Crosstalk between CTC, Immune System and Hypoxic Tumor Microenvironment

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Abstract Accumulating evidence indicate that the behavior of tumorigenic cells is highly influenced by their microenvironment. In this regard, microenvironmental hypoxia plays a determinant role in the emergence of CTC (circulating tumor cells) and CSC (cancer stem cells). CTCs are believed to be indicators of residual disease and thus pose an increased risk of metastasis. In spite of being rare and exposed to immune attack, these cells are capable to escape the immune system of the host. Although CTC play a pivotal role in the metastatic cascade and their prognostic impact has been repeatedly demonstrated, little is known about their escape mechanisms to immune system of the host. Therefore a better knowledge of the immunogenicity of these cells and their cross talk with immune killer cells as well as with tumor microenvironment may represent an exciting new immunotherapy opportunity. In this chapter, we will discuss how hypoxia is involved in the regulation of tumor progression and induction of EMT and cancer stem cell like features. We will also illustrate the relationship between hypoxia and CTC and review how CTC interact with the cells of immune system (both innate and adaptive) in terms of their survival and EMT phenotype. We will attempt to outline how hypoxic stress may confer resistance to CTC by giving them EMT and CSC like phenotype. Finally we will discuss whether the inhibition of hypoxic signaling pathways in different compartments of the solid tumor microenvironment will have an impact on CTC number, resistant phenotype and CTC lysis by immune effectors.

Keywords Hypoxia · CTC · CSC · Immune system

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CTC and Tumor Microenvironment

Tumor Hypoxic Stress

Hypoxia

The term tumor hypoxia refers to a condition in solid tumors where the oxygen pressure is less than 5–10 mmHg. Hypoxia is a common feature of almost all of the solid tumors and one of the hallmarks of tumor microenvironment [37].

Solid tumors contain areas of variable oxygen concentrations. Accumulating evidence indicate that hypoxic zones in solid tumors have temporal fluctuations in oxygen concentration [8]. Tumor cells closest to a blood vessel have relatively high O₂ concentrations, which decrease as distance from the vessel increases. There are areas which are well oxygenated, poorly oxygenated and finally necrotic in which cancer cells have died due to inadequate oxygenation [15].

Hypoxia Inducible Factors (HIF's) and Tumor Cell Adaptation to Hypoxia

Tumor cells adapt to hypoxic microenvironment by the regulation of hypoxia inducible factor family of transcription factors (HIF's). This family is composed of three members namely HIF-1, HIF-2 and HIF-3. HIF-1 is a heterodimeric protein that is composed of a constitutively expressed HIF-1 β subunit and an O₂-regulated HIF-1 α subunit [46].

HIF-1 α in the presence of O₂ is hydroxylated on proline residue 402 and/or 564 by prolyl hydroxylase domain protein 2 (PHD2), resulting in its interaction with the von Hippel–Lindau (VHL) tumor suppressor protein, which recruits an E3 ubiquitin-protein ligase that eventually catalyzes polyubiquitination of HIF-1 α thereby targeting it for proteasomal degradation [35]. Under hypoxic conditions, hydroxylation is inhibited and HIF-1 α rapidly accumulates, dimerizes with HIF-1 β ,

binds to the core DNA binding sequence 50-RCGTG-30 (R, purine (A or G)) in target genes, recruits co-activators and activates transcription. In addition, O₂-dependent hydroxylation of asparagine-803 by factor inhibiting HIF-1 (FIH-1) blocks interaction of HIF-1 α with the co-activators P300/CBP under normoxic conditions. Both PHD2 and FIH-1 use O₂ and α -ketoglutarate as substrates and generate CO₂ and succinate as by-products of the hydroxylation reaction [37]. Similar to HIF-1 α , HIF-2 α is also regulated by oxygen-dependent hydroxylation. HIF-1 α and HIF-2 α are structurally similar in DNA binding and dimerization domains but differ in their transactivation domains. Consistently, they share overlapping target genes, whereas each also regulates a set of unique targets [23]. HIF-3 α lacks the transactivation domain and may function as an inhibitor of HIF-1 α and HIF-2 α and its expression is transcriptionally regulated by HIF-1 [26].

