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Histology and Histopathology

From Cell Biology to Tissue Engineering

Review

Ecology of melanoma cell

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Summary. Melanoma represents a cancer with increasing incidence worldwide and limited curability of advanced stages of the disease. Similarly to other types of tumors, the microenvironment is an important factor that participates in the control of melanoma biological properties. This review summarizes data regarding the role of the microenvironment, namely fibroblasts, keratinocytes and infiltrating immune cells, on melanoma growth and spreading. The role of embryonic microenvironment on melanoma cell biological properties is also discussed. The potential of therapeutic targeting of the melanoma microenvironment is demonstrated.

Key words: Neural crest, Melanoma, Cancer microenvironment, Cancer-associated fibroblast, Immune cell, Stem cell niche

Introduction

The number of people suffering from cancer of some type is increasing worldwide. This phenomenon seems to be associated with ageing and genomic instability in the elderly (Smetana et al., 2016). Melanoma follows this trend, and the incidence of this tumor is also raising. Compared to the situation before almost 40 years, the number of people suffering from this malignancy is approximately four times higher (Global Burden of Disease Cancer Collaboration, 2015; Dvořánková et al., 2017). Unfortunately, the mortality in advanced stages of this disease, despite the remarkable effort and progress in melanoma research, remains quite stable. Any new information providing deeper insight into the biology of the melanoma can therefore open the way to new therapeutic options that will cure or prolong survival of the patients with the advanced stage of this disease. One of the new therapeutic challenges could be manipulation of the "social networks" of melanoma cells.

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Ecology of normal and cancer cell

There are remarkable similarities between the macroscopic and microscopic world. As postulated by van der Ploeg (1982): Ecology is a study of relationships. These can be very complex or hardly recognisable. Indeed, each organism occupies a distinct landscape position (so-called niche) well defined by physical and biological factors such as geological conditions, sun exposure, temperature, water and nutrients enabling successful existence and reproduction. A very important role is attributed to predators (including, in broader sense, also pathogens and parasites) that prevent overpopulation by certain organisms based on their fitness in this niche. They are thus responsible for the maintenance of biological balance. A remarkably similar organisation is also respected in the body of each multicellular organism both under physiological conditions and in disease, including cancer. As parts of the organism, organs or even cells react differently to environmental influences, this concept has gained more and more relevance, particularly in the last few decades. Each distinct cell and cell type requires topologically as well as temporarily defined position due to, e.g., the demand for oxygen and nutrients. The immune cells in this context prevent overpopulation by genetically abnormal cells that can be at the beginning of the formation of a malignant tumor (Kareva, 2011; Cain et al., 2014; Amend and Pienta, 2015).

Adult tissue stem cells and cancer stem cells require a specific niche

Stem cells are present in virtually all adult tissues. They are particularly important for the tissue maintenance, participating in the continuous tissue self-renewal, and their role in tissue healing after trauma is also remarkable (Motlík et al., 2007; McCracken et al., 2016).

Stem cells can divide in unlimited numbers to replenish other cells; however, they can frequently remain quiescent for long periods of time until their proliferation is triggered by a normal need for tissue renewal, growth, or by disease or tissue injury. Stem cells adopt this quiescent state to preserve their key functional features. This concept of slow cycling has changed over time, and it has become increasingly apparent that the use of label retention alone is insufficient to identify adult stem cells. In later years, evidence has suggested the coexistence of quiescent and actively proliferating stem cell pools in high-turnover tissue compartments if necessary (Li and Clevers, 2010). Once stimulated, stem cells undergo asymmetric mitotic divisions, when usually one of the daughter cells retains the features of stem cell. As generally recognised, these adult tissue stem cells are usually monopotent under physiological conditions, or alternatively they exert restricted multipotentcy. The specific factors and conditions that allow stem cells in the tissues to remain unspecialized are still poorly understood. However, the stem cell population must balance the competing demands of proliferation, high/low differentiation maintenance in the tissue, as well as prevention of the genome from experiencing unnecessary risk during DNA replication. To eliminate e.g. certain exogenous harmful agents, stem cells possess multiple ATP-binding cassette transporters that have been identified as protective pumps against toxic agents. These molecules were shown to be expressed at high levels in stem cells and variably regulated during cell differentiation. The above-mentioned properties allow stem cells to withstand metabolic stress and preserve the genomic integrity over a lifetime. Further, somewhat higher resistance to anoikis than in other cells also represents an important feature of protection in some types of adult tissue stem cells (Dvořánková et al., 2005).

