REVIEW

Why target the tumor stroma in melanoma?

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Abstract Melanoma metastasis is fatal. Melanoma cells are often characterized by an activated extracellular signal-regulated kinase (ERK) pathway downstream of mutations in BRAF. Therapies targeting these BRAF mutations are useful for a while; however, patients ultimately develop resistance to these therapies. Recent evidence suggests that this resistance occurs when tumor cells leave their microenvironment and migrate on a stiff, activated tumor stroma; that is, this resistance is linked to the presence of an extracellular matrix reminiscent of a fibrotic microenvironment. These data suggest that agents targeting fibrosis might be used to treat melanoma. We therefore discuss what is known about the tumor stroma in melanoma. An emergent target, CCN2 (CTGF), that is required for fibrosis, may also be a good target for drug-resistant melanoma. Intriguingly, anti-CCN2 antibodies are currently under clinical development.

Keywords CTGF · CCN2 · Tumor stroma · Melanoma · Braf

Introduction

Melanoma, a cancer that develops from melanocytes, is the second most common invasive cancer in young adults, and the incidence of diagnosis among this group is only increasing (Reed et al. 2012). Of the three types of skin cancer (basal cell carcinoma, squamous cell carcinoma, and melanoma) it is the least common; it accounts for only 4% of skin cancers, but its

highly metastatic nature makes it so deadly that it accounts for over 80% of skin cancer deaths (Miller and Mihm 2006).

Melanoma progresses through several stages as it develops, and the survival rate of patients is highly variable based on the stage at which it is diagnosed. A lesion diagnosed before metastasis has occurred has The current staging system used to evaluate melanomas is the TNM staging system most recently updated by the American Joint Committee on Cancer in 2009 (Balch et al. 2009). The TNM system is named for the three factors measured: primary Tumor characteristics (such as thickness, ulceration, and mitotic rate), presence or absence of metastases in regional lymph Nodes, and presence or absence of distant Metastases (Balch et al. 2009). Proper staging is important in developing a prognosis for patients diagnosed. For example, a patient diagnosed while the tumor is thin and has not metastasized has a 95% 10-year survival rate, whereas a patient with multiple regional lymph node metastases has a 10-year survival rate of only 33% (Balch et al. 2009).

Under healthy conditions, melanocytes are found spread throughout the basal layer of the epidermis at a 1:10 ratio with keratinocytes where they extend their dendrites between keratinocytes to facilitate delivery of melanin (Cichorek et al. 2013). Melanocytes are also found in hair follicles, the uveal tract of the eye, and in lesser numbers in several other tissues (Shain and Bastian 2016). In the epidermis, each melanocytes and its associated keratinocytes are known as an Epidermal-Melanin Unit (Haass and Herlyn 2005). In this state, their association with keratinocytes and their E-cadherin mediated cell-to-cell contacts regulate their proliferation and prevent transformation; in fact, melanocytes grown in monoculture begin expressing surface markers consistent with those acquired during melanomagenesis; these markers disappear when the cells are then co-cultured with undifferentiated keratinocytes (Haass and Herlyn 2005). Typically melanocytes will escape the control of their associated

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keratinocytes in two circumstances: To divide, which occurs approximately twice a year, or as a result of a mutation (Shain and Bastian 2016). These mutations can be familial (such as mutations in *CDKN2A*) or somatic (such as mutations in *NRAS* or *BRAF*), but these mutations are frequently insufficient to drive melanocytes transformation on their own, and require additional mutations which are frequently acquired through UV exposure (Sample and He 2017).

Once sufficient mutations are accumulated, the affected melanocytes will undergo the first stage of melanoma development in which they decouple from their associated keratinocytes, proliferate and form a benign nevus – also known as a mole (Miller and Mihm 2006). The majority of nevi enter a senescence-like state in which very few of the melanocytes within them are able to proliferate further until they acquire further mutations, such as inactivating mutations of the p53 tumour-suppressor gene (Miller and Mihm 2006; Shain and Bastian 2016). In this state the nevus is typically symmetrical, smooth, evenly coloured, and does not penetrate the basement membrane (Miller and Mihm 2006).

