1996), whereas PlGF/VEGF

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Abbreviations: Flt-1, *fms*-like tyrosine kinase; sFlt-1, soluble *fms*-like tyrosine kinase; ITS, insulin-transferrin-sodium selenite medium supplement; KDR, kinase-insert-domain-containing receptor; PlGF, placenta growth factor; VEGF, vascular endothelial growth factor.

heterodimers are also able to activate KDR (DiSalvo *et al*, 1995; Cao *et al*, 1996b). More recently, $VEGF_{165}$ and PlGF-2 have been shown to bind also to neuropilin-1 and neuropilin-2, which function as receptors for axon guidance factors of the semaphorin family (Soker *et al*, 1998; Gluzman-Poltorak *et al*, 2000). Nevertheless, both neuropilin receptors lack tyrosine kinase activity, and binding of $VEGF_{165}$ to porcine aortic endothelial cells expressing only neuropilin-1 or neuropilin-2 did not affect cell proliferation or migration (Soker *et al*, 1998; Gluzman-Poltorak *et al*, 2000).

The role of VEGF in the process of tumor-induced angiogenesis is well established (reviewed by Ferrara, 1995), and several studies have focused on the intriguing possibility that VEGF could also directly affect tumor cell proliferation. VEGF tyrosine kinase receptors (Flt-1 and KDR) have been found in several human tumor cells, such as ovarian carcinoma (Boocock *et al*, 1995), AIDS-Kaposi sarcoma (Masood *et al*, 1997), prostate carcinoma (Ferrer *et al*, 1999), colon carcinoma (André *et al*, 2000), and melanoma (Gitay-Goren *et al*, 1993; Graeven *et al*, 1999).

In a melanoma cell line, the expression of KDR receptor has been correlated with a proliferative response to exogenously added VEGF (Liu *et al*, 1995). In this report, however, the role played by the other VEGF receptors as well as the production of PlGF by melanoma cells were not analyzed. We were interested in studying the possible role of PlGF in melanoma angiogenesis and progression. To this end, we have characterized a wide variety of primitive and metastatic human melanoma cell lines for the expression of VEGF, PlGF, and their receptors, analyzed the *in vivo* expression of PlGF in cutaneous melanomas, and evaluated the effect of exogenously added VEGF and PlGF on melanoma cell proliferation.

MATERIALS AND METHODS

Materials Culture media and supplements were purchased from Euroclone (Oud-Beijerland, Holland), except for the 0.85 g per liter $NaHCO_3$/minimum essential medium to grow WM115 cells, which was obtained from Gibco/BRL (Paisley, U.K.). Insulin-transferrin-sodium selenite medium supplement (ITS) and heparin were from Sigma (St. Louis, MO). Fatty-acid-free bovine serum albumin (BSA) was from Boehringer Mannheim (Mannheim, Germany). VEGF and PlGF homodimers and heterodimers used as standards in the enzyme-linked immunosorbent assay (ELISA) and as stimuli in the mitogenic assays were from R&D Systems (Abingdon, U.K.); $rhVEGF_{165}$ homodimer was expressed and purified from Sf21 insect cells; PlGF-1 was expressed in *Escherichia coli*; VEGF/PlGF heterodimer was originated by expression in *E. coli* of $VEGF_{165}$ and PlGF-1, followed by *in vitro* dimerization. Goat antibodies used in the ELISA analysis (R&D Systems) recognized all the VEGF or PlGF isoforms. The monoclonal antibodies used to neutralize VEGF (MAB 293) or PlGF (MAB 264) stimulated cell growth were also from R&D Systems. The goat polyclonal antibody raised against human PlGF and utilized for the immunohistochemical analysis was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines and normal human cells Nineteen human melanoma cell lines of primary or metastatic origin were used. Six were established in our laboratory (GR-Mel, ST-Mel, SN-Mel, PR-Mel, CN-Mel, and TVMBO). SK-Mel-28 (Shiku *et al*, 1976), WM115, and WM266-4 (Satyamoorthy *et al*, 1997) were purchased from the American Type Culture Collection (ATCC, Rockville, MD), and the remaining cell lines were generous gifts from other laboratories: 13443-Mel (Colombo *et al*, 1992) from Dr. G. Parmiani (Istituto Nazionale Tumori, Milan, Italy); PD-Mel, PNP-Mel, PNM-Mel, LCP-Mel, LCM-Mel, and GL-Mel from Dr. F. Guadagni (Istituto Regina Elena, Rome, Italy); M14 (Golub *et al*, 1976) from Dr. G. Zupi (Istituto Regina Elena); LB-24 (Wolfel *et al*, 1993) and 397-Mel (Topalian *et al*, 1989) from Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). Melanoma cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 μg per ml gentamycin, except for the ATCC cell lines WM115 and WM266-4, which were kept in culture as suggested by the supplier. Five of these cell lines were obtained from primary tumors (GR-Mel, ST-Mel, WM115, LCP-Mel, and PNP-Mel), six from cutaneous metastases (SN-Mel, PR-Mel, M14, 397-Mel, LB-24, and WM266–4), and eight originated from noncutaneous metastases, including lymph nodal and organ tissue metastases (CN-Mel, PD-Mel, 13443-Mel, PNM-Mel, LCM-Mel, GL-Mel, SK-Mel-28, and TVMBO).

Normal human melanocytes were isolated from human skin biopsies at the IDI-IRCCS laboratory of Dr. M. De Luca (Pomezia, Italy) and in our own laboratory, as previously described (De Luca *et al*, 1988), NIH/3T3 murine fibroblasts and RAJI (human Burkitt's lymphoma) cells were purchased from the ATCC, and the human microvascular endothelial cell line HMEC-1 was a generous gift of Dr. F.J. Candal (Centers of Disease Control and Prevention, Atlanta, GA) (Ades *et al*, 1992). Normal human keratinocytes were obtained from foreskins of healthy males, and cultured on a feeder layer of lethally irradiated 3T3-J2 murine fibroblasts (a gift from Dr. H. Green, Harvard Medical School, Boston, MA), as previously described (Zambruno *et al*, 1995).

