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The Role of the Microenvironment – Models for the Study of Melanoma

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1. Introduction

Melanoma is a progressive disease that claims many lives each year due to lack of therapeutics effective for the long-term treatment of patients. Currently, the best treatment option is early detection followed by surgical removal. Cutaneous melanoma continues to represent both a challenge and a big paradox among solid tumors: though considerable prognostic markers are available, little is known about their biological significance. Recent data on the effect of an anti-melanoma target therapy, ipilimumab (Hodi *et al*, 2010) and new anti-BRAF molecules (Flaherty, 2010) raised hope on the treatment of melanoma. However, although response rates with small molecule inhibitors are high, most are not durable. Moreover, for a large subset of patients, reliable predictive biomarkers especially for immunologic modulators have not yet been identified. Progress on the treatment of both early and advanced melanoma may depend on identifying additional molecular targets and on understanding the mechanisms leading to response or resistance.

Animal models have been critical in the study of the molecular mechanisms of cancer and in the development of new therapeutic agents; nevertheless, there is still much room for improvement. The most widely used *in vivo* model involves the injection of tumor cells in the flank of mice. The relevance of each particular model depends on how close it replicates the histology, physiological effects, biochemical pathways and metastatic pattern observed in the same human tumor type. Numerous models have been developed to study human tumorigenesis and its properties, such as proliferation, migration, invasion, neoangiogenesis, and metastasis, as well as for the study of anti-cancer treatments. Among others, these include *in vitro* systems such as focus formation in tumor cell culture explants and continuous cell lines grown on tissue culture plates, or, alternatively, anchorage-independent growth in soft agar. The foregoing experimental models are suitable for studying molecular pathways in cell-cell and cell-extracellular matrix interactions that would be difficult to dissect in the animal. However they will encounter their limitation in terms that they are not particularly amenable to the investigation of interactions of tumor cells with the surrounding microenvironment of adjacent normal human cell tissues and structures. It has

been shown that tumor progression is associated with extensive remodeling of adjacent tissues to provide a supportive environment for tumor growth, angiogenesis, invasion, and metastasis of cancer cells (Hanahan & Weinberg, 2000; Bissell & Radisky, 2001; Fidler 2002; Chambers *et al*, 2002)

In this chapter we will discuss the role of the microenvironment in the development of melanoma. Finally we will discuss the design of *in vivo* and *in vitro* models as tools for understanding the biology of melanoma as well as their utility for the evaluation of new treatments.

2. Melanoma anatomic clinical types

There are mainly four types of cutaneous melanoma:

Superficial spreading melanoma. The most commonly occurring melanoma, it accounts for approximately 70% of melanoma cases. It arises at the site of a pre-existing nevus. It affects men and women equally but it appears more frequently on the lower extremities in women and on the upper back in men. Superficial spreading melanoma often evolves during a period of 1 to 7 years.

Nodular melanoma. Up 15% of the cases and is more common among older males, particularly in trunk, head and neck. It is an aggressive type of melanoma due to its tendency to grow in depth invading the dermis and giving early metastasis. It evolves in months to less than 5 years.

Lentigo maligna melanoma. Occurs in 5% of the cases. It is diagnosed in the elderly on regions of the skin that were overexposed to the sun such as head, neck, and the dorsum of hands. This melanoma grows slowly in a radial fashion over a period of 5-20 years.

Acral lentiginous melanoma. Makes up 8% of the melanoma cases, but it is the most common melanoma diagnosed in dark-skinned people.

Cutaneous head and neck melanomas constitute 12%–21% of melanomas diagnosed annually and they have poorer outcomes relative to melanomas of other sites. Among head and neck melanomas, scalp/neck melanomas have a greater risk of melanoma-specific mortality (Tseng & Martinez, 2010).

Malignant melanomas may also arise from the melanocytes present at extracutaneous sites (noncutaneous melanomas) namely eye, anogenital regions, mucosal surfaces, nail beds, conjunctivae, orbit, esophagus, and leptomeninges. Unlike cutaneous melanomas, there is lack of association with sun damage, family history and precursor nevi in these neoplasms. Noncutaneous melanomas are rare neoplasms with a poor prognosis, they can metastasize to lymph nodes, bone, lung, liver, spleen, gastrointestinal tract, kidneys, adrenals, and subcutaneous tissue (reviewed in Hussein, 2008). In this chapter we will focus only in cutaneous melanomas.