Apart from strict oxygen dependant regulation of HIF's, HIF-1 α and HIF-2 α are regulated by several oxygen independent mechanisms [37]. It has been reported that mutation of VHL, PTEN, B-RAF, SDH, FH and MITF are able to regulate HIF-1 α under normal level of O₂ (hypoxia like normoxia). This stability of HIF-1 α under normoxia leads to its increased transcriptional activity and may have a huge impact on cell biology and cancer development (Fig. 1).

A large body of clinical data shows a positive correlation between increased hypoxic expression of HIF-1 α and HIF-2 α and patient mortality. Both HIF-1 α and HIF-2 α have common target genes as well as their respective target genes. The genes induced by hypoxia dependent HIF-1 α and HIF-2 α play important roles in regulating different aspects of tumor biology such as angiogenesis, cell survival, chemo- and radio-resistance, proliferation, invasion and metastasis, pH regulation and metabolism, resistance to immune system and maintenance of cancer stem cells [37].

Hypoxia Promotes Tumor Progression

Hypoxia, EMT and CTC

Epithelial-to-mesenchymal transition (EMT) is a complex molecular process during which epithelial cells lose their cell polarity, the cell-cell adhesion, apico-basal polarity and acquire a mesenchymal, and motile phenotype. The hallmarks of EMT are the down regulation of epithelial markers such as E-cadherin and cytokeratins and the up regulation of mesenchymal markers such as Vimentin and Fibronectin leading to an organized disassembly of epithelial cells contacts enabling motility and invasiveness [41]. Complex networks orchestrate EMT and several signaling pathways (TGF B, Wnt and Notch) and transcriptional

factors (Snail, Slug, Twist, and Zeb1/2) were directly involved in triggering EMT [42]. Hypoxic signalling through HIF was also implicated as important trigger and modulator of EMT. Infact, under hypoxic conditions, different signaling pathways, directly involved in triggering EMT like NF- κ B and Notch signaling, are activated. Epithelial cell lines regulate also the expression of major EMT-associated transcription factors under hypoxia [31]. Hypoxia regulated EMT has central role in cancer progression, resistance to apoptosis and drugs and in the tumor resistance to the immune response. In fact, Kudo-Saito et al. report that Snail expression in melanoma cells resulted in the impairment of dendritic cell maturation, expansion of T regulatory cells, and resistance to CTL-mediated lysis [22]. More recently, our group demonstrated that EMT impairs breast carcinoma cell susceptibility to CTL-mediated lysis through autophagy induction [1]. EMT has also an important role in metastasis formation. Infact, the mesenchymal characteristics acquired during EMT process allow isolated tumor cells to detach from the primary site, to invade surrounding tissues through lymphatic and blood vessels and to reach distant sites forming metastasis. Basing on this process, EMT can be at the origin of CTC presence and may be involved in their capacity to migrate into blood vessels. Several studies have demonstrated the upregulation of EMT markers in CTC supporting this hypothesis [43]. Infact, phenotypic characterization of CTC revealed EMT markers expression in many cancer models [9]. Kallergi identified CTC expressing vimentin and Twist in breast cancer patients. The presence of these CTCs cells, suggestive of EMT, was correlated with metastatic disease confirming that EMT is involved in the metastatic potential of CTCs [19]. More recently, Schlutze et al. demonstrate frequent presence of EpCAM-positive CTC in patients with intermediate or advanced hepatocellular carcinoma and its prognostic value for overall survival [36]. Interestingly, Baccelli et al. identified a population of blood circulating tumor cells (EpCAM+CD44+CD47+ MET+CTCs) from breast cancer patients that initiates metastasis in a xenograft assay [4]. One other study confirmed strongly a role for EMT in the blood-borne dissemination of human breast cancer. Infact, Yu et al. demonstrated that mesenchymal cells were highly enriched in CTCs. These mesenchymal CTCs occurred as both single cells and multicellular clusters, expressing known EMT regulators, including transforming growth factor (TGF)- β pathway components and the FOXC1 transcription factor. In an index patient, reversible shifts between these cell fates accompanied each cycle of response to therapy and disease progression [50]. As it is now well established that hypoxic tumor microenvironment and EMT process both favour the emergence of tumor variants with increased metastatic and resistance potential, further investigations are needed to better understand the relationship between

