Pathol. Oncol. Res. (2011) 17:569–577
DOI 10.1007/s12253-010-9350-y

RESEARCH

Embryonic Vasculogenesis in Nodular Melanomas and Tumour Differentiation

Bhanu Iyengar · Avantika V. Singh

Received: 13 March 2010 / Accepted: 16 December 2010 / Published online: 4 January 2011

Abstract The relationship of vasculogenic mimicry to pigment in nodular vertical growth phase [VGP] cutaneous melanomas is assessed in this study. 10 nodules each from 27 tumors, 15 pigmented and 12 amelanotic were sampled in proportion to the pigment level. Serial frozen and paraffin sections subjected to HE, Reticulin, PAS to assess the vascular pattern; Dopa Oxidase and Immunopositivity for HMB45, LN5 [laminin 5] & integrin[$\alpha_5\beta_1$], and EM [electron microscopy] to identify Weibel-Palade bodies within endothelial cells. The vascular pattern, pigment and the immunopositivity was mapped to assess the percentage VM [vasculogenic sinusoids] vs INC [incorporated microvasculature]. In pigmented melanomas, INC from preexisting stromal vessels is predominant. Amelanotic melanomas show embryonic vasculogenic mimicry, a self-propagating system of spaces within the sheets of tumors cells. Both INC and VM co-exist in tumors with both amelanotic and melanotic nodules. In areas with VM, loci of LN5 and $\alpha_5\beta_1$ integrin positive cells appear within the proliferating columns, positivity in these cells suggesting a switch to a more aggressive form. Irregular spaces appear lined by tumor cells, with initial hemopoeitic activity, coalesce and interlink into tubular networks. Spaces lined by tumor cells extend into an intricate network which then connects with the angiogenetic system. The tumor cells lining the vasculogenic spaces are positive for LN5, $\alpha_5\beta_1$ integrin. Statistically, INC is significantly higher in pigmented melanomas, whereas amelanotic melanomas show significantly higher VM. Pigmentation is correlated positively with INC and negatively with VM. INC and VM are negatively correlated with each other.

Keywords Vasculogenic mimicry · Incorporated microvasculature · Pigmented · Amelanotic · Laminin · Integrin

B. Iyengar (✉) · A. V. Singh
Pigment Cell Center, Iyengar Farm,
Brijwasan Road, PO Kapashera,
New Delhi 110037, India
e-mail: bhanu_i@yahoo.com

Introduction

All tumors require a blood supply for growth and metastasis [1]. Most human cancers persist in situ for months in a prevascular phase. However, the tumors require vascularisation, for the progression to an enlarging tumor with the ability to metastasize beyond 2–3 mm^3 [2]. The development of the tumor microcirculation includes both the production of new blood vessels [angiogenesis] and their remodeling [3].The process of new vessel formation [neoangiogenesis] includes proliferation, sprouting, and migration of endothelial cells within normal tissues adjacent to the tumor.

The formation of patterned vascular channels found in the most aggressive primary intraocular [uveal] melanomas and their metastases are different from endothelial-derived angiogenic vessels. Highly invasive melanoma cells reconstitute *in vitro* the patterned matrix-associated vascular channels seen in human melanomas in the absence of endothelial cells and fibroblasts. Tissue sections from aggressive human intraocular [uveal] strongly suggest that aggressive melanoma cells may generate vascular channels that facilitate tumor perfusion independent of incorporated tumor microvasculature. Endothelial cells were not identified within these channels by light microscopy, by transmission electron microscopy, or by using an immunohistochemical panel of endothelial cell markers [4]

Melanomas are uncommon tumors in India where the ratio of incidence of vitiligo versus melanomas is the reverse of that seen in Australia or Europe. The following is the study of 27 melanomas collected over a period of 5 years. Melanomas, unlike most other rapidly growing tumors show very little or no evidence of necrosis on gross or microscopy even in the rapidly growing amelanotic tumors where the cells quickly lose access to the stromal vessels. This work has been taken up to study the pattern of vascularisation in cutaneous pigmented and amelanotic melanomas in the vertical growth phase VGP to elucidate the possible mechanism involved.

Material and Methods

Twenty seven nodular melanomas in the vertical growth phase [VGP] were received from the Cancer Surgery Unit of Safdarjung Hospital, New Delhi, fixed in 10% formol glutaraldehyde. Fifteen were pigmented and 12 were amelanotic. Ten blocks were taken from each of 27 tumors to make a total of 270 blocks. Nodules were sampled in the ratio of pigmented to amelanotic areas in the entire tumor. As the specimen were received and sampled the blocks were arranged in a grid, according to the pigment level.

Each block was subjected to: Frozen sections, Paraffin sections. Serial sections 5 μm thick [20-40] were cut from each block and maintained for routine Histochemistry, [HE, Reticulin, PAS to assess the vascular pattern]; Enzyme histochemistry: Dopa Oxidase method; Immunohistochemistry by the Avidin/ Biotin system using the monoclonal antibodies HMB45, from BioGenex, LN5 [laminin 5] from Kappa Zymed & integrin[$\alpha_5\beta_1$] from Dakopats [5–7]. As negative control all slides included a serial section stained with no mAb. The same mAb were used simultaneously against known positive sections from human skin as positive controls.

Presence of pigment; a positive DOPA reaction; and HMB-45 positivity are criteria for diagnosis. In the absence of pigment a positive dopa reaction, HMB45 positivity and the presence of premelanosomes on electron microscopy [EM] is diagnostic of amelanotic melanomas. These criteria form the basis of diagnosing each tumor included in this study. EM was done to locate Weibel-Palade bodies [WPB] for the identification of capillary endothelial cells.

