Regulation of Gene Expression in the Tumor Environment

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Editor

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Preface

This is the second volume of the book series entitled *"The Tumor Microenvironment."* This volume will focus on the "regulation of gene expression in tumor and non-tumor cells in the tumor microenvironment."

It is now becoming very clear that the development and progression of tumor towards the malignant (metastatic) phenotype depend tightly on the interaction between the tumor cells and the tumor microenvironment. Tumor cells respond to stimuli generated within the tumor microenvironment for their growth advantage while the tumor cell themselves reshape and remodel the architecture and function of their extracellular matrices. The term tumor microenvironment is a wide umbrella consisting of stromal cells such as fibroblasts and endothelial cells and infiltrating immune cells including T and B cells, macrophages, and other inflammatory cells (PMNs). These different components of the tumor microenvironment could have stimulatory and inhibitory effects on tumor progression by regulating the gene expression repertoire within the tumor cells on one hand and the stroma cells on the other. In this volume we have seven contributors who will discuss several different aspects on the cross talk within the tumor microenvironment components leading to the acquisition of the metastasis phenotype. It is our hope that these state-of-the-art studies will shed further light on our understanding of these complicated processes.

Contents

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Chapter 1
Regulation of Melanoma Progression by the Tumor Microenvironment: The Roles of PAR-1 and PAFR

Gabriel J. Villares and Menashe Bar-Eli

Abstract The interaction of tumor cells and the host stroma (microenvironment) is essential for tumor progression and metastasis. The melanoma tumor microenvironment has emerged within the last decade as a significant player in melanoma progression from the radial growth phase to the vertical growth phase by providing the necessary elements for growth, invasion and survival. Two receptors involved in this transition that are not only activated by factors from the tumor microenvironment but also in turn secrete factors into the microenvironment are the Protease Activated Receptor 1 (PAR-1) and the Platelet Activating Factor Receptor (PAFR). Thrombin, which is abundant in the microenvironment milieu, activates PAR-1 causing cell signaling via G-proteins resulting in upregulation and secretion of gene products involved in adhesion (integrins), invasion (MMP-2) and angiogenesis (IL-8, VEGF, PDGF, bFGF). PAF, which is secreted by platelets, macrophages, neutrophils, endothelial cells and keratinocytes within the tumor microenvironment, will activate PAFR and signal through p38 MAPK to phosphorylate the CREB/ATF-1 transcription factors. Phosphorylation of CREB/ATF-1 results in overexpression and secretion of MMP-2 and MT1-MMP. Since only metastatic melanoma cells express activated CREB/ATF-1, we propose that they are better equipped to respond to PAF than their non-metastatic counterparts. These two G-protein coupled receptors that play major roles in melanoma progression highlight the crucial interactions between the tumor microenvironment and melanoma cells in the acquisition of the metastatic phenotype.

Keywords Melanoma progression · Metastasis · Invasion · Angiogenesis · Thrombin · Protease activated receptor-1 · Platelet activating factor · Tumor microenvironment · Transcription factors · Metalloproteinase · G-protein coupled receptor

Melanomas, as with all other cancers, are not comprised of a group of stand-alone cells with similar characteristics or capabilities. They are, however, comprised of

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a group of heterogeneous cells that co-exist and interact with an infrastructure of other cells(keratinocytes, fibroblasts, endothelial cells, inflammatory cells) and stromal components, all together known as the tumor microenvironment [1]. The tumor microenvironment is comprised of diverse cell types and elements such as extracellular matrix components (lamin, collagen), growth factors (VEGF, bFGF, thrombin), proteases and interleukins involved in invasion (MMP-2, IL-8, uPA) as well as varying concentrations of oxygen [2]. Furthermore studies have shown that inflammatory cells within the tumor microenvironment contribute to malignancies by releasing growth factors and chemokines [3]. It seems evident that the interaction of tumor cells and the host stroma (microenvironment) is, therefore, essential for tumor progression and, eventually metastasis. Following these same lines, the melanoma tumor microenvironment has emerged within the last decade as a key player in melanocyte transformation and transdifferentiation by providing these necessary elements for growth, invasion and survival [2].