Hypoxia-CTC interactions and tumor progression

Fig. 1 The impact of hypoxia on CTC's EMT and CSC like phenotype. CTC escape immune attack in the blood stream by upregulating hypoxia (HIF-1a, BNIP3, CA-IX and GLUT1), EMT (Snail, Twist, EpCam,

Vimentin and E-cadherin), and CSC (Nanog, OCT4, SOX2 and CD133) related genes. CTC; Circulating tumour cells and CSC; cancer stem cells.

CTCs, hypoxia, EMT process and tumor progression will provide insights into the resistance mechanisms in patients and enable monitoring therapeutic efficacy.

Hypoxia, Cancer Stem Cells (CSC) and CTC

Tumor growth is dependent on the presence of a subpopulation with stem-like properties called cancer stem cells (CSC) within the tumor [34]. CSC, which are in an undifferentiated state, undergo self-renewal and when implanted in immune-deficient mice are able to develop tumors and to re-establish the bulk tumoral heterogeneity [32]. CSC also have the property to resist to conventional anti-tumor therapies, which makes them a probable cause of tumor recurrences after treatment [31]. Therefore, their eradication in the tumor is a therapeutic challenge that justifies a better understanding of their emergence and persistence in the tumoral tissue.

In this regard, hypoxia and HIFs have been described to induce tumor cell dedifferentiation toward an immature phenotype and similarly to maintain tumor cells with stem-cell properties [20]. Several reports show the role of hypoxia and HIFs in promoting a stem-like phenotype through the expression of genes such as *OCT4*, *SOX2*, *NANOG* required for self-renewal maintenance in stem cells or the activation of the Notch- signaling pathway that regulate cell self-renewal and differentiation [3]. The ability of hypoxia to increase the stem cell-like subset inside a tumor cell population reflects the plasticity and the dynamics of the CSC compartment and the role of micro-environmental stimuli in shaping this particular subset.

Some of the effects of hypoxia on tumor cell differentiation are directly mediated by the HIFs. Li et al. reported that targeting HIF-1 α and HIF-2 α in CD133⁺ glioma stem cells decreased their survival and their tumorigenic and angiogenic potentials [24, 16]. These findings support a preferential targeting of HIF-2 α for selective eradication of CSC without adverse effects on

normal progenitor cells. HIF-1 α is not outdone since a recent study by Wang et al. using human leukemia showed a selective activation of HIF-1 α in CSC under normoxic conditions due to VHL deficiency, and that blocking HIF-1 α activity was able to eliminate leukemia stem cells without affecting the normal hematopoietic stem cells [47].

Therefore hypoxia via the HIF factors leads to the development of tumor cells with stem-cell like features from non stem cell-like tumor cells.

CSC contribute to the metastatic and invasive processes which constitute the major cause of cancer mortality. Given that CTC also contribute to metastasis, one could speculate that CTC are enriched for CSC as compared to the tumor from which they arise. Increasing evidence show that CTC exhibit CSC features. Indeed, in castration-resistant prostate cancer, the majority of CTC were found to express the stem-cell like marker CD133 which is a cell-surface protein associated with prostate cancer stem cell phenotype [44]. In hepatocellular carcinoma, CTC displaying a CD45⁺ICAM-1⁺ surface phenotype exhibited tumor induction and sphere-forming capacities, indicative of CSC properties [25]. In breast carcinoma, the presence of CTC in blood sample was found to significantly correlate with stage and grade of disease, as well as the expression of ALDH1 in CTC [33]. CTC with a putative CSC like phenotype [40] and NANOG, OCT3/4, SOX2 genes were also expressed in CTC from breast cancer patients blood samples, and their expression in CTC correlated with the stage of the disease [2]. Analyzing the level of expression of CSC markers in CTC may therefore improve the establishment of the prognosis for cancer patients.

Since hypoxia and HIF factors can directly increase the expression of CSC markers and induce CSC properties, one would predict that hypoxia and HIFs might contribute to CTC generation and CTC CSC like phenotype resistant to immune system.