The vascular pattern and the positivity of the different markers was assessed and mapped within a grid. The percentage of capillaries and incorporated microvasculature [INC] vs vasculogenic sinusoids [VM] was calculated and plotted against the level of pigment in each case and depicted in the graphs.

Results

Pigmented Tumors: (Fig. 1a)

Of the 27 tumors 15 are pigmented. Four cases showed no defined amelanotic nodule throughout the tumor while in 11 cases, 10% to 50% nodules were poorly pigmented, the rest being pigmented. The total pigmented area forms 76.7% while amelanotic is formed by 23.3%. Capillaries and incorporated microvasculature [INC] are seen in 88.7% areas of which 12% are in poorly pigmented areas. Combined capillary and vasculogenic vascularisation [VM] is seen in 11.3% in amelanotic areas. Combined INC and VM vascularisation seen in 37.8% of amelanotic

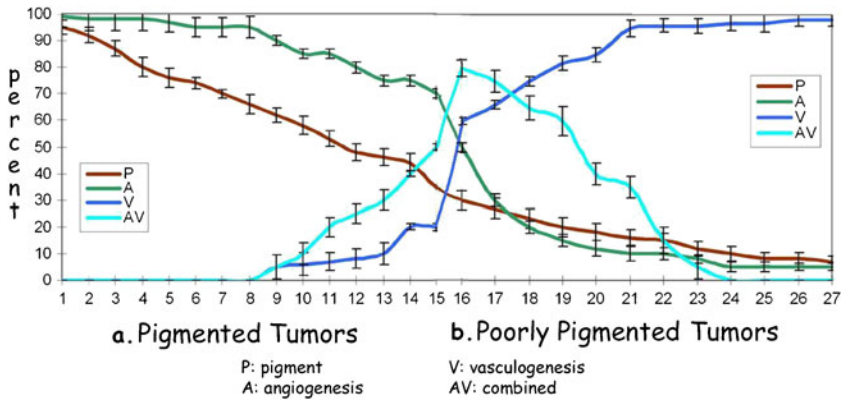

Fig. 1 **a** Pigmented melanomas: Pigmentation and INC are positively correlated with each other [Pearson's correlation coefficient=0.941; $p<0.0001$], whereas pigmentation is negatively correlated with VM [Pearson's correlation coefficient=−0.853; $p<0.0001$]. INC and VM are negatively correlated with each other [Pearson's correlation coefficient=−0.942; $p<0.0001$]. **b** Amelanotic melanomas: Pigmentation and INC are positively correlated with each other [Pearson's correlation coefficient=0.907; $p<0.0001$] whereas pigmentation is negatively correlated with VM [Pearson's correlation coefficient=−0.954; $p<0.0001$]. INC is also negatively correlated with VM in amelanotic tumours [Pearson's correlation coefficient=−0.943; $p<0.0001$].

areas in the pigmented tumors. There are no areas having only VM.

Amelanotic Tumors: (Fig. 1b)

In the 12 amelanotic tumors there were one to four pigmented nodules in eight tumors, and none in four tumors. The total amelanotic area is formed by 83.3% while pigmented area forms 16.7%. The predominant vascularisation is by VM in 68.3% areas. Combined INC and VM neovascularisation is seen in 31.7% areas. All the 16.7% pigmented areas and 18% of the amelanotic areas show both INC and VM. None of the areas shows pure INC.

Statistical Correlations

When the level of pigmentation was compared between pigmented and amelanotic melanomas, pigmentation was significantly higher in pigmented than in amelanotic cases [Mann-Whitney U statistic [MW-U=180.00; $p \leq 0.001$]. INC was also significantly higher in pigmented than in amelanotic melanomas [$t=16.567$ with 25° of freedom, $p \leq 0.001$]. In contrast, VM was significantly higher in amelanotic melanomas compared to pigmented melanomas [MW-U=0, $p \leq 0.001$].

Pigmented Melanomas

Pigmentation and INC are positively correlated with each other [Pearson's correlation coefficient=0.941; $p<0.0001$], whereas pigmentation is negatively correlated with VM [Pearson's correlation coefficient=−0.853; $p<0.0001$]. INC and VM are negatively correlated with each other [Pearson's correlation coefficient=−0.942; $p<0.0001$].

Amelanotic Melanomas

Pigmentation and INC are positively correlated with each other [Pearson's correlation coefficient=0.907; $p<0.0001$] whereas pigmentation is negatively correlated with VM [Pearson's correlation coefficient=−0.954; $p<0.0001$]. INC is also negatively correlated with VM in amelanotic tumours [Pearson's correlation coefficient=−0.943; $p<0.0001$).

Vascularisation

Incorparated Microvasculature [INC]: (Fig. 2)

In the pigmented areas, the tumor growth is toward preexisting blood vessels around the peripheral margins. Tongues of tumor cells extend towards and encircle marginal blood vessels and the surrounding fibrous connective tissue. The vessels ultimately get incorporated within the tumor substance to sprout endothelial buds which enter and branch within the adjacent tumor substance. These vessels are still connected to the existing vasculature and the general circulation (Fig. 2a).

INC is the main form of vascularisation in pigmented tumors forming 88.7%. The marginal layers of the tumor cells at the stroma-tumor interphase are positive for integrin [$\alpha_5\beta_1$] [85.7%] and LN5 [98.4%], both required for preparing the ECM for spread of the tumor cells as well as the vascular network (Fig. 2d,e). This induces proliferation of the adjacent blood vessels, which throw out endothelial buds. The endothelial buds cannelise, acquire a silver positive basement membrane, and a surrounding connective tissue matrix. As the blood vessels mature they show extensive branching into the tumor substance. Tumor cells interact with the neovasculature to ensheath the vessels and grow out into several layers as seen on reticulin staining (Fig. 2a-c).