3. The role of the microenvironment in the development of cutaneous melanoma

The microenvironment is a key factor in tumor progression. Poorly aggressive melanoma cells acquired a vasculogenic phenotype when they were exposed to a microenvironment preconditioned by aggressive metastatic melanoma cells (Seftor *et al*, 2006). The opposite effect has also been studied long ago by Illmensee & Mintz (1976) who showed that teratocarcinoma cells could be normalized by the blastocyst environment. Melanoma

metastatic cells could be reverted to its cell type of origin, the neural-crest-derived melanocyte using an embryonic chick model (Kasemeier-Kulesa *et al*, 2008). Thus this evidence shows that whether a melanocyte remains normal or progresses to a neoplastic state would depend on the microenvironment, indicating that clues to understand the etiology of melanoma should be found at the tissue level of organization (Sonnenschein & Soto, 1999)

The frequency of melanoma tumors appears to be site -specific but yet this phenomenon is not well understood. Thus, we will analyze whether the characteristics of the skin of various anatomic sites might account for the different types of melanoma. Particularly we will focus on the cellular components, reactivity and biomechanical properties of the skin in distinct anatomic sites.

The human skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue. The dermal–epidermal junction is undulating in section; ridges of the epidermis project into the dermis. The junction provides mechanical support for the epidermis and provides a partial barrier against exchange of cells and large molecules. A fatty layer underlies the dermis, the panniculus adiposus, usually designated as "subcutaneous". The major cellular components of the epidermis are keratinocytes, melanocytes, Langerhans cells, Merkel cells, and dendritic cells, and in the dermis, fibroblasts, mast cells, macrophages and micro-vascular cells (endothelial cells and smooth muscle cells) and adipocytes in the underlying layer (McGrath *et al*, 2010).

Skin fibroblasts from different anatomic sites have distinct phenotypes, a phenomenon called topographic differentiation, which is maintained even after fibroblasts are isolated and cultured *in vitro*. A differential expression of HOX proteins which are involved in site-specific organization and in the migration of epidermal cells through integrins and cadherins was described. HOXA13 was expressed in toe and foreskin fibroblasts while HoxD9 was expressed in upper arm fibroblasts (Chang *et al*, 2002). Fibroblasts derived from soles and palms differ from those at other body sites. Interestingly, expression of keratin 9 was not observed in cultured non-palmoplantar keratinocytes when cultured alone or with non-palmoplantar fibroblasts. However, those keratinocytes expressed keratin 9 mRNA when co-cultured with palmoplantar fibroblasts, indicating that the phenotype of keratinocytes is anatomically-dependent. Failed migration of melanocytes and pigmentation in palms and soles appears to be regulated by the fibroblasts (Yamaguchi *et al*, 1999, 2005). Indeed, melanocyte density in the trunk skin of Caucasians, Asians and African-Americans was found to be similar, but was five-fold lower in palmoplantar skin.

Fibroblasts and keratinocytes also seem to participate in the development of melanomas from pre existent nevi (Coleman & Lugo, 1998; Fiuraskova *et al*, 2005), such as in the case of superficial spreading melanomas.

A type of dendritic cells, the Langerhans cells, are thought to play an important role in skin immunity. Studies in inflammatory ear skin explants of mice showed that migrating Langerhans cells were able to stimulate CD8+ T cell responses (reviewed in Stoitzner 2010). Another study showed that the antigenic response was dependent on Langerhans cells when the antigen was applied to the flank, but not when applied to the ear skin of mice (Wang *et al*, 2008). In the human the number of Langerhans cells in hair-bearing skin (chest, scalp and abdomen) is higher compared to hairless skin (palm and sole). On the other hand, higher numbers of Langerhans cells were found in the dorsum of the hand and foot compared to palm and sole (Thomas *et al*, 1984). The role of Langerhans cells is still

controversial, pointing out that it might be equally strongly dependent on both the anatomic site and the condition of the skin.

Mast cells play an active role in inflammatory and allergic reactions. They also participate in tissue remodeling; through the releasement of histamine they control the growth of the epidermis by inhibiting the growth of keratinocyte epithelium. Histamine induces the production of MMP-9 in keratinocytes thereby facilitating the migration of immune cells, such as T cells, across the vascular basement membrane or into the epidermis during skin inflammation (reviewed in Harvima, 2008). Morever, human skin mast cell extracts have proven to enhance gel contraction in 3-D cultures of skin fibroblasts possibly by inducing collagen production and organization (Garbuzenko *et al*, 2002). A study showed that mast cells distribution in normal adult skin is site dependent. Higher numbers were found in the distal extremities, forearm and lower leg, compared with those in the trunk, upper leg, and upper arm. Mast cells on the face were not counted (Janssens *et al*, 2005).