These tissue stem cells require a precisely defined microenvironment, the niche, to preserve their stem cell properties. In this regard, the stem cell rate and timing of proliferation may not be directly linked to their stemness, but rather to their microenvironment (Weigelt and Bissell, 2008). The niche modeling *in vitro* is not easy and, therefore, the propagation of stem cells in clinically relevant quantities represents one of the barriers to clinical employment of adult tissue stem cells (except for hematopoietic stem cells) in routine medical practice. One of the most relevant examples of the stem cell niche related to the topic of the article is the so-called bulge region of the human hair follicle, where epidermal stem cells, as well as neural crest-originated stem cells, are located (Dvořánková et al., 2017). Epidermal stem cells can differentiate into keratinocytes and also into cells of the sebaceous gland and hair. Their role in wound healing was also well recognized (Lavker et al., 2003). Another stem cell population in this location migrated to the hair follicle bulge from the neural crest. This embryonic neuroectodermal structure is a developmental source of multiple cell lineages spreading through the whole body (Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004; Shyamala et al, 2015). Among many others, neural crest can give origin to epidermal melanocytes (Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004). Such coexistence of two different stem cell pools of different origin is rather unique across all mammalian species. Certain collaboration between both stem cell types was predicted and the role of transcription factor NFIB was postulated (Chang et al., 2013). As the hair follicle repeatedly cycles from anagen to catagen during postnatal life, the synergistic and highly orchestrated coexistence of both stem cell types in this particular niche in the outer root stealth of the hair follicle can be expected behind this periodic process to achieve full structural and functional integrity of this unique organ. The existence of a specific niche is, therefore, an important prerequisite for successful complete regeneration and function of the hair follicle apparatus. In parallel, a similar role can be essential in genetically altered stem cells in malignant tumors (Lau et al., 2017), and its therapeutic manipulation seems to be a highly promising approach in cancer therapy.

Melanocytes cooperate with keratinocytes in protection of their genetic information

Melanocytes are located between mitotically active epidermal keratinocytes of the basal layer, where they produce and release melanosomes containing light-absorbing melanin. This pigment is later transferred to keratinocytes. The engulfed melanosomes protect the nuclei of keratinocytes from UV-caused DNA damage as a parasol, thus preventing formation of mutations with oncogenic potential (Colombo et al., 2011; Merkel and Gerami, 2016). Both cell types closely collaborate after UV irradiation, and therefore a functional epidermal pigmentary unit was postulated (Archambault et al., 1995).

Melanoma

Cutaneous melanoma is a malignancy with increasing incidence and unfavorable prognosis in advanced stages of the disease resistant to therapy (Bastian, 2014; Kalal et al., 2017). Its occurrence is usually associated with previous exposure of unprotected skin to UV irradiation from sun light or artificial sources (D'Orazzio et al., 2013). The exposure can precede by many years the onset of the tumor, and particularly high