INC is significantly higher at the margins as quantified by counting the blood vessels at the margins and well within the tumor growth. On an average 8.18 vessels/HPF are observed near the invasive margins while there are 1.9 vessels/HPF within the tumor. At the margins a maximum of 14 and a minimum of five vessels/ HPF are observed. In the areas of main tumor growth a maximum of four and a minimum of one are observed. Thus as there is a significant difference between angiogenic vessels at the invasive margins and within the tumor, in a rapidly growing tumor the central portions recede from the margins and are deprived of vascularisation.

Vasculogenic Mimicry [VM]: (Fig. 3a–d)

There is no necrosis in amelanotic tumors in spite of rapid proliferation with low differentiation. The rapidity of growth outstrips the vascularisation by INC as observed in the pigmented areas. The bulk of the tumor is composed of sheets of uniform cells, most areas being rapidly distanced from the available blood vessels at the periphery.

In many areas there is a disruption of the sheets of cells. Collections of LN5 and integrin [$\alpha_5\beta_1$] positive cells form a loose network within the sheets and chords of tumor cells. Irregular spaces appear within these areas which expand into islands of hemopoeisis, blood filled spaces with megakaryocytes, myeloid and erythroid series of cells with. These spaces abut directly on to the tumor cells (Fig. 3a).

On tracing the spaces further, blood filled lakes are seen with red cells derived from the hemopoeitic areas within the tumor. The lining tumor cells become flattened to resemble endothelial cells (Fig. 3b–c). The spaces narrow down to form sinusoid like tubes lined by the flattened endothelial-like cells positive for HMB45 (Fig. 3d).

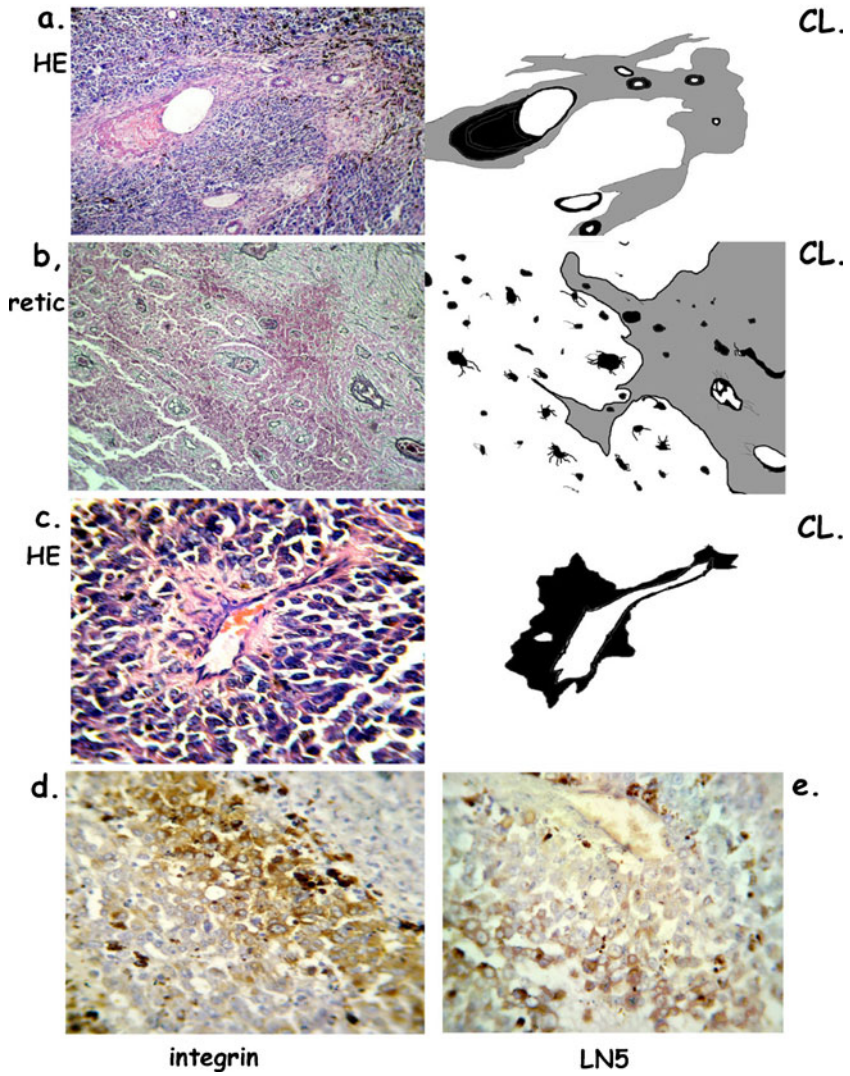

Fig. 2 Vasculaure in pigmented tumors along with camera lucida [CL] tracings: **a** pre existing stromal vessels enclosed by the extending columns of tumor cells. Pigment can be seen in the surrounding tumor cells [HEX40]; **b** Capillaries grow out of the pre-existing marginal and stromal blood vessels to extend into the margins of the tumor [reticX40]; **c** well formed thin-walled vessel within the tumor [HEX100]. **d** Marginal tumor cells showing $\alpha_5\beta_1$integrin positivity [mAbintX100]; **e** LN5 positivity at the tumor/stroma interphase [mALN5X100]

These tubes narrow down to form a network of innumerable capillaries closely associated with the tumor cells as can be seen in the figure. In many areas within this rich network, the lining cells show further differentiation into primitive fibroblastic activity in the abluminal area and show WPB within. HMB45 positivity highlights the melanocytic origin of the capillary network (Fig. 3e).

LN5 [71.4%] and integrin [$\alpha_5\beta_1$] [57.1%] positivity is seen at the margins of amelanotic areas abutting on the surrounding tissue. The reactivity of the stromal vasculature is similar to that seen in the more differentiated areas.