UV radiation and aging are factors known to predispose to the development of cutaneous melanomas. This could be attributed to the onset of an inflammatory condition, particularly in the case of UV radiation, as well as to changes in the biomechanical properties of the skin. The role of inflammatory reactions in the development of cutaneous melanoma was demonstrated in a recent study in volunteers older than 40 years of age. The results indicated that the use of aspirin for 5 years or more reduced the risk of cutaneous melanoma by almost half (Curiel-Lewandrowski *et al*, 2011). UVB is a key factor during extrinsic skin aging, it induces collagen cleveage changing the structure of the skin. Because the collagen network can not be completely reconstituted *de novo*, skin elasticity is impaired in the long term. Indeed, in a study where human dermal fibroblasts cultured in 3-D collagen gels were irradiated with UVB it was shown that collagen degradation inhibited the synthesis of hyalorunic acid by the fibroblasts (Röck *et al*, 2011). Thus it is most likely that photoaging alters the extracellular matrix, which in turn induces changes on the phenotype of the embedded cells such as keratinocytes, fibroblasts and dendritic cells.

The biomechanical properties of the skin such as thickness and extensibility decrease with age, most likely due to a degeneration of the collagen network and the loss of glycosaminoglicans. This is particularly true in sun exposed areas on the face and neck (Escoffier *et al*, 1989; Cua *et al*, 1990), coincidently with the site of preference of melanomas with poorer prognosis. In a different study shoulder skin resulted to be thicker than thighs and calves skin (Smalls *et al*, 2006).

4. Building models to study the biology of melanoma

4.1 *In vivo* models

Since the microenvironment plays a key role in cancer development, accurate models for the study of melanoma should take into account the differential features of the anatomic sites of the skin. The most widely used *in vivo* model for the study of melanoma is the inoculation of tumor cells in the flank of mice. In a previous work we investigated whether the dermis of a different anatomical site of mice would offer different characteristics than those of the flank. Thus, we chose the dorsal region of the foot and compared it to the flank in terms of cellular influx and reactivity (Speroni *et al*, 2009a). We further compared the acute and chronic inflammatory response in the selected areas of the skin in order to gain better understanding of the reactivity of both anatomic sites.

4.1.1 The inflammatory response in distinct anatomic sites of the skin of mice
4.1.2 Nonspecific inflammatory response

We tested the acute inflammatory response of the two anatomic sites induced by an extract of lung homogenate in Balb/c mice. The release of histamine and bradykinin by mast cells induces edema after 2-3 h that persists for up to 24 h post injection. A 0.1 mg/ml lung homogenate (Van den Brenk *et al*, 1974) was prepared and 0.05 ml of this suspension was intradermically injected in the dorsal region of the foot and in the flank of Balb/c mice. Swelling was recorded as skin thickness using a dial micrometer (Pocotest, GESSH) and 3 mm^2 of skin from the injected site were excised and weighted. Controls were inoculated with PS.