Hypoxia and the Emergence of CTC

Hypoxia selects tumor cells with more aggressive phenotype and decreased apoptotic potential [15]. The hypoxic tumor microenvironment contributes both directly and indirectly to increased metastases by regulating number of genes that are implicated in EMT process. Clinical evidence also suggests that patients with tumours that contain more extensive hypoxic and anoxic areas have higher rates of metastases [6].

The relationship between CTCs and tumor hypoxia remains largely unknown. Ameri K. et al., using the human triple negative breast cancer cell line MDA-MB-231, generated orthotopic xenografts in mice that produced CTCs [10] and resulted in lung metastases. These xenografts were found to be profoundly hypoxic and produced CTCs that were captured and cultured. They examined the response of CTCs and parental MDA-MB-231 cells to hypoxia (O₂ levels of

0.2 %) and compared the ability of both cell types to develop tumor xenografts in vivo. These CTCs demonstrated an altered response to hypoxia compared with the parental MDA-MB-231 cells from which they were derived and a greater aggression in vivo. One altered response was ATF4 induction (anoxia-induced factors) seen only in hypoxic CTCs but not in parental MDA-MB-231 cells. In addition to expressing the anoxia-induced factors at greater levels, CTCs also demonstrated an altered regulation of hypoxia-HIF1 α target genes. Circulating tumour cells did induce BNIP3, CAIX, and GLUT1, but the induction of these factors was much lower than in parental MDA-MB-231 cells. In contrast, induction of apelin was much higher in CTCs, compared with parental MDAMB-231 cells [10].

In another study, Kallergi and colleagues investigated HIF-1 α and vascular endothelial growth factor expression in circulating tumor cells of metastatic breast cancer patients. They showed that CTCs from these patients express VEGF both at mRNA and protein level. Moreover, they also demonstrated that VEGFR2 is also expressed on CTCs' membrane suggesting an autocrine loop involving VEGF and VEGFR2. More interestingly, double and triple staining experiments on CTCs showed that VEGF co-expressed with HIF-1 α and VEGF2. Hence, the production of VEGF was probably under the regulation of HIF-1 α and/or pFAK as suggested by the observed significant correlation between the expression of these molecules [18]. CTCs expressing HIF-1 α and their target genes (BNIP3, CA-IX, VEGF and GLUT1) might be resistant to immune system because of an altered regulation of hypoxia-HIF1 α target genes.

Apart from above mentioned few preliminary reports, the relationship between CTCs and tumor hypoxia has not been fully investigated and much needs to be explored. The impact of hypoxia on CTC number, CTC phenotype, CTC CSC like properties is still unexamined. It is still unclear whether hypoxia driven CTC's resistant phenotype (EMT and CSC like) helps these cells evade the immune system. Whether hypoxic tumor microenvironment is involved in modulating and determining the metastatic ability of CTCs, as well as their progression once secondary tumor metastases are established remains largely unknown.

CTC and Immune System

Immune Escape Mechanisms from CTL/NK Effectors Inherent to CTC

CTC are continuously facing a very hostile environment in the blood stream. Tumors are most probably shedding millions of CTC into the blood stream but only few survive and can be detected at any given time. There may be a

rapid clearance of CTC in blood by natural killer (NK) cells, monocytes /macrophages, and neutrophils. CTC are therefore under evolutionary pressure to develop a phenotype that can protect or hide them from the immune system. Few reports analyzed the sensitivity of CTC to cell-mediated cell death. Given that CTC express EMT and CSC markers, both involved in a decreased sensitivity to cytotoxic immune effectors [31], one could expect that CTC are resistant to cell-mediated cell death as compared to the primary tumor from which they originate.

Notwithstanding the EMT and CSC phenotypes, a significant increased expression of cell-surface CD95 was found in peripheral T helper cells (CD3⁺/CD4⁺) in the blood of CTC-positive breast cancer patients [13]. Cytotoxic T-cells (CD3⁺/CD8⁺) also exhibited an increase of CD95 expression, but not significantly. The upregulation of CD95 at the cell surface of T helper cells may favor the induction of helper T cell apoptosis via the interaction with tumoral CD95L, and may therefore represent a mechanism of tumor cell escape. Also, direct association with lymphocytes and aggregation of platelets around CTCs has also been shown to protect circulating tumor cells from NK cell-mediated lysis [38].