Combined INC and VM: (Fig. 4)

There is a transition between the two extreme forms of vascularisation, correlating with the level of pigmentation, INC being prominent in pigmented tumors [88.7%], while de novo VM is prominent in amelanotic tumors [68.3%]. Pigmented tumors do not show exclusive INC while amelanotic tumors do not show pure INC areas, 31.7% showing a combination. (Fig. 5)

In the pigmented tumors 14 of 150 sections [9.33%] and in the amelanotic tumors 38 of 120 sections [31.7%] show a combination of classical INC as well as VM. LN5 and integrin [$\alpha_5\beta_1$] positive cells are seen within the sheets and chords of tumor cells. Irregular crevices appear within these groups of tumor cells to form channels. Cells lining VM channels show 91.4% integrin $\alpha_5\beta_1$ and 93.8% LN5 positivity indicating that these are aggressive and invasive cells. Single or small groups of tumor cells separate from the main mass to spill into these spaces. The lining tumor cells flatten to form capillary channels which then connect with the vascular network.

These areas coexist with well formed INC channels surrounded by expanding layers of tumor cells. Simultaneous INC is observed, at the margin between the tumor and the stroma. 19.25% combined vascularisation is seen in the entire group.

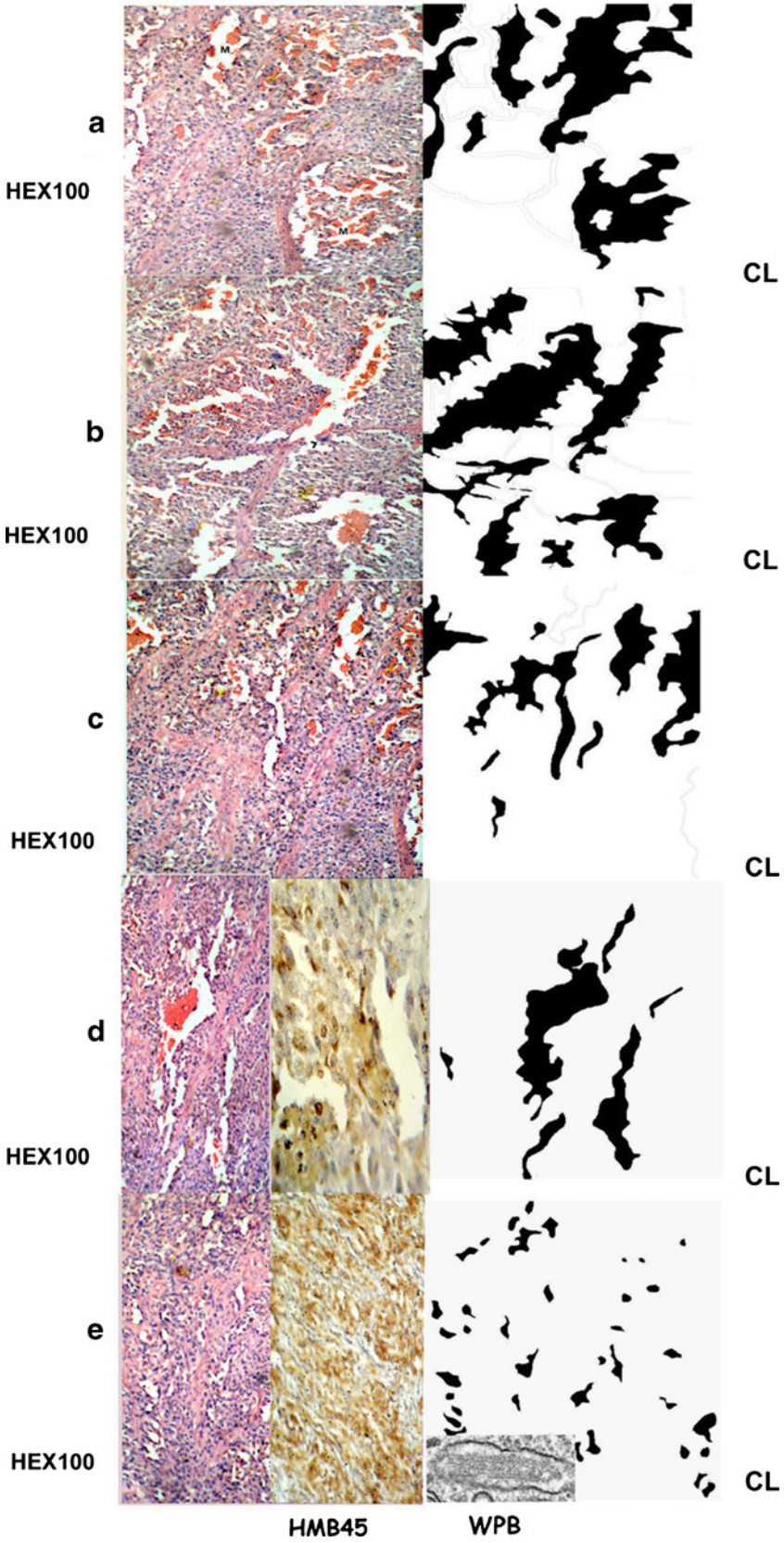

Fig. 3 Composite picture tracing the development of the self-propagating vascular system in amelanotic melanomas. Camera lucida [CL] diagrams are given alongside to trace the progression of vasculature. **a** Cavernous spaces appear with sheets of amelanotic cells. These show hemopoeitic activity with megakaryocytes **[>>]**, erythroid and myeloid cells **[M]**. **b** Irregular cavernous spaces lined by viable tumor cells elongate to form sinusoids. **c** There is further refinement with flattening of the lining tumor cells. **d** Blood filled cavernous as well as endothelial tubes are seen. Inset shows HMB45 positivity of lining cells. **e** Endothelial tubes proliferate to form a capillary network with the appearance of young fibroblastic stroma. Inset shows HMB45 positivity of the surrounding cells