Once melanocytes within a benign nevus acquire sufficient mutations to escape its senescence-like state, melanoma progresses to the second stage of development and becomes a dysplastic nevus. Typically at this stage the lesion becomes asymmetrical, with abnormal coloration patterns and the melanocytes within it become irregularly and abnormally shaped (Miller and Mihm 2006). While a benign nevus has very little proliferation, once its senescence-like state has set in, it is believed that dysplastic nevi engage in constant, slow proliferation that is offset by attritional factors which are sometimes strong enough to reverse the development of the lesion entirely (Shain and Bastian 2016).

The third stage, known as the radial growth phase is characterized by a cancerous histological phenotype, rapid growth across the epidermis with only minor incursions across the basement membrane, and constitutive activation of ERK signaling to drive rapid clonal cell proliferation (Haass and Herlyn 2005; Miller and Mihm 2006; Shain and Bastian 2016). Explanted cells from lesions in the radial growth phase, or any earlier phase, are typically unable to produce colonies in soft agar, as they are typically reliant on exogenous growth factors (Miller and Mihm 2006; Steck 2014). Since the cells are largely unable to penetrate deeper than the basement membrane, surgical excision up to this stage remains highly effective (Steck 2014).

However, crucially once the lesion breaches the basement membrane and begins to expand through the dermis it has reached the vertical growth phase (Nesbit and Herlyn 1994; Miller and Mihm 2006). This phase is characterized by a loss of E-cadherin expression, increased N-cadherin expression, and increased $\alpha V\beta 3$ integrin expression that results in induction of MMP2, to break down the collagen of the basement membrane, and BCL-2, to prevent apoptosis in the absence of

cell-cell contacts, which collectively permit explanted cells to colonize soft agar (Kuphal et al. 2005; Miller and Mihm 2006; Shain and Bastian 2016). Additionally, the shift from E-cadherin to N-cadherin expression allows melanoma cells to interact with fibroblasts and vascular endothelial cells to better facilitate migration and intravasation (Haass and Herlyn 2005). It has been theorized that this newfound ability to communicate and attach to fibroblasts might be instrumental in facilitating the transition from the radial growth phase to the vertical growth phase (Haass and Herlyn 2005). At this point the tumor is fully competent to invade and metastasize to distant organs, though continuing accumulation of mutations may speed this process (Steck 2014). Once metastasis to distant organs has occurred, the tumor has entered its final stage is now termed metastatic melanoma.

Traditional chemotherapies are ineffective

Despite major advances in therapies for other cancers, melanoma remains difficult to manage. Although other cancers are vulnerable to chemo- and radio-therapies, melanoma is largely unaffected by these traditional standbys (Soengas and Lowe 2003). Treatment with dacarbazine, temozolomide, or fotemustine have very low response rates (with complete response in less than 5% of patients and most responses lasting only ~1.5 months) and toxic combinatorial treatments not showing any significant increase in survival (Thompson et al. 2005; Palathinkal et al. 2014). Surgical excision is highly effective but only when melanomas are caught early; while resection of non-metastatic lesions provides an approximately 90% 5-year survival rate, even the presence of microsatellite metastases (discrete lesions greater than 0.05 mm in diameter separated from the primary lesion) decreases this survival rate from 90% to 36% (Honsi et al. 2005; Cummins et al. 2006). While conventional chemotherapies have failed, and surgical excision is of little use in late stage melanoma there are several promising therapies.