Preparation of conditioned medium and cell extracts for quantification of VEGF and PlGF polypeptides Conditioned media from human melanoma cells and normal human melanocytes in culture were obtained by incubating semiconfluent cell cultures for 24 h in 0.1% BSA/RPMI medium without fetal bovine serum. These conditions did not significantly affect cell viability, and the percentage of adherent cells was maintained between 93% and 99%. Supernatants were concentrated at least 10-fold in Centriplus concentrators (Amicon, Beverly, MA). Cells were detached from the flasks with phosphate-buffered saline (PBS)/ethylenediamine tetraacetic acid (EDTA) or, when necessary, with trypsin/EDTA. Cytokine secretion values were normalized by the total number of cells. To quantify unreleased VEGF or PlGF, cell extracts were prepared by washing cells once in PBS and resuspending them in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM AEBSF, 1% Nonidet P-40, pH 8.0). After 30 min of incubation on ice, cell lysates were centrifuged and supernatants were directly used for ELISA analysis.

ELISA Quantification of the amount of VEGF and PlGF homodimers and heterodimers in the conditioned medium and cell extracts was performed as previously described (Harlow and Lane, 1988) using goat anti-VEGF or anti-PlGF IgGs (R&D Systems) at a concentration of 10 μg per ml in PBS to coat Maxisorp Nunc immunoplates (Nunc, Roskilde, Denmark). Detection of the cytokines was performed with biotinylated goat anti-VEGF or anti-PlGF IgGs (R&D Systems) and streptavidin-alkaline phosphatase conjugate (1:10,000) (Boehringer Mannheim). For the detection of VEGF/PlGF heterodimers, plates were coated with anti-VEGF antibody and detection was carried out with biotinylated goat anti-PlGF IgGs. The reaction was stopped and optical density at 405 nm was measured in a Microplate reader 3550-UV (Bio-Rad, Hercules, CA). This assay allowed detection of VEGF and PlGF polypeptides at concentrations equal to or greater than 100 pg per ml.

Immunohistochemical analysis Sections of melanoma specimens 4 μm thick were deparaffinized, rehydrated, and treated for 20 min with 0.3% H_2O_2 in PBS. Sections were preincubated with 3% BSA/PBS for 1 h, and then incubated with the C-20 anti-PlGF antibody (Santa Cruz Biotechnology) at a concentration of 4 μg per ml for 2 h at room temperature in 3% BSA/PBS. Specimens were subsequently treated for 45 min with biotinylated antigoat IgGs (1:150) (Vector Laboratories, Burlingame, CA) diluted in 3% BSA/PBS, and with peroxidase-conjugated avidin (Vectastain *Elite* ABC kit, Vector Laboratories) for 1 h. Immunoreactivity was visualized by peroxidase reaction, using 3-amino-9-ethyl carbazole (AEC, Vector Laboratories) in H_2O_2 as substrate, and specimens were counterstained with hematoxylin. Negative controls were obtained by omitting the primary antibody. In a previous analysis on PlGF expression during wound healing (Failla *et al*, 2000) we demonstrated the suitability of the Santa Cruz polyclonal antibody sc-1880 (C-20) for immunohistochemistry, checking its specificity by Western blotting against human recombinant VEGF and PlGF, and confirming the data by *in situ* hybridization analysis.

The study was approved by the Ethical Committee of the Istituto Dermopatico dell'Immacolata, IDI-IRCCS, Rome, Italy.

Reverse transcriptase polymerase chain reaction (RT-PCR) Total cellular RNA from the different cell lines and cultured normal human cells (melanocytes and keratinocytes) was prepared using an RNeasy Midi kit from Qiagen (Hilden, Germany), following the manufacturer's directions. Three micrograms of total RNA per sample were used for reverse transcription by the AMV enzyme (Boehringer Mannheim), for 60 min at 42°C, in 25 μl. Five microliters of this cDNA preparation were used for each PCR amplification reaction by 1 U of Dynazyme II DNA polymerase (Finnzymes OY, Espoo, Finland), utilizing the following primers and annealing conditions: forward primer 5′-CCATGAACTTTCCTG-

CTGTCTT-3′ and reverse primer 5′-TCACCGCCTCGGCTTGTC-3′ for VEGF (annealing for 30 s at 60°C); forward primer 5′-CTCCTA-AAGATCCGTTCTGG-3′ and reverse primer 5′-GGTAATAAATAC-ACGAGCCG-3′ for PlGF (annealing for 30 s at 55°C); forward primer 5′-CACAGGAAACCTGGAGAATCAGACGACAAG-3′ and reverse primer 5′-TGGTCGACCATGACGATGGACAAGTA-3′ for KDR (annealing for 1 min at 58°C); forward primer 5′-GAAGGAAGGGAG-CTCGTCATTC-3′ and reverse primer 5′-TACCATATGCGGTACAA-GTCAGG-3′ for the extracellular Flt-1 region (annealing for 1 min at 60°C); forward primer 5′-CAAGTGGCCAGAGGCATGGAGTT-3′ and reverse primer 5′-GATGTAGTCTTTACCATCCTGTTG-3′ for the Flt-1 carboxi-terminal end (annealing for 1 min at 60°C); forward primer 5′-ATGGAGAGGGGGCTGCCG-3′ and reverse primer 5′-CTATCGCG-CTGTCGGTGTA-3′ for neuropilin-1 (annealing for 30 s at 52°C); forward primer 5′-ATGGATATGTTTCCTCTCACC-3′ and reverse primer 5′-GTCCAGCCAATCGTACTTGC-3′ for neuropilin-2 (annealing for 30 s at 55°C). RNA integrity and the correct reverse transcription of the samples were assessed by testing each cDNA preparation for the amplification of the housekeeping gene glyceraldehyde-phosphate dehydrogenase (GAPDH). Controls were performed excluding AMV reverse transcriptase from the reactions. Amplification products obtained after RT-PCR were purified using the QIAquick gel extraction kit (Qiagen), and sequenced using the dye terminator cycle sequencing kit, in an Applied Biosystems PRISM 377 DNA Sequencer (Perkin Elmer, Foster City, CA).

