The Role of Neutrophilic Inflammation, Angiotropism, and Pericytic Mimicry in Melanoma Progression and Metastasis

Jennifer Landsberg¹, Thomas Tütting¹, Raymond L. Barnhill²,³ and Claire Lugassy²,³

Angiotropism in melanoma correlates with ulceration and poor prognosis. It has been shown to be a marker of pericytic mimicry, that is, the spreading of tumor cells in a pericyte location along abluminal vascular surfaces. Such extravascular tumor spread may represent another form of tumor plasticity with reversion to a neural crest cell migratory phenotype. In a murine melanoma model, it has recently been demonstrated that neutrophilic skin inflammation promotes angiotropism and metastatic spread of primary melanomas. This review discusses the role of neutrophilic inflammation in angiotropism and pericytic mimicry in melanoma progression, metastasis, tumor cell plasticity, and tumor therapeutic resistance.

INTRODUCTION

Melanoma is one of the most aggressive cancer types because melanoma cells tend to metastasize early into multiple organs that can lead to death of patients. Recent experimental work in a murine melanoma model demonstrated that neutrophilic inflammatory responses in the skin can promote angiotropism, pericytic mimicry, and metastatic spread of primary melanomas (Bald et al., 2014). Angiotropism and pericytic mimicry could promote intravasation leading to intravascular metastasis, and/or extravascular migration of melanoma cells and metastasis to local or more distant sites. Thus, in addition to the classical pathway of “intravascular metastasis” of circulating tumor cells, emerging evidence supports an additional pathway of tumor spread along the abluminal vascular surfaces, without entrance into vascular channels. This process has been termed “extravascular migratory metastasis” (EVMM) (Lugassy et al., 2013a).

This review will discuss the new field of angiotropism and pericytic mimicry, and its links with tumor inflammation, particularly neutrophilic infiltrates, in melanoma progression and metastasis. It will especially articulate the idea that angiotropism and pericytic mimicry of melanoma cells represent a form of tumor cell plasticity with reversion to an embryonic phenotype. It will emphasize the analogies with extravascular migration and invasion programs occurring during the neural crest cell migration in the embryo (Lugassy et al., 2013a), and the role of inflammatory mediators in angiotropism and melanoma progression (Bald et al., 2014). Finally, this review will briefly discuss the potential role of angiotropism in resistance and recurrence after successful targeted therapy with a Braf inhibitor (Lugassy et al., 2014). Because lymph angiotropism of tumor cells has not been described thus far, this review will not discuss the role of lymphatics in melanoma progression and dissemination.

DESCRIPTION AND DEFINITIONS OF ANGIOTROPISM AND PERICYTIC MIMICRY IN MELANOMA

Dating back more than 15 to 20 years, Lugassy and Barnhill have conceived and developed the field of angiotropism, pericytic mimicry, and extravascular migration in melanoma (Lugassy et al., 2013a). Specifically, the term “angiotropism” represents a histopathological image, the term “pericytic mimicry” refers to the replacement of pericytes by angiotropic tumor cells, and the term “extravascular migration” is used to describe a mechanism of progressive tumor migration outside vessels versus the “express” intravascular dissemination. Angiotropic melanoma cells are defined histologically as melanoma cells closely associated with the endothelium of vascular channels in a pericytic location, and are generally detected at the advancing front of the tumor and a priori without intravasation (Barnhill and Lugassy, 2004) (Figure 1a–b). Ultrastructurally, angiotropic melanoma cells are linked to the endothelium by an amorphous matrix confirmed by immunohistochemistry to contain laminin (Lugassy et al., 1997, 1999). Current data have shown that angiotropism involves both microvessels and larger vessels (Lugassy et al., 2014); however, additional studies are needed to better discern the involvement of mature vessels versus neovessels.

PROGNOSTIC SIGNIFICANCE OF ANGIOTROPISM, PERICYTIC MIMICRY, AND NEUTROPHILIC INFLAMMATION IN MELANOMA

Several studies have demonstrated that angiotropism in melanoma is an independent prognostic marker predicting risk for metastasis (Bald et al., 2014; Barnhill et al., 2002; Lugassy et al., 2011; Van Es et al., 2008; Wilmott et al., 2012).
In addition, independent histopathological studies have demonstrated that angiotropism is significantly associated with metastasis, ulceration, and Breslow thickness (Lugassy et al., 2011; Wilmott et al., 2012). Ulceration of primary melanomas is an adverse prognostic factor and is part of the American Joint Committee on Cancer staging guidelines for melanoma (Balch et al., 2009). It has been hypothesized that ulceration may be associated with tumor-related inflammation and reduced antitumor immunity (Jewell et al., 2015). In addition, immunohistochemical staining of neutrophils in 186 primary melanomas confirmed the strong association between ulceration and neutrophilic inflammation (Jensen et al., 2012). Neutrophils, which are present in large numbers in ulcerated melanomas and are known for their proangiogenic properties, likely play an important functional role in tumor-promoting angiogenesis (Mentzel et al., 2001). Finally recently, in an unselected sentinel-node stage melanoma patient cohort (n = 178), we verified that angiotropism and lymph node metastases are associated with ulceration and prominent neutrophilic infiltration in primary melanoma (Bald et al., 2014).