BRAF inhibitors work to a point

BRAF is a protein kinase of the RAF family that functions downstream of RAS to help stimulate cell growth and survival by phosphorylation of MEK to activate ERK (Chan et al. 2017). In 2002 large-scale sequencing of the RAS-RAF-MEK-ERK-MAPK pathway revealed a mutation in the BRAF gene resulted in constitutive activation of the pathway (Davies et al. 2002). The vast majority (92%) of these mutations were in the same codon: BRAFV600E, which prevents folding of BRAF in its inactive state and instead results in a new conformation that has 10–12 fold increased kinase activity (Davies et al. 2002; DeLuca et al. 2008). Present in approximately 60% of melanomas, BRAF mutations drive

melanoma progression from the earliest stages, even contributing to the formation of a benign nevus (Miller and Mihm 2006). While it is insufficient on its own to generate a cancerous lesion, once a melanocyte acquires secondary mutations BRAF becomes a major driver of disease progression (Miller and Mihm 2006; DeLuca et al. 2008). Since the discovery of the BRAFV600E mutation, several inhibitors of its activity have been developed. The two most promising inhibitors are vemurafenib and dabrafenib, both of which have gone through phase III clinical trials. While they both show significant increases in both overall and progression-free survival, they also have approximately 50% response rates, and both only increased overall survival by ~4 months (DeLuca et al. 2008; Lim et al. 2017). While intrinsic resistance to BRAF inhibition is only present in approximately 10% of BRAFV600E mutant melanomas, acquired resistance is rapidly acquired by almost all patients (Lim et al. 2017). The exact mechanism behind this resistance is still being studied, but potential mechanisms include expression of splice variants with reduced inhibitor affinity, BRAF allele amplification, overexpression of alternative RAF isoforms, and activation of alternative ERK signaling pathways (Lim et al. 2017). One of the side-effects of the inhibition of BRAFV600E is a paradoxical activation of wild-type BRAF in non-mutant cells. This has been shown to occur not only in wild-type BRAF melanomas, but also in the cancer associated fibroblasts of the stroma which results in increased contractility and matrix deposition (Hirata et al. 2015). When BRAFV600E cells are grown in the presence of cancer associated fibroblasts, or when plated on an ECM layered rigid substrate, they displayed BRAF inhibitor resistance that was disrupted by inhibition of integrin β 1 or FAK indicating that the stiff matrix created by activation of the fibroblasts in the stroma was conferring BRAF resistance through an alternate ERK signaling pathway that relied upon integrin-mediated attachment to ECM (Hirata et al. 2015). Disruption of this activated, stiff matrix (tumor stroma) might be a potential strategy to prevent BRAF inhibitor resistance.

Novel therapeutic approaches: Targeting the stroma?

In melanoma, the tumor stroma is relatively understudied. As a tumor develops it not only alters the cells carrying oncogenic mutations, but also begins to recruit host tissue cells to form an active stroma that participates in the development and progression of the disease (Le Bitoux and Stamenkovic 2008). As the tumor grows it begins to secrete cytokines that recruit and activate several different cells types which develop into macrophages, mast cells, adipocytes, and carcinoma-associated fibroblasts which begin to secrete cytokines, disrupt existing extracellular matrix, and deposit new extracellular matrix in a fashion that can play a significant role in the progression of the disease

(Le Bitoux and Stamenkovic 2008). Understanding how the tumor stroma acts to promote melanoma may be useful in uncovering novel antifibrotic targets.