Mitogenic assay The capability of VEGF and PlGF homodimers and heterodimers to stimulate human melanoma cell growth was tested by measuring cellular methyl-^{3}[H]-thymidine uptake or by counting the number of cells following incubation with these growth factors. For the methyl-^{3}[H]-thymidine uptake experiments, cells were seeded on 96-well plates (3×10^3–5×10^3 cells per well, depending on the cell line), in 100 μl of complete RPMI medium, and kept overnight at 37°C in a CO_2 incubator. Subsequently, medium was changed with serum-free RPMI containing 0.1% BSA and cells were maintained at 37°C for two further days. Medium was then replaced with RPMI containing 0.1% BSA, 1 μg per ml heparin, and one of the growth factors or the medium supplement ITS, in quadruplicate samples. Samples with only BSA/heparin medium were included to determine basal radioactivity uptake. After 15 h incubation, 1 μCi of methyl-^{3}[H]-thymidine (73 Ci per mmol, Amersham, Buckinghamshire, U.K.) was added to each well, and the plates were further incubated for 5 h. Labeled cells were transferred to Unifilter GF/C microplates (Canberra Packard, Meriden, CT) and the excess of nonincorporated thymidine was washed out, using a Filtermate 196-cell harvester (Canberra Packard). Radioactivity retained in the filter plates was then counted in a Top-Count microplate scintillation counter (Canberra Packard). The specific inhibition of VEGF- or PlGF-induced methyl-^{3}[H]-thymidine uptake was performed by preincubating these cytokines or ITS (as negative control) with the respective monoclonal antibodies, for 1 h at room temperature. Preincubation of the cytokines with a control monoclonal antibody (anti-HLA DP from Becton & Dickinson, San Jose, CA) was carried out simultaneously.

For direct cell number counting, cells were seeded in 24-well plates (2×10^4 cells per well), allowed to attach to the plastic overnight, and serum-starved in 0.1% BSA/RPMI for 24 h before stimulation. Stimuli (10 ng per ml of the cytokines and 5 μg per ml ITS as control) were added in the presence of 1 μg per ml heparin in 0.1% BSA/RPMI. Seventy-two hours after the addition of the different cytokines, cells were detached from the plate by incubating with PBS/EDTA, and viable cells were counted.

RESULTS

***In vitro* secretion of VEGF and PlGF homodimers and VEGF/PlGF heterodimers by human melanoma cells** Cell culture supernatants were collected to quantify, in an enzyme-linked immunoassay, the amount of VEGF and PlGF homodimers and VEGF/PlGF heterodimers secreted by different human melanoma cell lines (**Table I**). Normal human keratinocytes, secreting both VEGF and PlGF polypeptides (Ballaun *et al*, 1995; Failla *et al*, 2000), were used as positive control, and mouse NIH/3T3 fibroblasts were employed as negative control.

VEGF homodimers (VEGF/VEGF) were detected in the supernatant of all the metastatic melanoma cell lines, at concentrations ranging between 298 and 5743 pg per 10^6 cells. Among the primary melanoma cells, three out of five cell lines (ST-Mel, LCP-Mel, and PNP-Mel) secreted VEGF at levels ranging between 1000 and 1800 pg per 10^6 cells, whereas the other two cell lines did not release (WM115) or released extremely low levels (GR-Mel) of VEGF/VEGF. PlGF homodimers (PlGF/PlGF) were also secreted by human melanoma cells (**Table I**), although at lower levels than VEGF/VEGF. In 10 out of 14 metastatic cell lines PlGF/PlGF were released at concentrations ranging from 22 to 454 pg per 10^6 cells. On the other hand, only two out of five cell lines from primary melanomas secreted medium to low levels of PlGF (PNP-Mel, 152 pg per 10^6 cells, and LCP-Mel, 19 pg per 10^6 cells). VEGF/PlGF heterodimers were detected in the supernatant of human melanoma cells as well (**Table I**), following the pattern of PlGF secretion. As VEGF is generally produced in large amounts by melanoma cells, the PlGF synthesis level could represent the limiting factor for heterodimer assembly. Among the melanoma cell lines utilized here, three pairs of cell lines from primary and metastatic tumors from the same patient were analyzed. The WM115 cell line (originated from the primary tumor of a patient showing the first metastases 9 mo after removal of the primary lesion) did not release detectable VEGF or PlGF homodimers. Its metastatic counterpart, the WM266-4 cell line, obtained from a skin metastasis 18 mo later, secreted the highest levels of VEGF and PlGF homodimers and heterodimers. Otherwise, when primary and metastatic tumors were surgically removed simultaneously (cell line pairs LCP-Mel/LCM-Mel and PNP-Mel/PNM-Mel), levels of VEGF and PlGF released by cell lines from the primary lesions and their metastatic counterparts did not differ substantially. Neither PlGF/PlGF nor VEGF/PlGF were detected in the

Table I. Release of VEGF homodimer, PlGF homodimer, and VEGF/PlGF heterodimer by human melanoma cells and normal human melanocytes in culture

	VEGF/VEGF[a]	VEGF/PlGF[a]	PlGF/PlGF[a]
Controls			
NIH/3T3	ND[b] (5)[c]	ND	ND
Keratinocytes[d]	422 ± 36 (3)	50 ± 11	106 ± 5
Melanocytes			
MK54	ND (3)	ND	ND
MK57	54 ± 31 (4)	ND	ND
MK291	ND (3)	ND	ND
Primary melanomas			
GR-Mel	10 ± 10 (4)	ND	ND
ST-Mel	1842 ± 276 (5)	9 ± 6	ND
WM115	ND (4)	71 ± 20	ND
LCP-Mel	1563 ± 263 (5)	ND	19 ± 7
PNP-Mel	1030 ± 146 (4)	176 ± 30	152 ± 13
Cutaneous metastatic melanomas			
PR-Mel	325 ± 62 (4)	32 ± 11	38 ± 14
SN-Mel	759 ± 66 (4)	22 ± 11	5 ± 5
M14	635 ± 56 (6)	5 ± 5	ND
397-Mel	1052 ± 155 (4)	45 ± 15	81 ± 12
LB-24	298 ± 31 (4)	205 ± 18	335 ± 46
WM266-4	5743 ± 599 (3)	1142 ± 5	454 ± 77
Noncutaneous metastatic melanomas			
TVMBO	3570 ± 475 (4)	100 ± 34	117 ± 16
PD-Mel	628 ± 68 (4)	ND	ND
13443-Mel	792 ± 116 (4)	148 ± 26	293 ± 58
LCM-Mel	2338 ± 387 (4)	27 ± 9	22 ± 6
PNM-Mel	608 ± 11 (4)	97 ± 9	114 ± 14
GL-Mel	1224 ± 74 (4)	23 ± 3	66 ± 12
CN-Mel	1691 ± 102 (4)	8 ± 5	5 ± 5
SK-Mel-28	598 ± 72 (4)	100 ± 29	82 ± 16