sensitivity was observed in the skin of children (Volkmer and Greinert, 2011). However, the sun non-exposed skin is not completely devoid of the risk of melanoma development (Brash, 2015). From the molecular point of view, malignant melanoma is highly heterogeneous even at the single lesion level. It has been well documented by expression of embryonic protein Nodal that the percentage of positive cells reflects the transition from radial growth to the dangerous vertical phase (Seftor et al., 2014). Some of these Nodal-producing cells have properties of melanoma (initiating) stem cells and their properties are strongly modulated by the micro-environment (Nguyen et al., 2015). The interaction between nerve growth factor and CD271 receptor expressed on melanoma stem cells seems to participate in their stemness maintenance (Redmer et al., 2014). This seems to be particularly important for the extensively metastatic behavior in melanoma cells. Factors such as morphogen EDN3 produced by cells of the melanoma microenvironment in the zebrafish model seem to be crucial for cancer cell metastastation. This morphogen influences expression of MiTF, a master regulator gene of melanocyte development and melanoma oncogene. With respect to this, more positive cells proliferate, and the cells with a low level of MiTF are more active in migration (Vachtenheim and Ondrušová, 2015; Kim et al., 2016).

Similarly to other types of tumors, malignant melanoma represents a complicated ecosystem (Tirosh et al., 2016) where mutual interactions between distinct cell types are worthy of careful analysis.

Grafting of cells from advanced stage of melanoma to vertebrate embryos

As mentioned above, the neural crest-originated cells represent an important source of many cell types through the vertebrate body. This migration through various structures of the developing organism requires proper timing and tight regulation of both epithelial to mesenchymal transition and the reverse process (Thiery and Sleeman, 2006). In recent years, remarkable similarities between melanoma cells and neural stem cells were even demonstrated (Handoko et al., 2013; Ivanov and Hei, 2015). This is highlighted by the low differentiation status, a phenomenon typical in melanoma. Transplantation of melanoma cells to fish/avian embryos can illustrate this migratory activity, and grafted cells are later detected in these models in locations typical of neural crest progeny. Despite the successful survival of melanoma cells in various host structures in experiments, the malignant potential of malignant melanocytes seems to be abrogated or minimized (Lee et al., 2005; Kulesa et al., 2006; Hendrix et al., 2007; Díez-Torre et al., 2009). Furthermore, the conditioned media from embryonic stem cell cultures also reduce the growth potential of melanoma cells in experiments and shift their phenotype to more differentiated stages (Kim et al., 2011a; Kodet et al., 2013). Such influence of embryonic microenvironment on cancer cells seems to be a general feature applicable to other models as well. In a seminal trial, teratocarcinoma cells injected into mouse blastocysts did not impair development of a normal embryo and tumor cells contributed to the formation of animal structures (Mintz and Illmensee, 1975). Such melanoma cell grafting experiments raised intriguing questions regarding their differentiation plasticity. Next to that, the dominant regulatory role of the microenvironment with respect to cancerous cell phenotype was documented on several levels. Indeed, the idea that the embryonic microenvironment is able to control properties of malignant cells is very old (Pierce, 1983). In the particular case of malignant melanoma, the dependence of the properties of malignant cells on micro-environment-regulated signaling was proposed. Analysis of the embryonic microenvironment surrounding the malignant cells grafted to the embryo suggested that the Nodal-Notch 4 regulatory axis could participate in the control of melanoma cells by the embryonic microenvironment. Such microenvironmental effectors acting dominantly, presumably at the epigenetic level, can override genetic instructions in melanoma cells in order to suppress the aggressive behavior. This regulation bears the potential of being a target for future tumor therapy (Strizzi et al., 2011).

Crosstalk between keratinocytes and melanoma cells (Fig. 1)

As mentioned above, the well-orchestrated close interaction of normal melanocytes and surrounding keratinocytes is a prerequisite for the maintenance of the epidermal layer. In parallel, some mutual crosstalk can also be conserved in the case of the malignant melanocytic population within the epidermis. Melanoma growth has an inhibitory effect on expression of connexins in epidermal keratinocytes (Haas et al., 2010). This phenomenon can positively influence the spreading of melanoma because both connexins and E-cadherins on the keratinocyte surface play an inhibitory role in this behavior (Hsu et al., 2000; Ableser et al., 2014). On the other hand, laminin production by keratinocytes enhances melanoma cell migration (Chung et al., 2011). Interaction of melanoma cells and basal cell keratinocytes can reduce MiTF expression in melanoma cells via production of Notch ligand by keratinocytes. This phenomenon seems to participate in the switch of radial phase growth to the vertical growth phase in the tumor. This illustrates increasing invasiveness of the malignant melanocytic clone (Golan et al., 2015).