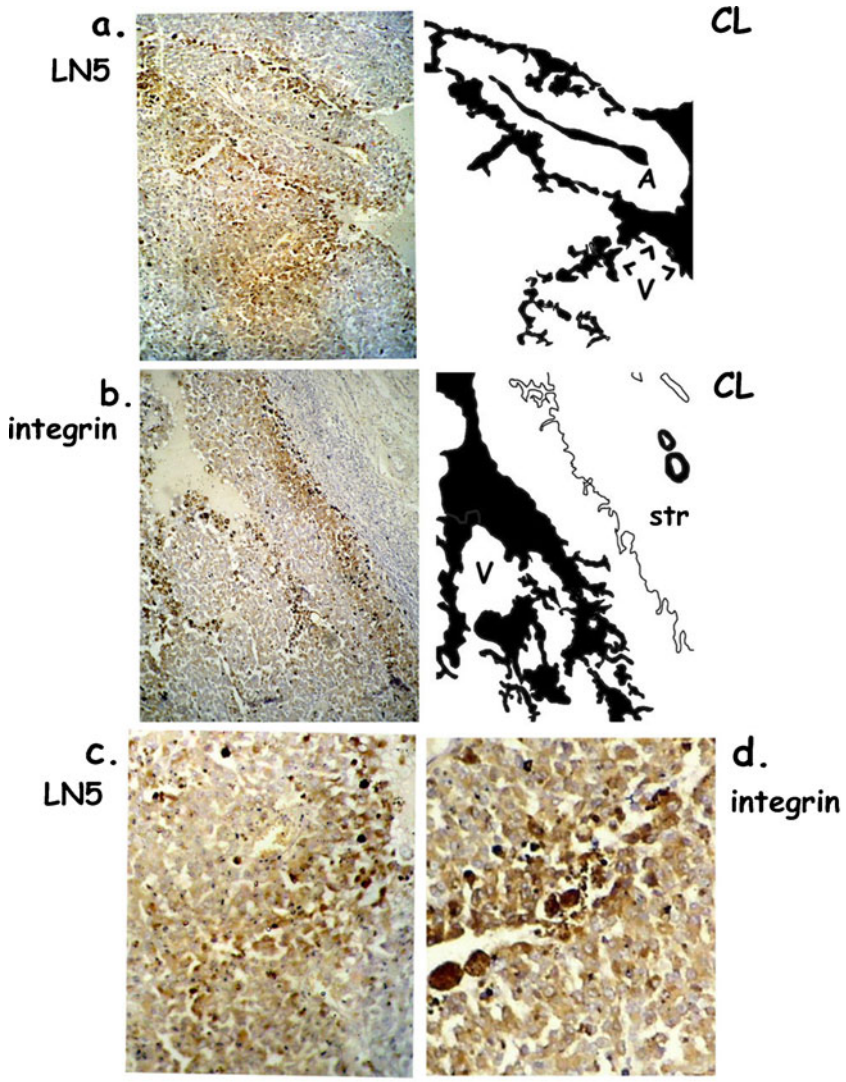

Fig. 4 Combined vasculature in tumors with both pigmented and amelanotic nodules with CL diagrams: **a** area showing an angiogenetic capillary with VM within the surrounding tumor showing LN5 positivity; **a** is an angiogenic vessel with a mantle of tumor cells and **V** indicates the VM areas; **b** $\alpha_5\beta_1$integrin positive cells line the vasculagenic network connecting; **c** laminin5 positive cells at sites of vasculogenesis [mAbLN5X100]; **d** serial section shows cells positive for $\alpha_5\beta_1$integrin [mAbintX100]

Discussion

From these observations it is evident that there are diverse modes of vascularisation in melanomas. These depend on the size, stage and differentiation (as assessed by pigment) and proliferation of the tumor. Melanoma progression is characterized by an increase in both metastatic frequency and the vascular density of the tumor tissue. Metastasis is invariably preceded by angiogenesis. Vertical growth phase [VGP] melanoma [nodular melanoma] or the ability of melanoma cells to undergo proliferation in three dimensions is a highly angiogenic and proliferative lesion [8, 9].

Tumor vascularization is a vital process for the progression of a neoplasm from a small localized tumor to an enlarging tumor with the ability to [2]. Tumors regulate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors [10]. This imbalance may be caused by either hypoxia [low oxygen tension] or genetic alterations that activate oncogenes and/or inactivate tumor suppressor genes. VEGF can be directly activated by the oxygen sensing, transcription factor hypoxia induced factor-1 alpha [HIF-1a].

The successful development of tumor vasculature is due to the remodeling of preexisting vasculature in host tissues surrounding the growing tumor mass and starts concurrently with tumor cell invasion. Such vessels enlarge and actively engage in sprouting and branching to meet the metabolic demands of adjacent malignancy [10, 11]. Angiogenesis is also defined as the sprouting of blood vessels from preexisting ones and begins by focal reduction of vascular intercellular interactions and interactions between vascular cells and the extracellular matrix [ECM] [2, 12].