[a]Values are expressed as pg per 10^6 cells, as measured by the enzyme linked immunoassay described under *Materials and Methods*, and represent the arithmetic mean ± the standard error of the mean.

[b]ND, not detectable.

[c]The number of independent determinations is given in parentheses.

[d]The results represent the arithmetic mean ± the standard error of the mean for three different cultures of human keratinocytes.

supernatant of cultured melanocytes from three different healthy donors (**Table I**), and only one of them released quite low levels of VEGF/VEGF (54 pg per 10^6 cells). The possibility that culture conditions could affect VEGF and/or PlGF secretion levels was further analyzed. Similar amounts of PlGF/PlGF or VEGF/PlGF were secreted by melanoma cells cultured in the presence or the absence of serum or in the melanocyte culture medium (data not shown). Levels of VEGF/VEGF released, however, were increased up to 50% when cells were maintained in the presence of serum or in the melanocyte culture medium, compared with cells cultured in 0.1% BSA/RPMI (data not shown).

Detection of VEGF and PlGF isoforms expressed by human melanoma cells VEGF and PlGF isoform expression was analyzed in human melanoma cells by RT-PCR, and the patterns obtained were compared with those of the positive control normal human keratinocytes (**Fig 1**). NIH/3T3 cells were used as negative control. The pattern obtained in human melanoma cells included the same four bands shown in human keratinocytes (**Fig 1**): $VEGF_{121}$ (442 bp), $VEGF_{165}$ (575 bp), $VEGF_{189}$ (647 bp), and an additional unidentified 514 bp fragment. The size of this band indicated that it could correspond either to $VEGF_{145}$, which contains the exons 1–5, 6a, and 8 (Poltorak *et al*, 1997), or to an artifactual heteroduplex DNA formed from the $VEGF_{121}$ and $VEGF_{165}$ amplification products during PCR amplification (Eckhart *et al*, 1999). Surprisingly, VEGF transcript expression was also strong in the cell line GR-Mel, which secreted almost undetectable amounts of VEGF/VEGF and did not release VEGF/PlGF. The amplification products corresponding to the two major isoforms, $VEGF_{121}$ and $VEGF_{165}$, were also faintly detected in one of the normal melanocyte cultures (MK57).

PlGF isoforms were visualized as the 184 bp (PlGF-1) and 248 bp (PlGF-2) amplification products (Maglione *et al*, 1993). Both PlGF transcripts were detected in all the 14 melanoma cell lines positive for PlGF polypeptide secretion (**Fig 2**). Faint bands corresponding to PlGF-1 and PlGF-2 mRNA were observed in amplificates from three melanoma cell lines (ST-Mel, M14, and CN-Mel) that did not release appreciable amounts of the protein, either as homodimer or heterodimer, probably because of the lower sensitivity of the ELISA with respect to RT-PCR detection. Unexpectedly, GR-Mel cells, which do not release PlGF to the culture medium, were positive for PlGF amplificates. RT-PCR positivity for VEGF and PlGF in GR-Mel cells cannot be explained by a delayed cytokine release, because after as long as 6 d of culture VEGF and PlGF secretion was still undetectable (data not shown). Accumulation of the protein into a cellular compartment could not be detected in GR-Mel cell extracts (data not shown).

In vivo* PlGF expression in human melanoma specimens** To verify that the PlGF expression we observed in melanoma cells was not induced by culture conditions but was representative of the *in vivo* situation, we analyzed primary as well as metastatic melanomas by means of immunohistochemistry using an antihuman PlGF antibody (**Fig 2**). Sixteen primary cutaneous melanoma specimens (three, Breslow thickness ≤ 0.75 mm; four, Breslow thickness > 0.75–1.5 mm; four, Breslow thickness > 1.5–3.0 mm; five, Breslow thickness > 3.0 mm) and four skin metastases were analyzed (**Table II**). A low percentage ($< 20\%$) of PlGF-immunopositive melanoma cells was detected in one out of three primary tumors with Breslow thickness of 0.75 mm or less, whereas in the other two no immunolabeled tumor cells could be scored (**Fig 1*A). Cytoplasmic staining was observed in nine out of 13 primary tumors with Breslow thickness greater than 0.75 mm (**Fig 2*B*–*D***). These specimens showed different levels of immunopositivity, in terms of staining intensity and percentage of labeled tumor cells (see **Table II**). All the cutaneous metastases analyzed displayed PlGF immunoreactivity (**Fig 2*E***), although with different levels of expression. Interestingly, staining was also observed in the keratinocytes near melanoma cells, in both PlGF-negative and PlGF-positive melanoma specimens (**Fig 2*A*, *E***).