In melanoma, there are several cell types within the tumor microenvironment that influence melanoma progression. For example, keratinocytes, which are found within normal skin, form interactions with melanocytes that are mediated by E-cadherins. Keratinocyte-regulated expression of E-cadherins affects the phenotypic behavior of melanocytes [1]. Disturbances in normal keratinocyte–melanocyte adhesion may contribute to malignant transformation by releasing melanocytes from contact-mediated regulatory controls leading to the advancement of melanoma [4]. Furthermore, keratinocytes induce several pro-angiogenic interleukins (IL-6, IL-8) as well as pro-inflammatory factors (PAF), which may also lead to melanoma progression [3].

Fibroblasts, once thought to play a minimal role in tumorigenesis, have been found to play an important role in potentiating tumor growth. A bi-directional model between melanoma cells and fibroblasts has been proposed in which melanoma cells first produce growth factors such as PDGF, bFGF and TGF-β to activate fibroblasts and endothelial cells and, subsequently, fibroblasts produce a series of growth factors (IGF-1, HGS/SF, bFGF, TGF-β) that further supports the growth and proliferation of melanoma cells [1, 4]. These paracrine signaling loops act to create an environmental niche conducive to tumor growth [1].

As can be seen, transformed melanocytic cells will recruit and interact with host cells in the microenvironment. These cells will then become activated and in turn elicit survival, proliferation and invasion signals [4]. The progression of melanoma from radial growth phase to vertical growth phase is accompanied by a myriad of molecular changes that are involved in this transition. Two of the factors involved in this transition that are not only activated by the tumor microenvironment but also in turn affect the microenvironment are the thrombin receptor (PAR-1) and the Platelet Activating Factor Receptor (PAFR).

1.1 PAR-1

Thrombin is a serine protease abundant in the tumor microenvironment milieu, which not only plays a crucial role in blood coagulation but also initiates various

cellular responses through the activation of the thrombin receptor, PAR-1 [5]. In fact, activation of coagulation factors have been implicated in tumor growth and are hallmarks of advanced cancers [5, 6]. Studies have also demonstrated that tissue factor (TF) is constitutively expressed in melanoma cells and can activate thrombin in a coagulation independent manner, thereby promoting melanoma metastasis [7, 8]. In fact, the hypoxic tumor microenvironment also induces TF expression by endothelial cells, tumor associated macrophages and myofibroblasts, thereby also augmenting thrombin in the tumor microenvironment [6].

Furthermore, thrombin-treated tumor cells (including melanoma) enhance their adhesion to platelets and fibronectin *in vitro* [9]. Thrombin also promotes endothelial cell alignment in Matrigel *in vitro* and angiogenesis *in vivo* [10]. It induces the differentiation of endothelial cells into capillary structures in a dose-dependent manner on Matrigel [10]. Furthermore, in the *in vivo* Matrigel system of angiogenesis, there is a 10-fold increase in endothelial cell migration infiltration in response to thrombin. In lung epithelial cells, thrombin was also found to stimulate the expression of PDGF [11]. Blocking of the coagulation pathways at the level of tissue factor, factor Xa, or thrombin, inhibits metastasis of human melanoma cells in SCID mice [8].

Thrombin can also activate several signal transduction pathways through its receptor. The thrombin receptor is a 7-pass transmembrane G-protein coupled receptor. Unlike typical ligand-receptor interactions, thrombin does not activate PAR-1 upon binding. Rather, it cleaves the N-terminus of PAR-1 at serine 42. Upon cleavage, the new amino terminal peptide acts as a tethered ligand that will now bind to the body of the receptor thereby causing cell signaling via G proteins resulting in upregulation of gene products involved in adhesion ($\alpha_{IIb}\beta_3$, $\alpha v\beta_5$, $\alpha_v\beta_3$ integrins) [12–14], invasion (MMP-2) [15], and angiogenesis (IL-8, VEGF, bFGF, PDGF) [11, 16–18]. This suggests that activation of the thrombin receptor may facilitate tumor invasion and metastasis through the induction of cell adhesion molecules, matrix degrading proteases, and stimulating the secretion of angiogenic factors into the melanoma tumor microenvironment, thus contributing to the metastatic phenotype of melanoma.