**EXPERIMENTAL MODELS OF ANGIOTROPISM AND PERICYTIC MIMICRY**

Angiotropic melanoma cells replace pericytes in the vasculature, a process that has been described as pericytic mimicry (Lugassy et al., 2013a). Several experimental models have indicated that angiotropism promotes pericytic mimicry, the spreading and invasion of tumor cells along the abluminal vascular surfaces in a pericytic location. In vitro, it has been shown that cocultures of melanoma cells with endothelial tubules resulted in angiotropism and pericytic mimicry of melanoma cells (Lugassy et al., 2002). Live cell imaging enabled quantitative analysis of migration (cell path length and velocity) in single melanoma cells along vascular tubules (Zadran et al., 2013). Ex vivo studies using rat aortic rings (Bald et al., 2014; Lugassy et al., 2004) also resulted in angiotropism and pericytic mimicry of melanoma cells. In a chick chorioallantoic membrane assay (CAM) (Lugassy et al., 2007), the spreading of melanoma cells along the abluminal vascular surfaces was clearly demonstrated, and histopathology confirmed the angiotropism of melanoma cells without intravasation, as observed in human angiotropic
melanoma (Figure 1d,e). In a murine genetically engineered melanoma model, the distance of melanoma spread along dermal blood vessels was more than 25 mm and histopathology showed striking angiotropism, confirming pericytic mimicry (Bald et al., 2014) (Figure 1d–j).

EXTRAVASCULAR MIGRATORY METASTASIS

It is generally assumed that cells migrate passively intravascularly through the blood and/or lymph. In this perspective, angiotropism and pericytic mimicry could induce intravascular and intravascular tumor dissemination (Bald et al., 2014). In addition, it has been hypothesized that a continuous migration of angiotropic melanoma could constitute an alternative mechanism of tumor spread distinct from intravascular dissemination and termed extravascular migratory metastasis or EVMM (Lugassy et al., 2000). By this mechanism, tumor cells could spread to nearby, or to more distant sites, without entering vascular channels, representing an alternative metastatic pathway to “the inefficient intravascular metastatic process” (Talmadge and Fidler, 2010).

Importantly, vascular mimicry, a plastic, transendothelial phenotype, has recently been shown to promote intravascular metastasis (Wagenblast et al., 2015). Of interest, we have observed a switch between vasculogenic mimicry and pericytic mimicry in 3D culture when melanoma cells are cocultured with endothelial tubules rather than being cultured alone (Lugassy et al., 2013a and 2013b). It is therefore conceivable that such a “phenotype-switch” could occur when cells implicated in vascular mimicry are connected with endothelial-lined vasculature, and that both phenomena participate in EVMM.

Pertinent to EVMM are the origin of melanocytes from the neural crest and the strong analogies of EVMM with neural crest cell migration (Figure 1c), especially the migration along vessels in the embryo (reviewed in Lugassy et al., 2013a), suggesting that EVMM recapitulates neural crest stem cell migration to reach secondary sites.

ANGIOTROPISM AND PERICYTIC MIMICRY: A FORM OF MELANOMA CELL PLASTICITY

Several data strongly suggest that pericytic mimicry of angiotropic melanoma cells in their vascular niche represent tumor plasticity and induction of stem cell properties, in particular neural crest cell (NCC) migration (Lugassy et al., 2013b). Indeed, migration of NCC along the abluminal vascular surfaces has been recently confirmed by other authors (Lugassy et al., 2013b). Notably, frozen primary melanomas, previously utilized for gene expression profiling, were analyzed for angiotropism as a differential marker. This microarray analysis identified 128 genes differentially expressed in angiotropic versus nonangiotropic melanomas (Lugassy et al., 2011). From the results of this microarray analysis, 7 genes were linked to NCC migration: TCOF1, NEIL3, AHNAK, KCTD11, HMMR, CEBPA, and AQP3. In addition, in the study of Lugassy et al. (2013b), the interaction between the abluminal surface of endothelial cells and angiotropic melanoma cells triggered a differential expression of 28 genes. Among them, 20 have demonstrated properties linked to (besides inflammation mentioned below) cancer cell migration, cancer progression (CCL2, ICAM1, SELE, TRAF1, IL6, SERPINB2, and CXCL6), epithelial-mesenchymal transition (CCL2 and IL6), embryonic/stem cell properties (CCL2, PDGFB, EVX1, and CFDP1), and pericytic recruitment (PDGFB). In particular, 3 of these genes are associated with neural crest development (CCL2, PDGFB, EVX1, and CFDP1). These data suggest that angiotropism and pericytic mimicry induce the expression of genes linked to embryonic/NCC migration. Finally, laminins are essential for early embryonic development and organogenesis, and during the course of NCC migration, specific laminins are expressed by the basal surfaces of the epithelia lining these pathways (Durbeej, 2010). Using a chicken CAM melanoma model, it has been shown that several genes related to laminin were overexpressed in angiotropic melanoma cells (Lugassy et al., 2009). The presence of laminin between melanoma cells and endothelial cells raises the possibility that exposed cryptic promigratory sites on laminin trigger tumor cell migration along the abluminal surface (Lugassy et al., 2013a).