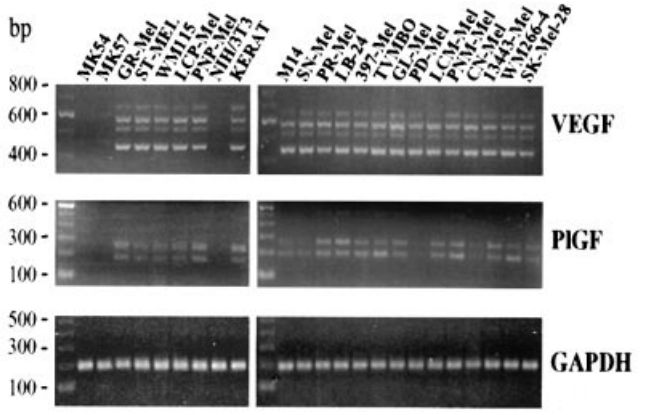

Figure 1. RT-PCR analysis of VEGF and PlGF isoform expression by human melanoma cells. Primers described in *Materials and Methods* were used to amplify cDNA obtained from each cell line. PCR for GAPDH was performed using the same cDNA preparation, to confirm integrity of the RNA samples. PCR products were separated in 2% agarose gels and a 100 bp DNA ladder (Gibco/BRL) was used as molecular weight marker. Results are representative of three different experiments.

Expression of VEGF and PlGF receptor genes in human melanoma cells The expression of receptors for VEGF and PlGF (KDR, Flt-1, neuropilin-1, and neuropilin-2) in the 19 melanoma cell lines was evaluated by RT-PCR analysis. **Figure 3** shows the results obtained for the tyrosine kinase receptors KDR and Flt-1. Normal human keratinocytes and the human microvasculature endothelial cell line HMEC-1 were used as negative and positive controls. PCR amplification of the KDR and Flt-1 cDNAs coding for the receptor extracellular domains resulted in the visualization of a 412 bp fragment and a 927 bp fragment, respectively. None of the three normal melanocyte cultures analyzed expressed either KDR or Flt-1 transcripts (two of them are shown in **Fig 3**), whereas both KDR and Flt-1 mRNAs were detectable in 16 out of the 19 melanoma cell lines tested. Among the three remaining melanoma cell lines, only M14 cells were negative for both receptors, whereas the PD-Mel cell line expressed Flt-1 but not KDR, and LCP-Mel cells expressed KDR but not Flt-1 transcript. The Flt-1 signal observed in **Fig 4** might represent the soluble form of this receptor (sFlt-1). Hence, RT-PCR amplification of a 497 bp fragment from the cDNA region coding for the Flt-1 C-terminus was performed (Masood *et al*, 1997). Flt-1 mRNA expression was confirmed in all but one of the melanoma cell lines (i.e., LCM-Mel cells), in which Flt-1 molecules expressed might therefore correspond to the soluble form of this receptor (data not shown).

Figure 4 shows the results relative to neuropilin-1 and neuropilin-2 mRNA expression. HMEC-1 cells were included as positive control for the expression of both receptors, and RAJI and NIH/3T3 cells were used as negative controls for neuropilin-1 and neuropilin-2, respectively (**Fig 4**). One of the melanocyte samples presented a weak neuropilin-1 signal, whereas the five cell lines originating from primary melanomas, as well as 13 out of the 14 cell lines from metastatic melanomas, were all positive. Similar results were obtained for neuropilin-2.

Effect of $VEGF_{165}$ and PlGF-1 homodimers and heterodimers on human melanoma cell proliferation The finding that human melanoma cell lines secrete PlGF and VEGF and express their receptors raises the possibility of an autocrine stimulation loop. To test this hypothesis, melanoma cell growth in response to exogenously added homodimeric or heterodimeric forms of $VEGF_{165}$ and PlGF-1 was evaluated. Growth factors were used at a concentration of 20 ng per ml, and the effect on melanoma methyl-^{3}[H]-thymidine uptake was compared with that of the cell growth supplement ITS (5 μg per ml). **Figures 5(*A*)** and **5(*B*)** show