Changes in melanoma behavior also require remodeling of the keratinocytic landscape in the vicinity of the invading melanoma clone. A highly interesting phenomenon was observed in nodular melanomas invading into the dermis, where overlying the epidermis acquired hyperplastic - pseudoepitheliomatous - features. Structural changes of the epidermis adjacent to the tumor

periphery included broad changes in the expression of keratins as markers of epidermal differentiation (McCarty et al., 2003; Drunkenmölle et al., 2005; Kodet et al., 2015). This feature can be induced by biomechanical factors, i.e., mechanical stress due to tumor growth (Valach et al., 2017). However, a series of *in vitro* experiments demonstrated that paracrine production of cytokines/chemokines such as FGF-2, CXCL-1, IL-8 and VEGF-A by melanoma cells is responsible for changes of the phenotype in co-cultured normal human keratinocytes (Kodet et al., 2015). We discussed above that extensive UV exposure of human skin plays a role in etiopathogenesis of melanoma by induction of critical mutations in melanocytes. However, keratinocytes are also seriously affected by UV light and after UV irradiation they produce substances such as basic FGF, endothelin-1, IL-6, IL-8, IL-11, TNF-α that can influence melanoma initiation and migration activity of melanoma cells (Brennen et al., 2005; Kim et al., 2011b; Li et al., 2013).

Next, an intensive interaction is also expected through the basement membrane of the epidermis. Normal keratinocytes, as well as cancerous keratinocytic proliferations, are able to stimulate fibroblasts to acquire an activated phenotype in response to tissue damage, injury or other pathologies. The importance of so-called cancer-associated fibroblasts (CAFs) has been widely acknowledged recently in various types of tumors, and a similar observation might also be true in the case of melanoma. Hereby we acquire another dimension of interactions in malignant melanoma. This aspect of melanoma-associated fibroblasts similar to CAFs (Kolář et al., 2012; Jarkovska et al., 2014) can also be a highly important topic in melanoma biology.

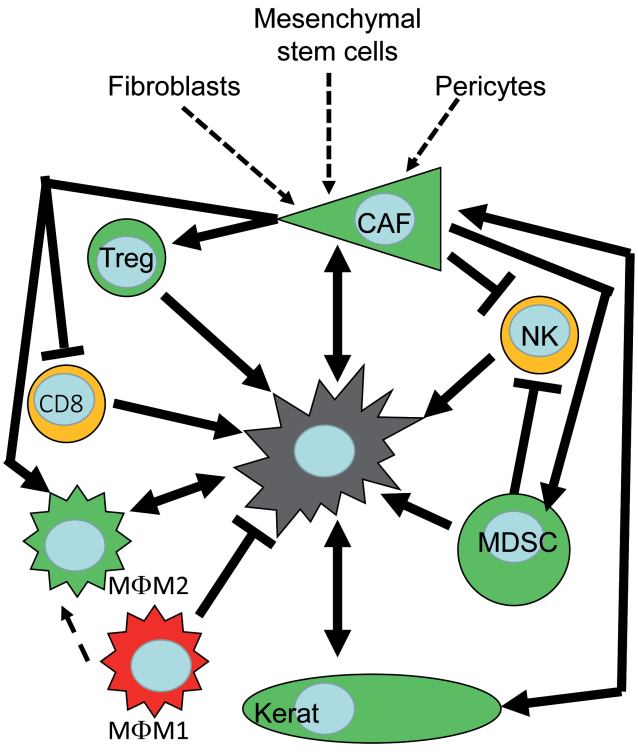

Fig. 1. Schematic representation of the ecosystem of melanoma cells, where cancer-associated fibroblasts (CAF), natural killer cells (NK), myeloid-derived suppressor cells (MDSC), keratinocytes (Kerat), M1 polarized macrophages (M1MΦ), M2 polarized macrophages (M2MΦ), CD8 cytotoxic T lymphocytes (CD8) and T regulatory lymphocytes (Treg) participate in formation of the melanoma cell niche. Cells that stimulate melanoma growth are marked in green and cells with an inhibitory effect are red. Yellow signal indicates cells with originally inhibiting activity attenuated by the activity of CAFs.