ROLE OF NEUTROPHILIC INFLAMMATION ON MELANOMA PLASTICITY, ANGIOTROPISM, AND PERICYTIC MIMICRY IN MELANOMA

Neutrophilic inflammation and endothelial cell activation in melanoma

Neutrophilic inflammation is a cocktail of growth factors, enzymes, and chemoattractants that, on release into the tumor microenvironment, have a significant impact on the existing vasculature (Tajzyman et al., 2013). One of these proangiogenic factors is the matrix metalloproteinase-9, a member of zinc-dependent endopeptidases that is involved in degradation of extracellular matrix and vascular remodeling. The proteolytic action of matrix metalloproteinase-9 mainly regulates the bioavailability of the vascular endothelial growth factor and the fibroblast growth factor 2 that are usually sequestered in an inactive form in the extracellular matrix (Vempati et al., 2014). It has been shown that matrix metalloproteinase-9 from myeloid cells is required for melanoma vasculogenesis (Ahn and Brown, 2008) and that matrix metalloproteinase-9 has a direct effect on melanoma cell migration (Orgaz et al., 2014). So far it has been difficult to verify experimentally that direct release of vascular endothelial growth factor from neutrophils is responsible for increased angiogenesis after neutrophil recruitment.

Neutrophilic inflammation and melanoma invasive state

Melanoma metastases involve melanoma cell plasticity leading to a phenotypic change from a proliferative to an invasive, migratory state. This principally reversible “phenotype-switch” model of metastasis includes a decrease of the differentiation status partly caused by decreased expression of the melanocyte master transcription factor microphthalmia-associated transcription factor (Hoek et al., 2008; Hoek and Godin, 2010). Investigations on how primary mouse melanomas resist an adoptive T-cell transfer therapy protocol targeting a melanocytic differentiation antigen revealed that proinflammatory mediators like tumor necrosis factor can shift the phenotype of melanoma cells toward a dedifferentiated state (Holzel et al., 2013; Landsberg et al., 2012). These data suggest that phenotype switching may be facilitated by a proinflammatory melanoma microenvironment.
Role of neutrophilic inflammation on angiotropism and pericytic mimicry

Experimental evidence that neutrophilic inflammation promotes angiotropism, pericytic mimicry, and metastatic spread came from a genetically engineered melanoma model, where neutrophilic inflammatory responses in the melanoma micro-environment, which were induced by repetitive exposure to sunburning doses of UV irradiation, led to selectively increased numbers of metastases in lymph nodes and lungs (Bald et al., 2014). Histopathologic analyses revealed strikingly increased angiotropism in UV-irradiated melanomas with increased local and systemic neutrophilic inflammation that correlated with the number of lung metastases (Figures 1f–1l and 2). Depletion of neutrophils or inhibition of their activation and recruitment via an high-mobility-group-protein B1-initiated, toll-like receptor 4- and MyD88-dependent innate signaling pathway significantly reduced both pericytic mimicry and the number of lung metastases. This provides the first experimental evidence that neutrophilic inflammation promotes metastatic spread through enhancement of reciprocal melanoma-endothelial interactions triggered by angiotropism.

In line with these data, it has been shown that a single UVB irradiation of SB-2 melanoma cells induces IL-8 mRNA and protein secretion and thereby enhances tumorigenicity and metastatic potential on transplantation into nude mice (Singh et al., 1995). UVB irradiation also induces IL-8 expression in different human melanoma cells and enhances motility of different human melanoma cells using time-lapse video-microscopy, suggesting that UVB can increase the aggressiveness of human melanoma cells among others by the major neutrophil chemoattractant IL-8 (Gebhardt et al., 2007). The fact that ulcerated melanomas show abundant neutrophils, reactive angiogenesis, and frequently angiotropism may provide a biological explanation for the clinical finding that ulceration of primary human melanomas is an independent adverse prognostic factor.