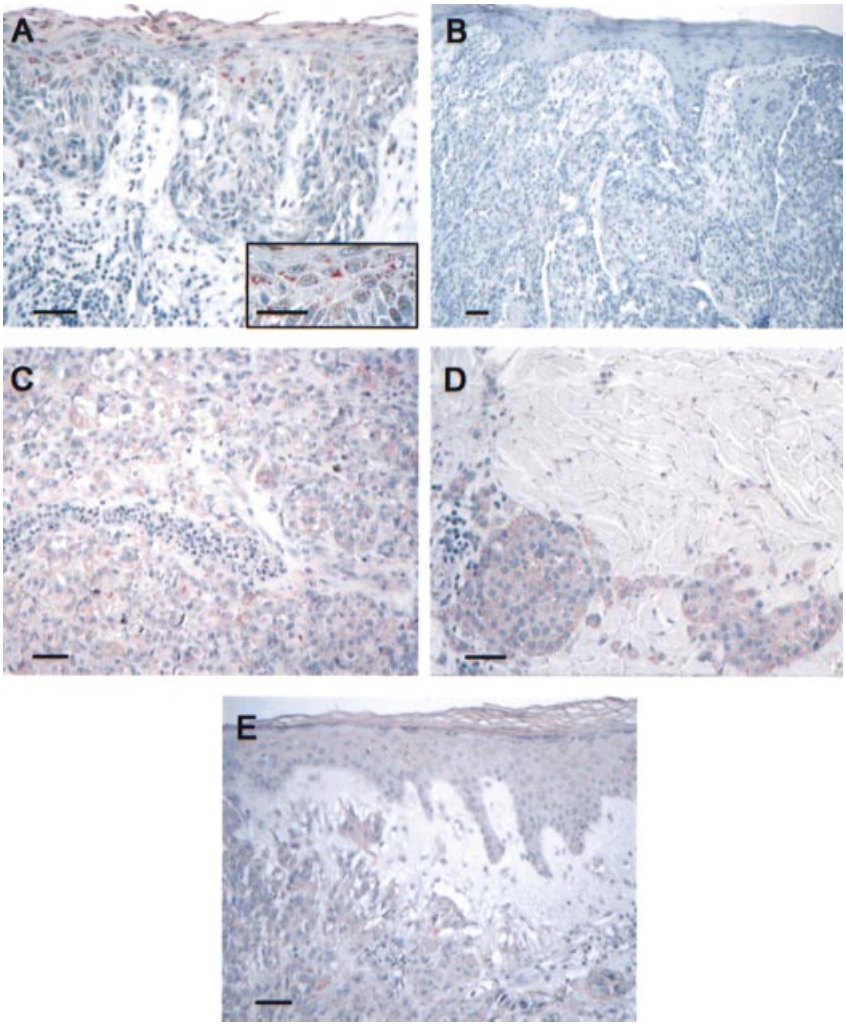

Figure 2. *In vivo* PlGF expression in primary melanoma and cutaneous metastases. (*A*) Primary cutaneous melanoma with a Breslow thickness of 0.4 mm, negative for PlGF. Some suprabasal keratinocytes in the epidermis show PlGF cytoplasmic staining with a predominant perinuclear localization (*inset*). (*B*) Primary cutaneous melanoma with a Breslow thickness of 2.7 mm, negative for PlGF. (*C*) Primary melanoma with a Breslow thickness of 5 mm, displaying PlGF cytoplasmic staining of tumor cells. (*D*) PlGF-positive melanoma cell nests in the deep dermis from the tumoral lesion shown in (*C*). (*E*) PlGF expression by tumor cells and epidermal keratinocytes in a cutaneous melanoma metastasis. *Scale bar*: 30 μm; *inset* in (*A*): 10 μm.

the results obtained with four representative melanoma cell lines. The absence of KDR and Flt-1 in M14 cells corresponded to the unresponsiveness of these cells to the VEGF and/or PlGF dimers, even if they expressed both neuropilin receptors and were stimulated by ITS (**Fig 5*A***). A 30%–50% increment in DNA synthesis was observed when melanoma cells expressing KDR and Flt-1 were stimulated by VEGF, PlGF, or PlGF/VEGF dimers (**Fig 5*A***), independently of the neuropilin-1 mRNA presence (i.e., GL-Mel cells). The stimulation rate obtained was in the same range as that described for VEGF by Liu *et al* (1995). The specificity of the response to the cytokines was tested by preincubation with antibodies anti-VEGF or anti-PlGF, which were then maintained in the culture medium during the experiment (**Fig 5*B***). A 70% inhibition of VEGF response was obtained using the anti-VEGF monoclonal antibody, whereas anti-PlGF monoclonal antibody completely abolished the response to PlGF. Stimulation by ITS was slightly modified by treatment with the specific antibodies (anti-VEGF and anti-PlGF, **Fig 5*B***). Interestingly, a significant reduction (about 20%) in basal methyl-^{3}[H]-thymidine uptake was observed when GL-Mel cells were incubated with anti-PlGF antibody, suggesting interference with an autocrine mechanism maintained by the melanoma-cell-produced PlGF (**Fig 5*B***). Conversely, anti-VEGF antibodies did not significantly affect basal cell growth (**Fig 5*B***), although the higher levels of endogenous VEGF retained in cell compartments in melanoma cells (result not shown) could be responsible for a reduced accessibility of VEGF proteins to the antibody. Cell growth was also analyzed by counting viable cells after 72 h in the presence of homodimeric or heterodimeric VEGF and PlGF or in the presence of ITS (**Fig 5*C***). The results confirmed the effect of VEGF and PlGF on melanoma cell proliferation, at levels comparable to those achieved by ITS.

DISCUSSION

The ability of cutaneous melanoma to induce angiogenesis (Denijn and Ruiter, 1993) and the critical role played by VEGF in melanoma progression (Erhard *et al*, 1997; Marcoval *et al*, 1997; Salven *et al*, 1997) are well established. By contrast, no data are available on PlGF expression and function in cutaneous melanoma. The practice of therapeutic inhibition of tumor angiogenesis, however, requires a detailed definition of the different angiogenic factors produced by tumor cells and an understanding of their relative biologic activity. We therefore decided to define the patterns of expression of VEGF, PlGF, and their receptors in human melanoma cell lines and to investigate whether these cytokines might be involved in an autocrine loop of cell growth stimulation. All but one of the human melanoma cell lines tested secreted moderate to high levels of VEGF. Here, we describe the expression in human melanoma cell lines of two PlGF isoforms (PlGF-1 and PlGF-2), and the release of PlGF polypeptides in the melanoma cell culture medium, as homodimer or heterodimer with VEGF. PlGF release appeared to be more frequent among cell lines derived from metastatic melanomas (ten of fourteen) than among cell lines derived from primary tumors (two of five, both of which were obtained from patients in an advanced stage of the disease, who had already developed lymph node metastases). Moreover, three of three normal human melanocyte cultures were

Table II. Expression of PlGF in cutaneous melanoma[a]

Specimen	Type	Breslow thickness	Clark level	PlGF immunoreactivity
11927/00	PM	0.3 mm	II	–
16762/99	PM	0.4 mm	II	–
12630/00	PM	0.6 mm	II	+/–
09890/00	PM	0.9 mm	IV	+/–
12711/99	PM	1.2 mm	IV	+
09713/00	PM	1.3 mm	IV	–
12467/00	PM	1.4 mm	IV	+
10840/00	PM	1.6 mm	IV	+/–
10486/99	PM	2.5 mm	IV	+/–
13044/00	PM	2.6 mm	IV	+
12765/99	PM	2.7 mm	IV	–
11521/00	PM	5 mm	IV	+
17630/99	PM	5 mm	IV	++
20693/99	PM	7 mm	IV	+/–
11270/00	PM	8 mm	IV	–
10564/00	PM	>10 mm	V	++
16261/99	CM			+
15345/99	CM			+
08340/99	CM			+/–
12747/00	CM			+/–

[a]PM, primary tumor; CM, cutaneous metastasis; ++, medium expression; +, low expression; +/– barely detectable expression or a few melanoma cells stained.