Human tumors are highly heterogeneous in vascular architecture, differentiation and functional blood supply. Vascular immaturity is a natural consequence of a genetically based unlimited expansion of tumor cells, compared to the well-regulated growth of different organs during

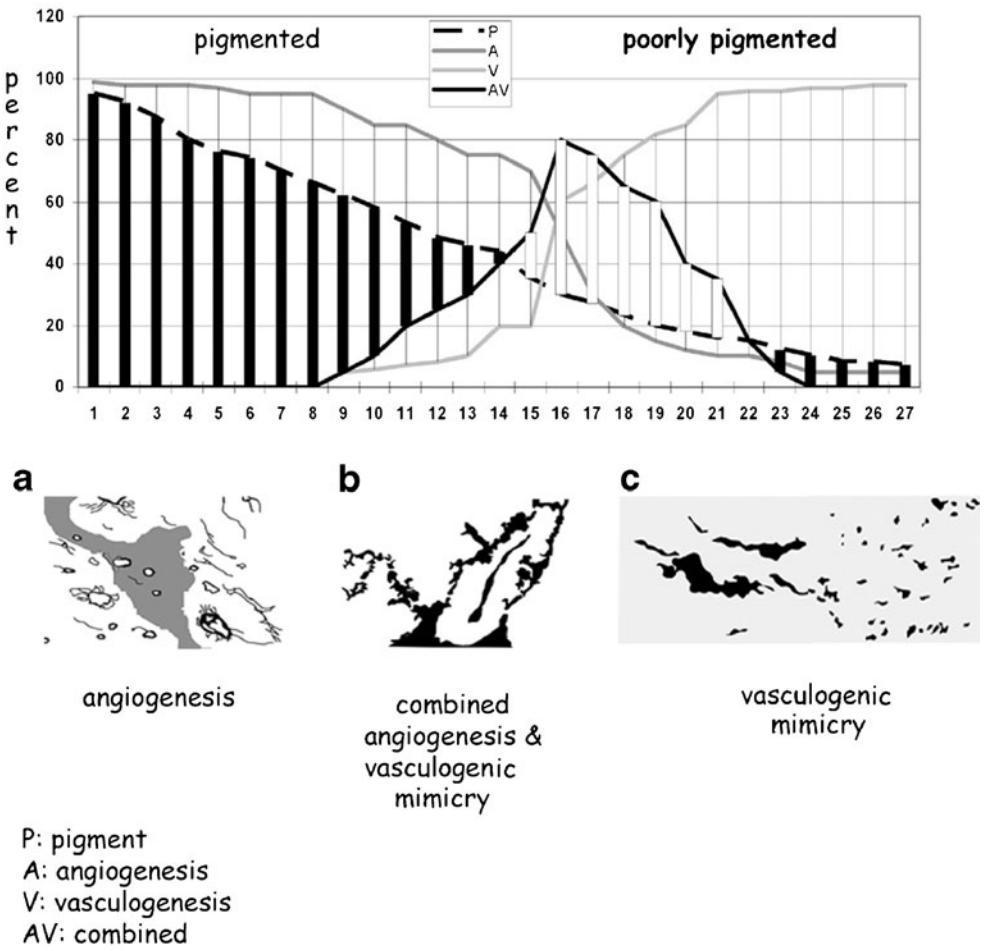

Fig. 5 Summary: Pigmentation is positively correlated with INC and negatively correlated with VM. INC and VM are negatively correlated with each other. This is diagrammatically represented below: INC is prominent in pigmented tumors (**a**); there is a graded shift from angiogenic to VM both being combined in tumors with 50% to 20% pigmentation (**b**). *de novo* VM is related to amelanotic tumors (**c**)

embryogenesis. Unlimited tumor expansion and therefore the continuous stimulation of vessel outgrowth prevent endothelial cells from generating a mature vasculature, but are instead continuously stimulated to expand the vascular compartment of the growing tumor [13].

To meet the demands of the growing population of tumor cells a complex interaction occurs between the advancing tumor and the surrounding blood vessels in the melanotic tumors. As seen in this study the tumor cells at the advancing edge produce LN5 and integrin [$\alpha_5\beta_1$] both of which are involved in the dissolution of the local ECM to allow the cells to move forth further. This is accompanied by the growth of endothelial buds towards the tumor margins to enter the substance and grow into larger vessels, thus initiating tumor angiogenesis in the pre-existing vasculature. Columns of tumor cells advance towards blood vessels and enclose them along with their surrounding stroma. These are gradually incorporated into the body of the main tumor and further extend capillary sprouts into the surrounding tumor mass. Tumor cells proliferate around these thin walled vessels with further expansion of the tumor, The above process of capillary incorporation, predominates in the pigmented areas of the tumor.

In the amelanotic rapidly progressive areas the central zone quickly loses contact with the stroma and the angiogenic vessels. Collections of LN5, integrin [$\alpha_5\beta_1$] positive cells form a loose network within the sheets and chords of tumor cells to form the nidus for VM. These form irregular spaces which, on close scrutiny, show haemopoeitic activity which includes erythroid, myeloid and megakaryocytic series of cells. The spaces continue into lakes of blood with flattened tumor cells which are positive for melanocyte markers, HMB45, indicating their melanocytic origin. The spaces extend to form a tubular network with an endothelial lining, which acquire WPB to further mature into a rich capillary network. The more mature areas show early connective tissue formation. Here the surrounding amelanotic cells are closely associated with the capillaries (Fig. 2).

During embryogenesis, the formation of primary vascular networks occurs via vasculogenesis with the formation of blood islands in the extraembryonic yolk sac [3]. Blood islands develop from aggregates of mesodermal cells at approximately 7 days post coitum of mouse development. They consist of an inner layer of primitive hematopoietic cells and a peripheral population of angioblasts which differentiate into endothelial cells.

A similar process occurs in the amelanotic melanoma under the stress of rapid proliferation, to give rise to hemopoeitic cells that normally derive from germ layers other than the neuroectoderm and neural crest [14–18]. First hemopoeitic cells form and then endothelial cells to self propagate a rich and abundant vascular network resembling the embryogenetic process.