Fibroblasts are also powerful players in the cancer microenvironment (Fig. 1)

Cancer-associated fibroblasts are able to influence the biological properties of different types of tumors in a significant manner (Plzák et al., 2010; Lacina et al., 2015). CAFs can be of different origin. Next to the most obvious local fibroblasts, other cell types, including mesenchymal stem cells (MSC) and pericytes, can be activated to support tumors (De Wever et al., 2008). On the other hand, the theoretical role of epithelial-mesenchymal transition in CAFs seems to be less probable, as demonstrated in animal experiments (Dvořánková et al., 2015). CAFs produce various types of extracellular matrix proteins such as collagens, fibronectin, tenascin, and also less usual molecules such as galectin-1. This endogenous lectin participates in the control of transition of fibroblasts to myofibroblasts and enhances expression and structural assembly of smooth muscle actin, which is a hallmark of CAFs (Valach et al., 2012). However, CAFs also produce a wide panel of paracrine factors, among them namely IL-6, IL-8 and CXCL-1 (Kolář et al., 2012). This seems to be highly relevant because of their above-mentioned role in the biology of melanoma. The correlation of increased IL-6 and IL-8 with respect to tumor progression has been well documented (Mouwad et al., 1996; Dhawan and Richmond, 2002; Yurkovetsky et al., 2007).

It is noteworthy to mention mesenchymal stem cells as one of the potential precursors of CAFs. An interesting phenomenon of the possible lateral propagation of stromal recruitment was depicted on cutaneous basal cell carcinoma-associated fibroblasts. CAFs isolated from the most common type of epithelial cancer induce unusual features in co-cultured mouse embryonic fibroblasts. Consequently, mouse fibroblasts acquire a similar phenotype to mesenchymal stem cells (Szabo et al., 2011).

Fibroblasts, including CAFs isolated from melanoma, have an effect on melanoma cells (Kodet et al., 2013). They stimulate the aggressive behavior of melanoma cells in hypoxic condition (Comito et al., 2012); presumably, IL-6 and IL-8 can also participate in this effect (Jobe et al., 2016). CAFs are able to stimulate production of proteolytic enzymes by melanoma cells

and thus facilitate their migration (Ntayi et al., 2003; Yin et al., 2012). CAFs from melanoma can also increase the resistance of tumor cells to anticancer therapy, including BRAF inhibitor therapy, by generating a safe harbor facilitating therapeutic escape. This phenomenon represents a common serious therapeutic complication in recent days (Flach et al., 2011; Whipple and Brinckerhoff, 2014; Fedorenko and Smaley, 2015). In the context of multilateral mutual microenvironmental interactions supporting melanoma progression, we have to acknowledge that CAFs isolated from melanoma also have a stimulatory role in co-cultured human keratinocytes (Kučera et al., 2015). On the other hand, melanoma CAFs have a strong inhibitory effect on the functions of NK cells invading the tumor (Balsamo et al., 2009), and thus CAFs reduce the local immune surveillance in the tumor ecosystem. Experimental depletion of fibroblast activating factor (FAP)-positive fibroblasts in the stroma of mouse melanoma can stimulate anticancer immunity by indirect activation of CD8-positive T lymphocytes (Zhang and Ertl, 2016). FAP-positive fibroblasts were therefore proposed as targets for anticancer therapy earlier (Kotačová et al., 2009). On the other hand, chronic inflammation represents an important and generally accepted condition that stimulates formation and supports the spread of melanoma, as demonstrated by participation of chemokines and their receptors in the disease (Richmond et al., 2009). It is a topic for discussion whether CAFs isolated from melanoma have a specific biological effect restricted to this type of tumor only. However, CAFs have no influence on the proliferation of glioblastoma cells, they stimulate their invasiveness, as evidenced *in vitro* (Trylcova et al., 2015). They are also able to shift the phenotype of breast cancer cell lines to a more aggressive phenotype *in vitro* (Dvořánková et al., 2012). These data collectively suggest the non-specific activity of CAFs isolated from melanoma, and thus at least their certain effect on tumors of ectodermal origin. These properties seem to be a suitable background justifying an attempt to develop a new broad-spectrum therapeutic strategy targeting CAFs.