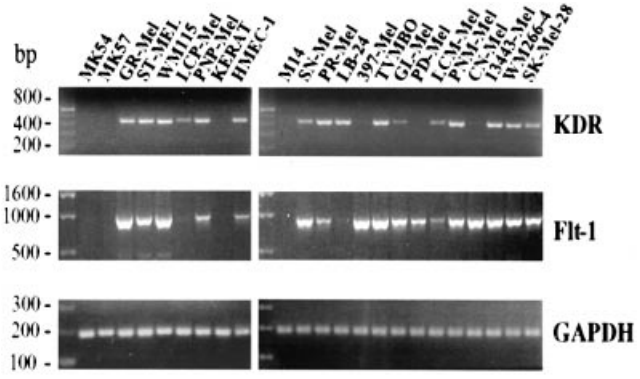

Figure 3. RT-PCR analysis of KDR and Flt-1 receptor expression by human melanoma cells. Primers described in *Materials and Methods* were used to amplify cDNA obtained from each cell line. PCR for GAPDH was performed using the same cDNA preparation to confirm integrity of the RNA samples. KDR and GAPDH PCR products were separated in 2% agarose gels, and a 100 bp DNA ladder (Gibco/BRL) was used as molecular weight marker. Flt-1 PCR was carried out using the primers for the extracellular domain of this receptor, and the amplification products were run in 1.5% agarose gels. As molecular weight marker, a 1 kb DNA ladder from Gibco/BRL was used. Results are representative of three different experiments.

negative for PlGF secretion. Differently from VEGF, PlGF secretion levels were not affected by culture conditions.

We could also prove that PlGF expression was not induced by culture establishment. Production of PlGF polypeptides was, indeed, demonstrated by immunohistochemical analysis of melanoma tumors. The *in vivo* results suggest that PlGF expression may be upregulated during melanoma progression, as already described for cervical squamous cell carcinomas (Kodama *et al*, 1997) and meningiomas (Donnini *et al*, 1999). Interestingly, a PlGF-specific staining was observed, *in vivo,* in the epidermal keratinocytes surrounding melanoma cells, both in primary tumors and in cutaneous metastases. Similarities between the stroma of tumors and wounds have led to the concept that the former may be

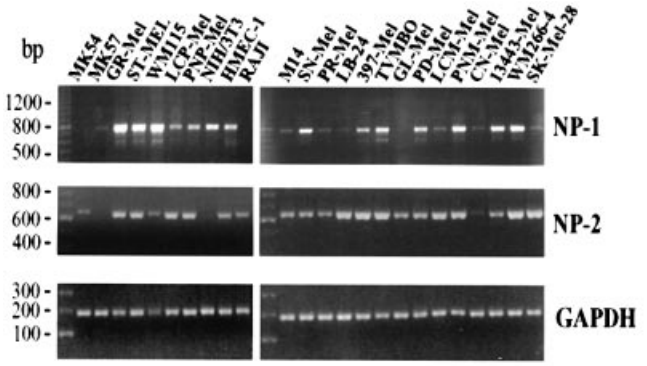

Figure 4. RT-PCR analysis of neuropilin-1 and neuropilin-2 expression by human melanoma cells. Primers described in *Materials and Methods* were used to amplify cDNA obtained from each cell line. PCR products for neuropilin-1 (NP-1) and neuropilin-2 (NP-2) were separated in 1% agarose gels and 100 bp DNA ladders from Pharmacia and from Gibco/BRL, respectively, were used as molecular weight markers. GAPDH PCR products, obtained using the same cDNA preparation to confirm integrity of the RNA samples, were separated in 2% agarose gels, and a 100 bp DNA ladder (Gibco/BRL) was used as molecular weight marker. Results are representative of three independent experiments.

experienced by the organism as a never healing wound (Dvorak, 1986). As PlGF expression is induced in keratinocytes migrating to cover the wound *in vivo* and is upregulated *in vitro* by cytokines secreted during wound healing (Failla *et al*, 2000), our data further support this hypothesis. The induction of PlGF expression in keratinocytes may be due to cytokines secreted by the melanoma cells themselves, and/or by the inflammatory infiltrate near the tumor lesion. Further analysis is required to clearly understand the biologic significance of this induction.

Several studies in the literature have focused on the presence in human melanoma cells of the VEGF receptors KDR (Gitay-Goren *et al*, 1993; Liu *et al*, 1995) and, more recently, Flt-1 (Graeven *et al*, 1999). In agreement with the previous reports, we demonstrated KDR and Flt-1 cDNA expression in 17 and 18, respectively, of 19 melanoma cell lines, and their absence in *in vitro* cultures of human melanocytes. Flt-1 upregulation in melanoma cell lines might lead to activation of signal transduction pathways that partially differ from those induced through KDR activation (Landgren *et al*, 1998). In addition, the coexpression of Flt-1 in PlGF-producing melanoma cell lines raises the possibility that this cytokine might affect melanoma cell biology either directly, through an autocrine mechanism, or indirectly, through modulation of the VEGF response.

Studies on the effect of exogenously added VEGF on melanoma cell growth have shown contradictory results (Liu *et al*, 1995; Graeven *et al*, 1999). In the experimental conditions used here, we observed an increase in methyl-^{3}[H]-thymidine uptake and cell number following VEGF or PlGF stimulation, as both homodimers or heterodimers, in several melanoma cell lines. This response correlates with VEGF receptor expression. Discrepancy with the results from Graeven *et al* could be related to differences in culture conditions, e.g., maintenance of melanoma cell lines in the presence of insulin or use of late passage cells (Graeven *et al*, 1999). The role initially played by VEGF and PlGF on melanoma cell growth could be lost with time, by adaptation to *in vitro* growth conditions.

At present, the ability of PlGF to trigger a mitogenic activity in endothelial cells is also controversial. Some studies suggested that PlGF might modulate VEGF response by forming VEGF/PlGF heterodimers with biologic properties different from those of the VEGF homodimer (Cao *et al*, 1996a) or by increasing VEGF availability to the KDR receptor through its release from common receptors (Flt-1, neuropilin-1, and neuropilin-2). Other studies described a direct PlGF mitogenic activity on human endothelial