In human melanoma cells, thrombin acts as a growth factor and is mitogenic, suggesting that signaling by PAR-1 is involved in the biological response of these cells [8]. PAR-1 can also be activated by ligands other than thrombin such as factor Xa, granzyme A, trypsin and plasmin [19–21]. In addition to melanoma, overexpression of PAR-1 has been observed in a variety of human cancers, such as breast, lung, colon, pancreatic and prostate [5, 22–26]. It has also been recently reported that PAR-1 in breast cancer cells can also be proteolytically cleaved and activated by membrane metalloprotease-1 (MMP-1) [27]. Our laboratory has previously demonstrated that PAR-1 is differentially expressed in melanoma cell lines with overexpression being found in highly metastatic cells as compared to non-metastatic melanoma cell lines [5, 28]. Moreover, we found that the overexpression of PAR-1 correlates with the loss of the activator protein-2α (AP-2α), which is a crucial event in the progression of human melanoma [28]. In fact, we observed an inverse correlation between AP-2 and PAR-1 from primary melanoma cell lines

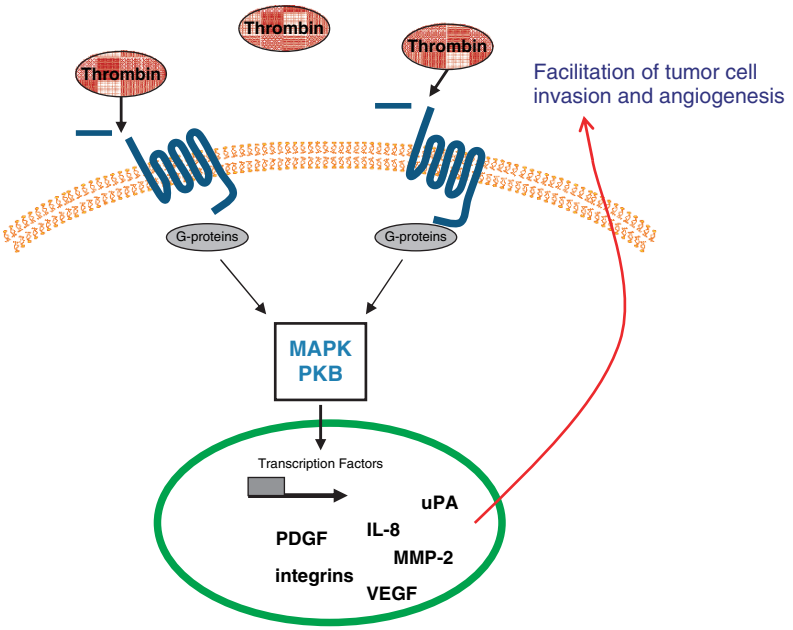

Fig. 1.1 Schematic representation of molecules involved in cell invasion and angiogenesis via activation of PAR-1, which is overexpressed in metastatic melanoma cells. Thrombin from the microenvironment cleaves the N-terminus of PAR-1 to activate the receptor. The tumor-promoting signals transduced by PAR-1 through G-proteins upregulate molecules involved in angiogenesis and invasion

up to highly invasive and aggressive melanomas [28]. Overexpression of PAR-1 is predominantly seen in patients with malignant melanoma tumors and in metastatic lesions as compared to common melanocytic nevi and normal skin [29]. Furthermore, our laboratory has found a significantly higher percentage of PAR-1 positive cells in metastatic melanoma specimens as compared to both dysplastic nevi and primary melanoma specimens [30] attesting to the role of PAR-1 in regulating tumor growth and metastasis of melanoma.

As can be seen, activation of PAR-1 in melanoma cells through different ligands present in the tumor microenvironment will subsequently cause activation of the angiogenic and invasive gene products that are released into the tumor microenvironment (Fig. 1.1). This will also cause activation of fibroblasts and endothelial cells that subsequently forms a more pro-invasive and proliferative environment for melanoma growth and metastasis.