Recently, Green et al. demonstrated that the expression of TLRs on monocytic dendritic cells DCs of CTC-positive patients was increased as compared to CTC-negative patients. Such uncontrolled and sustained TLR signaling in DC is able to create a suppressive microenvironment that allows tumor cell proliferation and immune evasion through expression of cytokines, chemokines, and growth factors. Thus, this study suggests that the presence of epithelial CTCs in metastatic breast cancer is associated with the presence of an immunologically vulnerable population and further susceptibility of host inability to effectively eliminate transformed cells [12].

Immune Escape From Anti-Tumor Immune Effectors CTL/NK Inherent to Tumor Cells and Transposable to CTC

Many studies have evidenced different mechanisms allowing tumors to escape or to interfere with the antitumor immune response [14]. The escape mechanisms can be classified into several categories, depending whether they are inherent to the tumor cells, the immune cells, or present in the tumor microenvironment. Since CTC arise from tumor cells, they may exhibit some of the immune escape mechanisms inherent to tumor cells.

Very recently, Green et al. evaluated the percent specific lysis using chromium-51 assay to illustrate the functional abilities of patient natural killer (NK) cells and found that those with greater than five CTCs per 7.5 mL blood had significantly decreased responses by their immune cells when compared with those patients who had 5 CTCs or less [11]. Gruber et al. demonstrated that CTC-positive patients presented a significant increase of peripheral CD95 (FAS)-positive T-

helper cells. As immune response is regulated by CD95 (APO-1/FAS)-CD95ligand interaction and tumor cells induce apoptosis via the CD95/CD95L (ligand) pathway, this might lead to tumor cell escape by apoptotic T-helper cells [13].

Interestingly, Steinert et al. showed that several CTC exhibited mutations in key genes such as KRAS or TP53 that could not be detected in the tumor. Gene expression analyses revealed both a pronounced upregulation of CD47 as a potential immune-escape mechanism and a significant down-regulation of several other pathways. This study suggests that upregulated immune-escape pathways, may be responsible for survival of CTC in circulation [39].

Escape to Recognition by Immune System

Antigen presentation by MHC molecules is crucial both for the initiation of the T cell response and the maintenance of this response. Alterations in processing and/or presentation of the antigenic peptide were reported frequently in many tumors. Complete loss of MHC molecules was observed in many types of cancers such as breast, prostate or lung cancers [28]. The decreased expression of TAP1 and TAP2 genes and decreased expression of components of the immunoproteasome such as subunits LMP2 and LMP7 are also frequent mechanisms of tumor escape, causing alterations of antigen presentation and processing. Finally, the loss of expression of tumor antigens is an important mechanism of escape to T cell-mediated immune response [14]. It would be interesting to study if CTC uses the decreased antigen production, processing or presentation mechanisms to escape tumor system.

Resistance to T Cell-Mediated Lysis

Tumor cell susceptibility to lysis by the immune system remains ineluctably a determining element of the effectiveness of anti-tumor immune responses. Several mechanisms of resistance to cytotoxic functions of CTLs and NK cells, involving especially the perforin- granzyme and the death receptor pathways have been previously described [14]. As EMT and CTC emergence are interrelated process and as almost CTCs express EMT markers, it is strongly suggested that CTC are resistant tumor cells to T cell-mediated lysis.

Resistance to the Perforin-Granzyme and Death Receptor Pathway Some tumors escape death induced through perforin-granzyme by expressing the Serpin PI9/SPI6, a cellular inhibitor of granzyme B [29]. Various alterations in mitochondrial signaling pathways (overexpression of Bcl-2, Bcl-XL, IAPs, and mutations of Bax, Bak, or Apaf-1) are frequently

observed in tumor cells and may alter the response to the perforin-granzyme pathway, mediating resistance to lysis. Similarly, resistance to apoptosis induced by FasL-Fas induced pathway has been demonstrated in many tumors [48, 14].