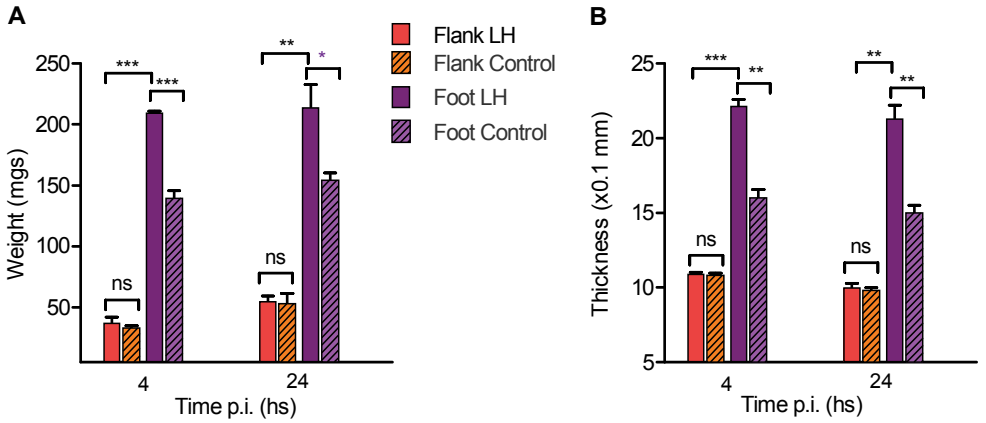


Fig. 1. Inflammatory response of the skin following lung homogenate (LH) injection. (A) weight and (B) thickness of the inoculated dermis of flank and dorsal region of the foot were recorded 4 and 24 h post injection (n=4). *, $p<0.05$; **, $p<0.005$; ***$p<0.001$ significant differences, ns, not significant; student t test

The injection of lung homogenate induced a significant inflammatory response in the dorsal region of the foot as evidenced by the significant increase in weight and thickness of the injected site. The edema persisted up to 24 h post injection. To the contrary, the skin from the flank did not show a significant response to the same inflammatory stimulus, weight and thickness were comparable to controls (Figure 1) (non published results).

The ability of the skin to respond to a chronic inflammatory stimulus was also tested. The injection of silica produces a granuloma due to a continuous lysis of macrophages in the injection site (Kessel *et al*, 1963). A suspension of 1 mg of silica in 0.05 ml of PS was intradermically injected in the flank and in the dorsal region of the foot of Balb/c mice. Local inflammation was recorded as thickness of the injected site using a dial micrometer (Pocotest, GESSH).

The response to silica (Figure 2) was significantly higher in the dermis of the dorsal region of the foot than in the dermis of the flank. Moreover in the dorsal region of the foot the response was sustained until 10 days after inoculation while in the flank was almost undetectable by that time. This result shows that the dermis of the dorsal region of the foot is more responsive to a chronic inflammatory stimulus than the dermis of the flank (non published results).

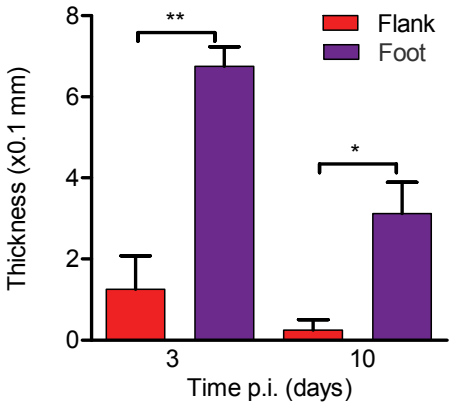


Fig. 2. Chronic inflammatory response to silica in the dermis of the flank and dorsal region of the foot (n=4) * $p<0.05$; ** $p<0.005$, Student t test between each group and control

4.1.3 Specific immune response

In a previous work we tested the immune response in both anatomic sites after injection of sheep red blood cells (SRBC). Seven days after immunization with SRBC, sera was obtained and antibody titer was analyzed by hemagglutination assay. We found that a significantly higher antibody titer was obtained when SRBC were inoculated in the dorsal region of the foot than in the flank (Speroni *et al*, 2009a).

Since different levels of inflammatory and immune response were observed, we further evaluated the growth of B16F0 melanoma in both anatomic sites.

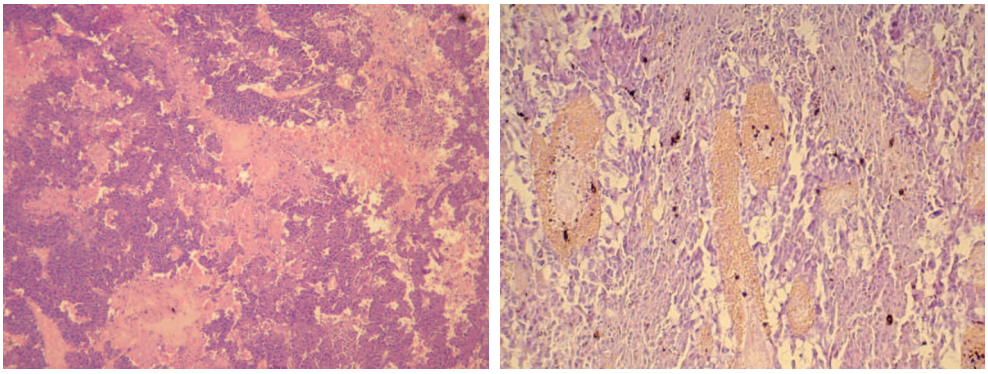
4.1.4 Melanoma growth in different anatomic sites of the skin of mice