1.2 PAFR

As mentioned previously, it has been shown through genetic and functional experiments that inflammatory cells such as tumor-infiltrating monocytes/macrophages, neutrophils, mast cells, eosinophils, and activated T-lymphocytes contribute to

malignancies by the secretion of growth and survival factors, proteases, pro-angiogenic factors and chemokines into the tumor microenvironment [31–34]. In fact, cancer cells promote the recruitment of inflammatory cells, thereby producing inflammatory mediators and angiogenic factors [3].

PAF is secreted into the tumor microenvironment by several cell types, including inflammatory cells, vascular endothelial cells and keratinocytes, which in turn also respond to PAF. Furthermore, platelets in response to thrombin can also secrete PAF. PAF binds and activates the Platelet Activating Factor Receptor (PAFR), a pro-inflammatory mediator, which is also a G-protein coupled receptor. PAFR, in a similar manner to PAR-1, activates signal transduction pathways including MAP kinase, PI3 kinase, PKA and Src pathways [3, 35–39]. Furthermore, our group and others have demonstrated that in human metastatic melanoma cells, PAF can stimulate the activity of p38 MAP kinase [39–41]. PAF activation of these signal transduction pathways results in upregulation of effectors of tumor growth, angiogenesis and malignant progression such as NF-κB, STAT-3 and MMPs [3].

Through the use of PAFR-overexpressing transgenic mice, it was shown that these mice exhibited keratinocyte hyperplasia soon after birth, accompanied by hyperpigmentation, increased melanocytes in ear and tail as well as consequent development of melanoma tumors later in life [42, 43]. These studies also suggested that the recruitment of melanocytes to the dermis was driven by keratinocytes and possibly accumulating fibroblasts and mast cells as the PAFR transgene expression was not seen in melanocytes but was present in keratinocytes. Furthermore the role of PAFR in human melanoma metastasis was further elucidated with *in vivo* experiments using the PAFR antagonist PCA4248. PCA4248 significantly inhibited experimental human melanoma lung metastasis in nude mice [3].

However, it has been shown that PAFR is expressed not only on the surface of keratinocytes but also our lab has shown that all cultured melanoma cell lines regardless of their metastatic potential express constitutively active PAFR [39, 44]. PAFR in melanoma cells is constitutively active in human melanoma cells and mediates gene expression [3].

Our lab also hypothesized that PAFR activation via PAF can phosphorylate and activate the transcription factors cAMP response element-binding (CREB) and activating transcription factor 1 (ATF-1). Expression of these two transcription factors correlate with the transition from radial growth phase to vertical growth phase of human melanoma cells and with their metastatic potential in nude mice [45, 46]. PAF induces CREB and ATF-1 via a PAFR-mediated signal transduction mechanism requiring the Gαq and adenylate cyclase. Furthermore, addition of PAF to the metastatic melanoma A375SM cells stimulated CRE-dependent transcription [39]. Studies have shown that PAF can transactivate membrane type 1-MMP (MT1-MMP) and TIMP-2 genes resulting in proteolytic activation of MMP-2 in human umbilical vein endothelial cells [47]. In human melanoma cells PAF also activated MMP-2 expression and gelatinase activity. Furthermore, MMP-2 activation correlated with an increase in PAF-induced MT1-MMP in human melanoma cells [3, 39].

We propose that all melanoma cells express PAFR regardless of their metastatic potential and secrete basal levels of MMP-2 and MT1-MMP. However, within the

melanoma tumor microenvironment where PAF secreting cells such as platelets, endothelial cells and inflammatory cells come into contact with melanoma cells, activation of the PAFR will cause phosphorylation of CREB and ATF-1 through the p38 MAP kinase and PKA signal transduction cascades. Consequently, this results in overexpression and secretion into the microenvironment of MMP-2 and MT1-MMP (Fig. 1.2). However, since only metastatic melanoma cells overexpress CREB