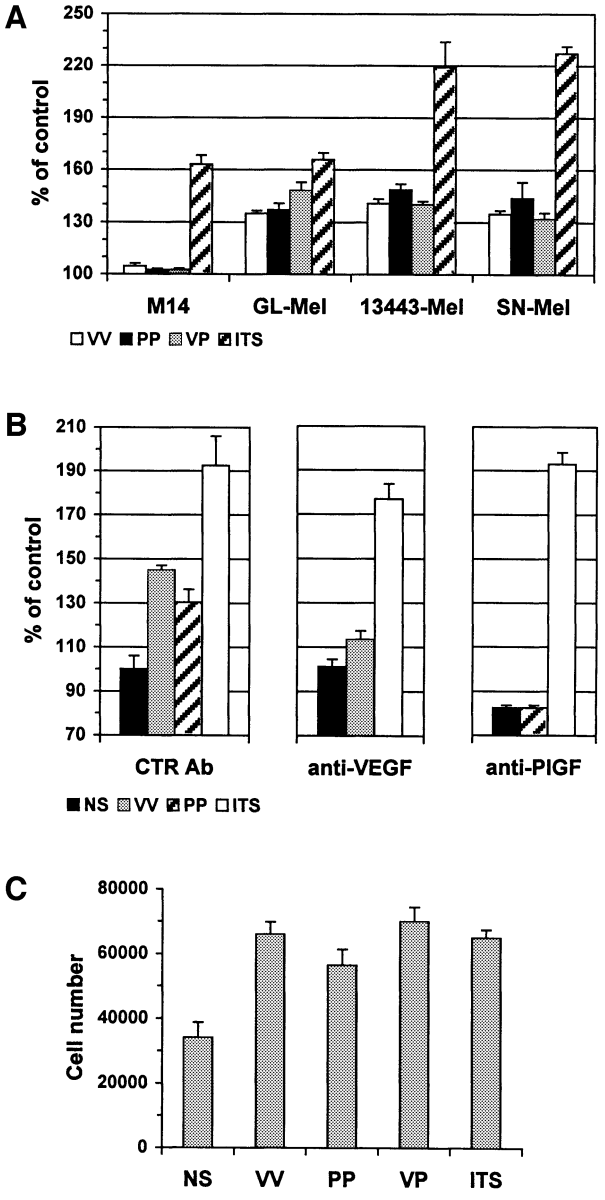

Figure 5. Effect of $VEGF_{165}$ and PlGF-1 homodimers and heterodimers on human melanoma cell growth. In the analysis of methyl-[^{3}H]-thymidine uptake (*A* and *B*) stimulation of melanoma cells was carried out for 20 h, as described in *Materials and Methods*. Results are expressed as the percentage of methyl-[^{3}H]-thymidine uptake in cells treated with the different growth factors compared to untreated cells. In both cases, a representative experiment performed in quadruplicate is shown, and data represent the arithmetic mean ± the standard error of the mean. (*A*) VV, VEGF/VEGF homodimer; VP, VEGF/PlGF heterodimer; PP, PlGF/PlGF homodimer. Basal levels of methyl-[^{3}H]-thymidine uptake for the different cell lines were as follows: M14, 7343 cpm; GL-Mel, 26075 cpm; 13443, 18897 cpm; SN-Mel, 63727 cpm. (*B*) The effect of antibodies anti-VEGF or anti-PlGF on stimulation of GL-Mel methyl-[^{3}H]-thymidine uptake was analyzed as described in *Materials and Methods*. NS, no stimuli added; VV, VEGF/VEGF homodimer; PP, PlGF/PlGF homodimer. The basal level of methyl-[^{3}H]-thymidine uptake for GL-Mel cells was 36900 cpm. (*C*) GL-Mel cells were incubated for 72 h in the presence of the different stimuli and then viable cells were counted, as described in *Materials and Methods*. Results are expressed as the total number of cells per well for cultures of treated or untreated cells. A representative experiment performed in triplicate is shown. Data represent the arithmetic mean ± the standard error of the mean. VV, VEGF/VEGF homodimer; VP, VEGF/PlGF heterodimer; PP, PlGF/PlGF homodimer.

cells (Ziche *et al*, 1997). Our results on melanoma cell stimulation by PlGF might be ascribed either to the direct activation of Flt-1 receptor or to the augmented availability of the VEGF produced by the melanoma cells themselves. The possibility of the establishment of a VEGF and/or PlGF autocrine loop in melanoma cells and the precise role played by PlGF in these cells are currently under study in our laboratory.

Recently, the capability of neuropilin-1 and neuropilin-2 to establish a receptor–ligand interaction with some VEGF and PlGF isoforms has been described (Migdal *et al*, 1998; Soker *et al*, 1998; Gluzman-Poltorak *et al*, 2000). Our study demonstrates neuropilin-1 and neuropilin-2 expression in 18 and 19, respectively, of the 19 melanoma cell lines analyzed, but the cytokine-neuropilin interaction does not seem to lead to increased cell proliferation. In fact, M14 cells (that lack KDR and Flt-1 but express neuropilin-1) were not responsive to $VEGF_{165}$, whereas GL-Mel cells (positive for KDR and Flt-1 expression and negative for neuropilin-1) were induced to proliferate by $VEGF_{165}$ treatment. It is also worth noting that a positive response was obtained using the PlGF-1 isoform, which binds with very low affinity to neuropilins. The role played by the binding of VEGF and PlGF to neuropilins, however, and the interference of this binding with KDR and/or Flt-1 activity, should be considered in further studies on melanoma cell stimulation by these cytokines.

In conclusion, our data show that a cytokine of the VEGF family, the PlGF, and the receptors neuropilin-1 and neuropilin-2 are expressed by human melanoma cells and support the hypothesis of a role of VEGF and PlGF in melanoma growth and progression. These findings raise the possibility of designing therapeutic strategies for human melanoma treatment aimed to specifically target the VEGF and/or PlGF interactions with their receptors.

Note added in proof While this work was being considered for publication, Graeven *et al* reported the *in vitro* expression and secretion of PlGF, as well as neuropilin-1 expression, in eight melanoma cell lines (this journal, July 2000, 115: 118–123). Our data confirm these findings in a wider cell line number from primary and metastatic melanomas, also demonstrating the *in vivo* production of PlGF in melanoma specimens. In contrast to what is reported by Graeven *et al*, who used a modified MTT colorimetric assay to measure cell proliferation and could not demonstrate a cell response to PlGF, our experiments with methyl-[^{3}H]-thimidine uptake and direct cell counting have shown a proliferative effect of PlGF on melanoma cells in culture.

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