CTC are reported to express survivin [49, 17] an anti-apoptotic protein that confers resistance to cytotoxic therapies and cytotoxic effectors [45]. Survivin expression in CTC may thus promote immune escape. Whether CTC can resist to the immune system by using the above mentioned pathways is a very interesting and unknown question.

Expression of Inhibitory Molecules

The expression by tumor cells of the B7-H1 molecule (also called programmed death ligand 1 (PD-L1)) that targets PD-1 on the surface of antigen-specific T cells has been described in many types of cancers [21]. The engagement of B7-H1 with its receptor induces the apoptosis of activated T cells [5]. Also, triggering by PD-L1-positive tumor cells of PD-1 on T cells increases tumor cell resistance to immune-induced death [3], demonstrating that cancer cells can use receptors on immune cells to induce resistance. The expression of HLA-G in ovarian carcinoma cells induces resistance to lysis mediated by CD8⁺ T cells [27]. Similarly, the soluble and membrane expression of another non-classical MHC molecule, HLA-E, by melanoma cells, decreases their susceptibility to lysis induced by CTL [7]. Whether CTC can express or up-regulate surface inhibitory molecules such as PD-L1 and HLA-G and E molecules remains unexplored.

EMT and Metastatic Tumoral Resistance

Metastatic tumoral resistance involves multiple factors against apoptosis mediated by cytotoxic inducers (TRAIL, FasL, TNF, and CTL) [30]. Down-regulation of ICAM-1 on metastatic melanoma cells is reported to decrease their sensitivity to CTL- and granzyme B-mediated lysis [14].

A study by Kudo-Saito and colleagues demonstrate that Snail-induced EMT accelerates cancer metastasis through not only enhanced invasion but also induction of immunosuppression by inducing Regulatory T cells and impairing dendritic cell maturation. In addition, although Snail expressing melanoma did not respond to immunotherapy, intra-tumoral injection with snail-specific siRNA significantly inhibited tumor growth and metastasis following increase of tumor-specific tumor-infiltrating lymphocytes and systemic immune responses [22].

CTC's are EMT+ cells and one may expect that CTC have acquired resistance to cytotoxic treatments including immune cytotoxic effector cells. It would be interesting to study if the

acquisition of this resistant EMT phenotype by CTC render them resistant to immune system.

Conclusions

Although CTC play a pivotal role in the metastatic cascade and their prognostic impact has been repeatedly demonstrated, little is known about their escape mechanisms to immune system of the host. NK cells and CTLs are important in host to eliminate tumor cells and in turn preventing the development of tumor cells into metastasis. However do these cells naturally recognize and protect against CTC invading cells remain poorly understood. It is well established that the tumor-killer cells and CTC are influenced in their behavior by tumor microenvironment that could be decisive in the development of the metastasis. In this context, CTC are under evolutionary pressure to develop a phenotype that can protect or hide them from the immune system. It is not known yet if the immune adaptive immune system is able to detect CTC by specifically recognizing the mutated proteins they may express. The recognition of CTC by the immune system of the host and their escape mechanisms is therefore an important issue in the field of tumor immunology that should be considered at the fundamental level and during immune intervention approaches.

In addition several questions are raised with regard the functional similarity between CTC and CSC: how similar phenotypically are these cells? If they are different, is relapse caused by the CSCs from the primary tumor or CTCs in metastasis that have become CSC-like? Do these cells accumulate mutations to evade immune destruction?

Most solid tumors, in the clinic and in experimental models, are characterized by poorly vascularized regions and many tumor cells flourish under hypoxic conditions. In addition, hypoxia has been shown to promote the EMT phenotype and the subsequent tumor invasion. In this regard it is tempting to speculate that hypoxia may favor the emergence of CTC and tumor metastasis. With this respect, the identification of immune-escape mechanisms as potential targets to disrupt the metastatic cascade in cancer is critical. Although our current understanding on how the immune system fights CTCs remains very fragmentary, a deeper understanding of the interaction between CSCs and their microenvironment is critical to the successful targeting of this population and may provide new targets for therapeutic intervention.

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