In tumors with fewer amelanotic areas INC is prominent while in tumors with predominantly amelanotic nodules VM is the main feature with a few areas of INC. In mixed nodules along with angiogenic vasculature, the surrounding sheets and chords of tumor cells show areas where there are collections of LN5 and integrin [$\alpha_5\beta_1$] positivity. These form a nidus for slit like spaces to form (Fig. 4c, d). These widen into sinusoidal spaces, lined by the HMB45, positive tumor cells which further flatten to continue into a network connecting with the surrounding angiogenic capillary network.

On casual observation these areas appear to be artefactual or due to necrosis. The presence of mitosis and the LN5/integrin[$\alpha_5\beta_1$] positivity in the bordering cells suggest that these are cells which have switched to an aggressive invasive phase. Thus as the melanocyte becomes poorly differentiated there is a switch over to the rapid fire vascularisation to facilitate rapid growth, mimicking embryonic conditions. LN5 and integrin $\alpha_5\beta_1$ positivity in the tumor melanocytes heralds this switch.

LN5 and integrin $\alpha_5\beta_1$ are important for the invasion of the stroma. In aggressive primary and metastatic melanomas, the tumor cells generate acellular microcirculatory channels composed of ECM and lined externally by tumor cells [4]. The name "vasculogenic mimicry" was given as aggressive tumor cells generate non-endothelial cell-lined channels delimited by ECM. The molecular profile of aggressive cutaneous and uveal melanoma cells is described as that of multiple phenotypes similar to a pluripotent, embryonic-like stem cell [4, 19].

Laminin 5 plays an important role in cell migration during tumor invasion and tissue remodeling. Akimoto et al. [2004] [20] reported Laminin5 β3 and γ2 chains expression in the cytoplasm of tumor cells at the cancer-stromal interface and at the invasive front in squamous cell carcinoma of the tongue and of colorectal carcinoma. Furthermore, strong expressions of these two proteins were observed in cancer cells invading in a scattered manner [21–23].

Goldfinger et al 1999 [24] proposed a model to explain the role of LN5 and its integrin receptors [$\alpha_v\beta_3$ & $\alpha_5\beta_1$] in epithelial wound healing. Upon wounding, production of LN5 is upregulated. This results in deposition of unprocessed α_3 laminin subunit in the form of arcs or circles at the leading edge of the wound. The integrin $\alpha_3\beta_1$ interacts with the unprocessed α_3 laminin subunit to help cell migration over the wound bed.

Results suggest that co-expression of the two β3 integrins, $\alpha_v\beta_3$ and αIIbβ3, in human melanoma cells enhanced cell survival and promoted growth in vivo [25]. Döme B, and his group, has outlined the role of αIIbβ3 and αvβ3 integrin in human melanoma growth and survival [26, 27].

It has been observed that α5 integrin promotes in vitro and in vivo survival of cells in metastatic melanoma [28, 29]. $\alpha_5\beta_1$ expression is correlated not only to migratory behavior of malignant cells but also to the need for normal human melanocytes to adhere, spread and migrate in the provisional matrix present in skin wounds. In the case of tumor cells, it is of advantage to have less $\alpha_v\beta_3$ and more $\alpha_5\beta_1$ integrin to facilitate rapid binding to blood clots and other matrix components. Thus a shift from $\alpha_v\beta_3$ to $\alpha_5\beta_1$ signals enhanced invasiveness increased motility [30–33].

Thus, biologically advanced melanoma cells can take over all or part of stromal functions. This constitutes the concept of "tumor dominant plasticity", which takes into account the flexible responses of malignant cells to micro-environmental pressures while maintaining dominance over the normal parenchymal as well as stromal cells. From this study it is observed that aggressive amelanotic melanomas can self propagate a vascular network for efficient and rapid progression and metastatic spread.

AW Griffioen and colleagues, have demonstrated that harmful endothelial cell anergy can be counteracted by inhibitors of angiogenesis. Anginex inhibits tumor growth and microvessel density significantly, the amount of infiltrated leukocytes [CD45], as well as the number of $CD8^+$ cytotoxic T lymphocytes, was enhanced markedly. The results suggest that immunotherapy strategies can be improved by combination with anti-INC [34–38]. However in view of the varying types of vascularisation evident in melanomas as seen in this study the therapeutic strategies have also to target the self propagating vascular networks within the tumor, not involving the angiogenic pathways.

Acknowledgement We are indebted to:

- The Institute of Pathology, [ICMR], New Delhi for the technical support;
- Dr. KK Pandey, formerly the Head of Cancer Surgery, Safdarjung Hospital, New Delhi;
- BITS, Pilani, supporting the MS-PhD program;
- Dr. Soumya Iyengar, Associate Professor, National Brain Research Center, Manesar, for the Statistical analysis.

References

1. Folkman J (1995) Clinical applications of research on angiogenesis. Seminars in Medicine of the Beth Israel Hospital, Boston. New Engl J Med 333:1757–1763
2. Folkman J (1971) Tumour angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