The dermis is a vascularized region composed primarily of large amounts of extracellular matrix, fibroblasts, and macrophages (Kanitakis 2002). It is divided into two layers: the papillary dermis, which lies just below the basement membrane and is named for fingerlike dermal papillae it forms in order to increase surface area of attachment with the basement membrane, and the reticular dermis (Kanitakis 2002). The papillary dermis consists of loose connective tissue that supports capillary networks which deliver oxygen and nutrients to both the dermis and the epidermis, as well as tactile sensors, while the reticular dermis is characterized by thicker connective tissue which provides strength to the dermis, and the presence of larger blood vessels which feed the capillaries of the papillary dermis (Kanitakis 2002; Oosterhoff et al. 2012). The reticular dermis also houses the roots of hair follicles, along with sebaceous and sweat glands (Kanitakis 2002). The vast majority of the mass of the dermis is made up of collagen fibres (mainly types I and III) with 90% of the fibres being collagen, providing the dermis with a high tensile strength (Kanitakis 2002; Arora et al. 2017). In addition to collagen, elastin and elastic fibres are present (mostly in the reticular dermis), comprising another 2–4% of the dermis, with the rest being composed of cells and a mixture of glycoproteins and proteoglycans known as ground substance (Kanitakis 2002, Arora et al. 2017). The extracellular matrix of the dermis is undergoing constant maintenance and has a high rate of turnover, with the synthesis of new connective tissue being conducted by spindle shaped cells called fibroblasts (Kanitakis 2002; McAnulty 2007). Fibroblasts regulate the extracellular matrix of the dermis by producing matrix metalloproteinases, which degrade extracellular matrix components, and by producing the majority of extracellular matrix components themselves (McAnulty 2007). In addition to their role in maintaining healthy extracellular matrix, fibroblasts are also key players in responding to tissue injury. When tissue is damaged, cytokines are released that recruit fibroblasts to the wound to assist in deposition of new extracellular matrix and they are activated into a myofibroblast phenotype that is characterized by expression of α -smooth muscle actin stress fibers which allow them to assist with contraction and closure of the wound (McAnulty 2007).

When tissue experiences an injury or insult it goes through several stages in attempting to resolve the damage: Hemostasis (when the injury has caused bleeding), Inflammation, Proliferation of new tissues, and finally Remodelling of the newly formed tissue (Buganza Tepole and Kuhl 2013). In the case of cancer, however, the insult is not easily resolved by these processes, and so the cancer cells persist, stimulating a wound healing response that is unable to resolve (Horimoto et al. 2012; Kalluri 2016). This results in sustained

inflammation, and the recruitment of a wide variety of cells to form an activated stroma, similar to what occurs in fibrotic diseases like Scleroderma. In fact, activated tumour stroma and fibrosis are both characterized by high numbers of activated fibroblasts and an increased deposition of extracellular matrix (Kalluri 2016). The link between cancer and fibrosis is reinforced by the fact that co-morbidity of cancer and scleroderma has been shown to occur quite frequently with the incidence of melanoma being 3.3 times as high among patients diagnosed with scleroderma as among the general population (Videtic et al. 1997; O'Byrne and Dalgleish 2001; Coussens and Werb 2002; Hill et al. 2003; Shah et al. 2010; Franks and Slansky 2012). In addition to this increased incidence, there is a notable temporal proximity between diagnoses of cancer and fibroproliferative diseases, with almost 50% of breast cancer diagnoses amongst scleroderma patients occurring very close to the diagnoses of scleroderma itself (Shah et al. 2010). There is considerable debate as to what is causing this increased association of not only the incidence, but also time of onset between cancer and fibroproliferative diseases. One theory is that the overactive fibroproliferative processes in diseases such as scleroderma not only lay down excessive extracellular matrix which can lead to organ failure, but they also produce a tumour stroma which is conducive to the progression of malignancy (O'Byrne and Dalgleish 2001; Rakoff-Nahoum 2006). Additionally, the inflammatory cascade produced by an initial injury or insult produces high amounts of oxidative stress, resulting in the production of reactive oxygen species that not only perpetuate the inflammatory cascade but also cause DNA damage leading to DNA breaks, single base mutations, and genomic instability which are known to lead to tumourigenesis (Le Bitoux and Stamenkovic 2008; Elinav et al. 2013). Cytokines like tumour necrosis factor α , interleukin 1 β , and TGF β 1 trigger signaling cascades that introduce mutations in the genes encoding p53 and Myc which contribute to oncogenesis (Elinav et al. 2013). These cytokines also induce nuclear factor- κ B signaling, which increases pro-inflammatory, proliferative, and survival gene expression from both tumour and stromal cells (Le Bitoux and Stamenkovic 2008; Kalluri 2016).