Immune cells: predators, bystanders or stimulators of melanoma growth and spreading?

As mentioned above (Balsamo et al., 2009), CAFs isolated from melanoma have an inhibitory effect on NK cell activity. MSCs attracted to the tumor stroma are hypothesized as one of the possible sources for CAF formation. MSCs also exhibit an immunomodulatory activity reducing the intensity of immune response (Watts and Cui, 2012; Poggi and Giuliani, 2016). These data suggest the immunosuppressive cues of tumor microenvironment inhibiting immune-system-mediated eradication of cancer cells. On the other hand, melanoma is frequently infiltrated by large numbers of inflam-matory cells of various types. When lymphocytes and dendritic cells prevail among these cells, the prognosis of patients seems to be better (Ladányi, 2015). However, a high presence of Treg lymphocytes in tumors is linked to a poor prognosis because of their immunosuppressive effect (Gray et al., 2017). CAFs originating from mesenchymal stem cells seem to be capable of Treg cell activation and thus be responsible for inhibition of the cytotoxic activity of CD8+ T lymphocytes (Duffy et al., 2011). Therapies eliminating Treg in melanoma patients such as cyclophosphamide, IL-2-based therapies as well as antibodies against Treg surface molecules seem to be promising (Ouyang et al., 2016). When myeloid-derived suppressor cells are present in melanoma, the immune response is also reduced and the prognosis of the patient is not favorable. These cells are also activated by mesenchymal stem cells (Giallongo et al., 2016). Therefore, the treatment with Ipilimumab is beneficial for these patients (Umansky et al., 2016). M2-polarized macrophages significantly improve progression of the disease by production of a panel of cytokines with a stimulatory effect on tumor growth. On the other hand, M1 macrophages have tumor suppression activity and potentiate efficiency of tumor immunotherapy via production of exosomes. Unfortunately, the possible shift of M1 to M2 macrophages has been described (Cheng et al., 2017; Falleni et al., 2017). The polarization of macrophages to the M2 type seems to be stimulated by mesenchymal stromal cells located in the melanoma stroma (Yamada et., 2016). Similarly to the ancient Roman two-faced God Janus, the immune cells infiltrating melanoma also offer two faces. The bright face offers a remarkable anti-tumor activity. The dark face stimulates tumor growth by production of bioactive substances and established immunosuppression. The therapeutic manipulation of immune cells and their equilibrium within the ecosystem present in malignant melanoma seems to be the most promising micro-environment-based therapeutic strategy yet.

Conclusion

The social networks of melanoma cells are broad and include extensive multilateral interactions with keratinocytes, CAFs, and immune cells. These interconnected networks can directly or indirectly influence the biological properties of melanoma cells, including melanoma stem cells. CAFs seem to play the role of a conductor within this microenvironmental orchestra, because they are able to influence melanoma cells directly or via their effect on keratinocytes and immune cells. Moreover, they also participate in the formation of an immunosuppressive micromilieu, so they support the tumor progression indirectly (Fig. 1). This complicated ecosystem represents a potential therapeutic target. An intervention against this system could influence not only tumor growth, but also the invasiveness of melanoma cells. Therefore, CAFs are likely to be one of the hottest future candidates for therapeutic targeting.

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