3. Risau W (1997) Mechanism of angiogenesis. Nature 387:671–674
4. Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, Trent JM, Meltzer PS, Hendrix MJC (1999) Vascular channel formation by human melanoma cells *in vivo* and *in vitro:* vasculogenic mimicry. Am J Pathol 13:739–752
5. Prophet ED, Mills B, Arrington JB, Sobin LH (1994) Laboratory methods in histotechnology. American registry of pathology, Armed Forces Institute of Pathology, Washington
6. Mikel UV (1994) Advanced laboratory methods in histology and pathology. American Registry of Pathology, Armed Forces Institute of Pathology, Washington
7. Pearse AGE (1985) Histochemistry theoretical and applied. Vol II: analytical Technology. Churchill Livingstone, London, pp 611–674
8. Liu W, Dowling JP, Murray WK, McArthur GA, Thompson JF, Wolfe R, Kelly JW (2006) Rate of growth in Melanomas: characteristics and associations of rapidly growing melanomas. Arch Dermatol 142:131–138
9. Shellman YG, Chapman JT, Fujita M, Norris DA, Maxwell IH (2000) Expression of activated N-ras in a primary melanoma cell line counteracts growth inhibition by transforming growth factor-ß. J Invest Dermatol 114:1200–1204
10. Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis. Cell 86:353–364
11. Papetti M, Herman IM (2002) Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol 282:C947–C970
12. Norrby K (1997) Angiogenesis: new aspects relating to its initiation and control. APMIS 105:417–437
13. Verheul HM, Voest EE, Schlingemann RO (2004) Are tumours angiogenesis-dependent? J Pathol 202(1):5–13
14. Shima DT, Mailhos C (2000) Vascular development biology: getting nervous. Curr Opin Genet Dev 10:536–542
15. Dickson BJ (2002) Molecular mechanisms of axon guidance. Science 298:1959–1964
16. Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL (1999) Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science 283(5401):534–537
17. Oishi K, Uchida MK (2003) Functional differentiation of neural stem cells into endothelial cells. Nippon Yakurigaku Zasshi 122 (Suppl):27P–29P
18. Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC, Gage FH (2004) Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. Nature 430 (6997):350–356
19. Huttenbach Y, Prieto VG, Reed JA (2002) Desmoplastic and spindle cell melanomas express protein markers of the neural crest but not of later committed stages of Schwann cell differentiation. J Cutan Pathol 29(9):562–568
20. Akimoto S, Nakanishi Y, Sakamoto M, Kanai Y, Hirohashi S (2004) Laminin 5 beta3 and gamma2 chains are frequently coexpressed in cancer cells. Pathol Int 54(9):688–692
21. Seftor REB, Seftor EA, Koshikawa N, Meltzer PS, Gardner LMG, Bilban M, Stetler-Stevenson WG, Quaranta V, Hendrix MJC (2001) Cooperative interactions of Laminin 5γ2 Chain, Matrix Metalloproteinase-2, and Membrane Type-1-Matrix/Metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. Cancer Res 61:6322–6327
22. Hendrix MJ, Seftor EA, Kirschmann DA, Quaranta V, Seftor RE (2003) Remodeling of the microenvironment by aggressive melanoma tumor cells. Ann NY Acad Sci 995:151–161
23. Hendrix MJC, Seftor REB, Seftor EA, Gruman LM, Lee LML, Nickoloff BJ, Miele L, Sheriff DD, Schatteman GC (2002) Transendothelial function of human metastatic melanoma cells. Role of the microenvironment in cell-fate determination. Cancer Res 62:665–668
24. Goldfinger LE, Hopkinson SB, deHart GW, Collawn S, Couchman JR, Jones JCR (1999) The a3 laminin subunit, a6b4 and a3b1 integrin coordinately regulate wound healing in cultured epithelial cells and in the skin. J Cell Sci 112:2615–2629
25. Trikha M, Timar J, Zacharek A, Nemeth JA, Cai Y, Dome B, Somlai B, Raso E, Ladanyi A, Honn KV (2002) 2002 Role for beta3 integrins in human melanoma growth and survival. Int J Cancer 101(2):156–167
26. Döme B, Paku S, Somlai B, Tímár J (2002) Vascularization of cutaneous melanoma involves vessel co-option and has clinical significance. J Pathol 197(3):355–362
27. Tímár J, Döme B, Fazekas K, Janovics A, Paku S (2001) Angiogenesis-dependent diseases and angiogenesis therapy. Pathol Oncol Res 7(2):85–94, Review
28. Koistinen P, Ahonen M, Kähäri VM, Heino J (2004) alphaV integrin promotes in vitro and in vivo survival of cells in metastatic melanoma. Int J Cancer 112(1):61–70
29. Mitjans F, Meyer T, Fittschen C, Goodman S, Jonczyk A, Marshall JF, Reyes G, Piulats J (2000) In vivo therapy of malignant melanoma by means of antagonists of alphav integrins. Int J Cancer 87(5):716–723
30. Pignatelli M, Stamp G (1995) Integrins in tumour development and spread. Cancer Surv 24:113–127
31. Ivaska J, Heino J (2000) Adhesion receptors and cell invasion: mechanisms of integrin guided degradation of extracellular matrix. Cell Mol Life Sci 57:16–24
32. Mitra A, Chakrabarti J, Chatterjee A (2003) Binding of alpha5 monoclonal antibody to cell surface α5β1 integrin modulates MMP-2 and MMP-7 activity in B16F10 melanoma cells. J Environ Pathol Toxicol Oncol 22(3):167–178
33. Zambruno G, Marchisio PC, Melchiori A, Bondanza S, Cancedda R, De Lucca M (1993) Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. J Cell Sci 105:179–190
34. Dirkx AEM, Egbrink MGA, Castermans K, van der Schaft DWJ, Thijssen VLJL, Dings RPM, Kwee L, Mayo KH, Wagstaff J, Boumater Steege JCA, Griffioen AW (2006) Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors. FASEB J 20:621–630
35. Castermans K, Griffioen AW (2007) Tumor blood vessels, a difficult hurdle for infiltrating leukocytes. Biochim Biophys Acta, Rev Cancer 1776:160–174
36. Molema G (2002) Tumor vasculature directed drug targeting: applying new technologies and knowledge to the development of clinically relevant therapies. Pharm Res 19:1251–1258
37. Griffioen AW, Tromp SC, Hillen HFP (2002) Angiogenesis modulates the tumour immune response. Int J Expert Pathol 79:363–368
38. Griffioen AW (2008) Anti-angiogenesis: making the tumor vulnerable to the immune system. Cancer Immunol Immunother 57:1553–1558