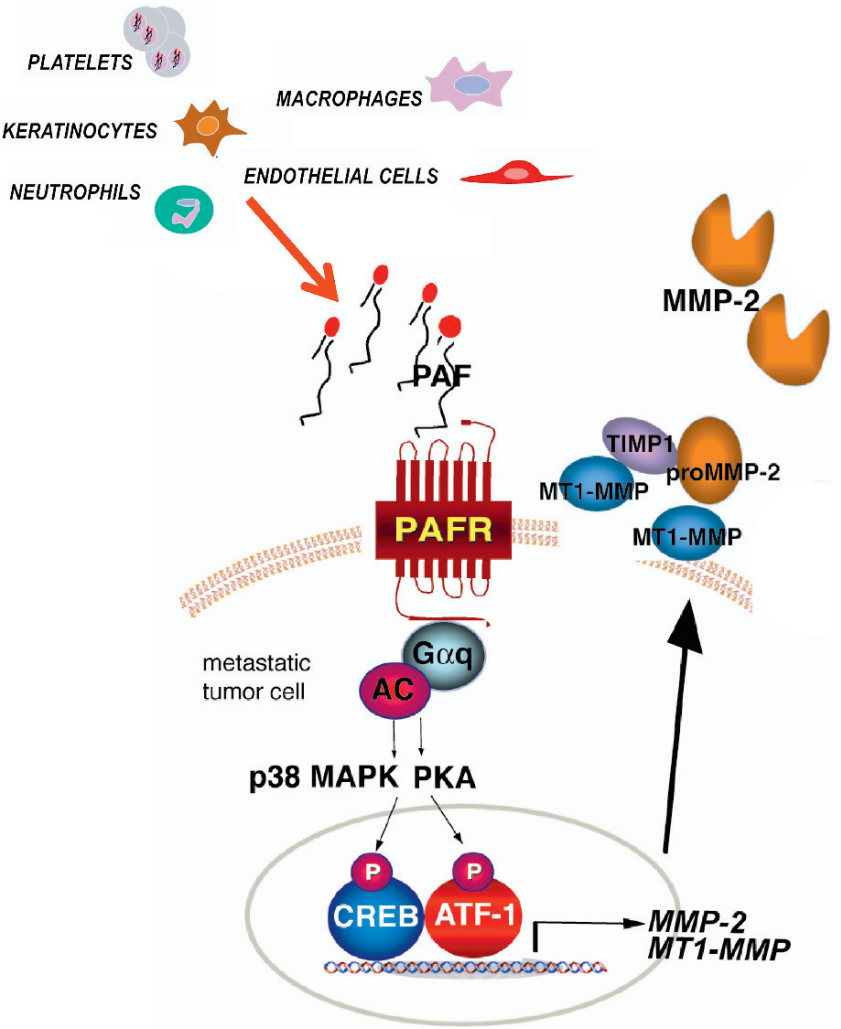

Fig. 1.2 A schematic for the stimulation of MMP-2 and MT1-MMP by PAF via activation of CREB/ATF-1. When melanoma cells come into contact with PAF-producing cells within the tumor microenvironment, PAFR is activated. Through G-proteins and adenylate cyclase, p38 MAPK and PKA phoshporylate CREB and ATF-1. This results in overexpression and secretion of MMP-2 and MT1-MMP

and ATF-1, they are better equipped to respond to the effect of PAF within the tumor microenvironment.

1.3 Conclusion

It is apparent that early inflammatory and angiogenic response and the remodeling of the extracellular proteins are essential factors in creating a microenvironment that sustains tumor growth and metastasis [48]. As we described in this chapter, all these different cell types and factors found within the tumor microenvironment play a significant role in homeostasis and behavior of melanocytes as well as directly affect melanoma growth and malignant invasion [1]. Thrombin, which is abundant in the tumor microenvironment, causes activation of PAR-1, which is found to be upregulated in metastatic melanoma cells. This activation promotes secretion of adhesion, angiogenic and survival factors into the tumor microenvironment allowing for increased metastatic potential of melanoma. Furthermore, PAFR is activated by PAF produced from an array of inflammatory cells, endothelial cells, keratinocytes and platelets found within the tumor microenvironment. Activated PAFR will cause upregulation of the CREB and ATF-1 transcription factors, which in turn increase the secretion of MMP-2 and MT1-MMP. Therefore, melanoma cells will be surrounded by these factors that increase the potential for basement membrane degradation and thereby increase their metastatic potential. Continuing to study the interactions between the tumor microenvironment and melanoma cells will drastically help us understand the mechanisms and key players involved in the transition of human melanoma from radial growth phase to vertical growth phase.

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