It has also been shown that the interaction of endothelial tubules and melanoma cells leading to angiotropism and pericytic mimicry in vitro triggered novel differential gene expression, including 10 genes linked to inflammation: CCL2, IL6, TRAF1, CXCL6, SELE, ICAM1, SLC7A2, C2CD4B, PDGFB, and SERPINB2 (Lugassy et al., 2013b). These data raise important questions about whether inflammation is the cause, the result, or both of the angiotropic phenotype of melanoma cells. Interestingly, in a murine model of brain metastasis from lung and breast cancer, SERPINB2 has recently been shown to promote cancer cell survival and vascular co-option (Valiente et al., 2014), a phenomenon most likely related to pericytic mimicry.

In addition, because laminin functions to link angiotropic melanoma and endothelial cells, it is interesting to note the role of neutrophil elastase and cathepsin G in laminin proteolytic modification and basement membrane alterations during inflammation (Heck et al., 1990). Furthermore, neutrophil elastase cleaves laminin-332 (laminin-5) generating peptides that are chemotactic for neutrophils (Mydel et al., 2008).

Using different in vitro experiments (Bald et al., 2014), we could also demonstrate that neutrophil-conditioned media or the proinflammatory cytokine tumor necrosis factor alone induces migration of murine and human cells toward endothelial cells in transwell assays. Inflammation also enhances the migration distance and velocity of melanoma cells on endothelial cell surfaces in 2D migration assays. An ex vivo 3D “ear crawl-in assay,” that was originally developed to study dendritic cell migration in the dermal interstitium, showed that human melanoma cells invade inflamed mouse ear tissue explants in close association with endothelial cells, that is, angiotropism.

In summary, neutrophil-derived tumor necrosis factor induces a more migratory and angiotropic melanoma cell phenotype in vitro and neutrophilic inflammation enhances

Figure 2. Graphical abstract illustrating how neutrophilic inflammation can promote angiotropism, pericytic mimicry, and metastasis of melanoma.

Neutrophilic inflammation shifts melanoma cells toward a migratory phenotype, promotes melanoma cell spreading in a pericyte location along abluminal vascular surfaces, and enhances the metastatic capacity. Further investigations are needed to clarify the relative importance of extravascular migratory metastasis versus intravascular dissemination in melanoma progression.
angiotropism and metastatic spread in vivo. These data support the idea that neutrophilic inflammation in ulcerated melanomas contributes to pericytic mimicry and metastasis by shifting melanoma cells toward the migratory angiotropic phenotype, activating endothelial cells, and fostering reciprocal melanoma-endothelial interactions.

**RESISTANCE TO BRAF INHIBITOR THERAPY, INFLAMMATION, AND ANGIOTROPISM**

Tumor cell plasticity leads to cancer cells with stem cell properties that are often resistant to therapy, and angiotropic melanoma cells in their vascular niche overexpress genes related to stem cell properties (Lugassy et al., 2013a). Resistance to (V600E) B-RAF kinase inhibitor emerges in part by melanoma cells upregulating platelet-derived growth factor receptor, beta polypeptide (PDGFRB) (Shi et al., 2011; Sun et al., 2014) and PDGFRB has been associated with angiotropic melanoma cells (Lugassy et al., 2013a). In a preliminary study, it has been shown that PDGFRB was expressed by angiotropic melanoma cells occupying a pericytic location in several metastatic samples from BRAF-resistant recurrences, versus the absence of such expression before the onset of resistance (Lugassy et al., 2014). Notably, the most frequent anti-BRAF therapy side effects are inflammatory dermatoses (Filitis and Mahalingam, 2013) characterized by a neutrophil-rich skin infiltrate, which is known to induce angiotropism and melanoma progression (Bald et al., 2014). Ongoing studies are aimed to verify the hypothesis that neutrophilic inflammation and angiotropism could be implicated in the resistance to BRAF inhibitor therapy.

**CONCLUSIONS**

Emerging evidence supports the idea that neutrophilic inflammation increases melanoma cell plasticity and facilitates the reactivation of migration and invasion programs that are observed in their neural crest precursor cells during embryonic development, in particular angiotropic migration. This inflammation-induced angiotropism and pericytic mimicry may provide a biological explanation for the clinical finding that ulceration of primary human melanomas is an independent negative prognostic factor. The emerging field of angiotropism and pericytic mimicry clearly mandates much more research as to (i) the precise molecular mechanism of this angiotumoral association and (ii) the relative importance of extravascular migratory metastasis versus intravascular dissemination in melanoma progression. Our ultimate goal is to define new molecular targets to reduce or prevent melanoma metastasis.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

We would like to thank Tobias Bald for helpful discussions. This work was supported by the following grants: German Research Foundation SP A12 in the SFB 832 and SP22 in the SFB704 to TT and BONFOR to JL. TT is the member of the Excellence Cluster Immuno Sensation at the University of Bonn, Germany.

**REFERENCES**


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/