The development of B16F0 melanoma in the dorsal region of the foot showed a more malignant phenotype than when growing in the flank (Figure 3). The tumor in the flank (Figure 3A) showed large and numerous necrotic areas with leukocytic infiltration. Cells were disposed in an acinar-like structure, and intratumoral vascularization was scarce or absent. On the other hand, melanoma growing in the dorsal region of the foot (Figure 3B) showed a different architecture. Cells were distributed in irregular masses. High vascularization, and numerous inflammatory cells and hemosiderin deposits were observed. Another important difference with tumors growing in the flank was the absence of necrotic areas. Macroscopic and histological post-mortem examination of serial lung sections from hosts bearing B16F0 tumors showed neither macroscopic nor microscopic metastatic foci. Conversely, lung metastases were observed in 100% hosts bearing melanoma tumors in the dorsal region of the foot that survived beyond day 14 p.i. (mean) (Speroni *et al*, 2009a).

Importantly the hosts of tumors in the dorsal region of the foot had a poor survival compared to the hosts of flank tumors (Speroni *et al*, 2009a).

Our results clearly show that, even though the tumor cell line was the same, when injected in the mice, these cells originate remarkably different tumors which malignancy is dependent on the anatomic site of the skin. The skin of the dorsal region of the foot displayed a higher inflammatory and immune response most likely due to a higher cellular influx (Speroni *et al,* 2009a) than the flank skin. It is clear that several limitations exist with mouse models,

particularly with the standard subcutaneous injection for the study of human cancer (Crnalic *et al*, 1997; Hirst & Balmain, 2004; Vitale *et al*, 2007). We have developed an alternative model to the standard inoculation of tumors into the flank of mice that, being equally easy to handle, might prove a better model for the study of melanoma progression and metastases (Speroni *et al*, 2009a; 2009b). Moreover, the model of tumor implantation in the dorsal region of the foot could be useful for the study of spontaneous metastases from tumors that do not originate metastases with the standard method of inoculating tumor cells in the flank.



(a) (b)

Fig. 3. Histological analysis of tumors. Sections stained with hematoxylin-eosin from B16F0 tumor growing in the flank (a) and in the dorsal region of the foot (b). Original magnification 10X

4.2 *In vitro* models

Building more complex *in vitro* models of melanoma could improve our understanding on the initiation and different stages of the disease. Fibroblasts, one of the principle cell types of the stromal compartment, have shown to induce melanoma progression and invasion. The interaction between melanoma cells and fibroblasts creates a tumor promoting environment by the up-regulation of chemokines, cytokines, and growth factors. Factors secreted by fibroblasts and melanoma cells modulate collagen synthesis and promote tumor cell chemotaxis and invasion (Van Kempen *et al*, 2007). Moreover the invasive capacity of human melanoma cells is enhanced by the presence of fibroblasts (Bartolome *et al*, 2004; Li *et al*, 2009; Wandel *et al*, 2002). This culminates in an environment that supports growth and invasion. Cells growing in a 3D (three-dimensional) architecture resemble the *in vivo* situation more closely than do cells in conventional 2D cultures (monolayer). Because they can be finely tuned they offer the unique opportunity to dissect the molecular pathways and signaling between cell-cell and cell-extracellular matrix interaction. Moreover they could serve as a useful tool for the assessment of the drug efficacy in the treatment of melanoma in different stages.

5. Conclusion

The microenvironment plays a key role in tumor development to an extent that it poses the question whether a shift of paradigm in biology is needed in order to truly understand the

phenomenon of cancer. The plasticity of cells is such that a cell adapts its phenotype according to what the systems imposes, i.e. the organization of the tissue and ultimately the self-organization of the system. In other words, the identity of a cell is not an intrinsic property of it but rather a condition dictated by spatio-temporal cues of the environment.

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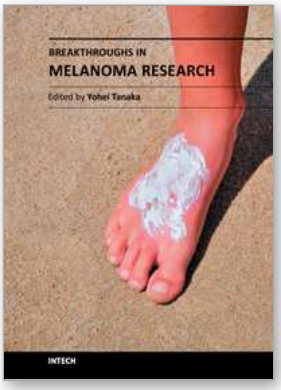

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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

How to reference

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