Once oncogenesis has occurred a diverse tumour stroma is recruited, most importantly including the recruitment of immune cells and the recruitment and activation of activated cancer associated fibroblasts (Le Bitoux and Stamenkovic 2008; Horimoto et al. 2012; Kalluri 2016). Recruited immune cells play several roles, with activated macrophages producing cytokines to increase inflammatory signaling throughout the stroma, recruit additional immune cells like leukocytes, inhibit immune response, increase pro-angiogenic signaling, and promote gene expression associated with survival (Le Bitoux and Stamenkovic 2008). Additional immune cells recruited by macrophages serve to amplify the signals secreted by macrophages, activate resident fibroblasts, and secrete proteolytic enzymes which can reduce physical barriers to tumour spread

and cause the release of sequestered growth factors from the extracellular matrix (Le Bitoux and Stamenkovic 2008; Horimoto et al. 2012).

The activated fibroblasts of the tumour stroma have proven to be key drivers in the progression of many different cancers. They have been shown to induce invasion of non-invasive cells in many cancer models, they secrete matrix metalloproteinases which help remodel stromal matrix to increase stiffness and align extracellular matrix components to facilitate invasion, they secrete TGF β allowing improved adhesion of cancer cells to the extracellular matrix, and they have been shown to be highly migratory (Horimoto et al. 2012, Kalluri 2016). Additionally, cancer associated fibroblasts secrete tenascin C and periostin, which have both been shown to be required for the development of a "cancer stem cell" phenotype that is able to take root in distant organs after metastasis and form metastatic sites (Horimoto et al. 2012). Cancer associated fibroblasts are a highly heterogeneous population: Although there are several markers which are absent in inactivated normal fibroblasts, such as fibroblast specific protein 1, vimentin, α smooth muscle actin, PDGF receptor α and β , these markers are neither exclusive to activated fibroblasts, nor expressed by all cancer associated fibroblasts with fibroblast specific protein 1 also identifying immune cells, vimentin being expressed widely in mesenchymally derived cells, and the PDGF receptors being expressed in perivascular cells (Kalluri 2016). Part of the reason for this heterogeneous marker expression pattern might be the highly heterogeneous populations that activated fibroblasts can arise from. Cancer associated fibroblasts have been seen to arise from bone marrow-derived precursors, mesenchymal stem cells, endothelial cells, resident fibroblasts, epithelial cells, pericytes, smooth muscle cells, and adipocytes (Bhattacharyya et al. 2012; Horimoto et al. 2012; Kalluri 2016). In fibrotic diseases, recruitment and proliferation of these cell types has been induced inflammatory cytokines, and differentiation has been seen to be caused by several different mechanisms. Resident fibroblasts can be differentiated into myofibroblasts by Transforming Growth Factor β 1 signaling or by mechanical strain being transduced through integrins (Koivisto et al. 2014). Blocking integrins α v β 5 or α v β 3 in vitro is able to prevent this differentiation, as is deletion of integrin β 1 in vivo (Liu et al. 2010; Koivisto et al. 2014). The protein Phosphatase and tensin homolog (PTEN), a tumor suppressor that inactivates adhesive signaling, is frequently mutated in cancers; PTEN can suppress myofibroblast differentiation of resident fibroblasts and loss of PTEN not only accelerates cancer progression, but when it is deleted from fibroblasts in vivo it results in spontaneous generation of fibrosis (Horimoto et al. 2012; Parapuram et al. 2015). The matricellular protein CCN2 (CTGF) is required for the fibrosis that occurs to the loss of PTEN; also, CCN2 expression by CAFs is required for melanoma metastasis (Parapuram et al. 2015; Hutchenreuther et al. 2015) strongly indicating a link between fibrosis and melanoma metastasis.

Indeed, CCN2 has emerged as a target for both fibrosis and cancer metastasis (Riser et al. 2015).

Summary

Melanoma metastasis is a major clinical problem. The role of the tumor stroma in this process is relatively underappreciated; however, CCN2 has emerged as a potential therapeutic target blocking activation of connective tissue both in fibrosis and in cancers.

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