Cancer stem cell and mesenchymal cell cooperative actions in metastasis progression and hormone resistance in prostate cancer: Potential role of androgen and gonadotropin-releasing hormone receptors (Review)

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Abstract. Prostate cancer (PCa) is the leading cause of male cancer-associated mortality worldwide. Mortality is associated with metastasis and hormone resistance. Cellular, genetic and molecular mechanisms underlying metastatic progression and hormone resistance are poorly understood. Studies have investigated the local effects of gonadotropin-releasing hormone (GnRH) analogs (used for androgen deprivation treatments) and the presence of the GnRH receptor (GnRH-R) on PCa cells. Furthermore, cell subpopulations with stem-like properties, or cancer stem cells, have been isolated and characterized using a cell culture system derived from explants of human prostate tumors. In addition, the development of preclinical orthotopic models of human PCa in a nonobese diabetic/severe combined immunodeficiency mouse model of compromised immunity has enabled the establishment of a reproducible system of metastatic progression *in vivo*. There is increasing evidence that metastasis is a complex process involving the cooperative actions of different cancer cell subpopulations, in which cancer stem-like cells would be responsible for the final step of colonizing premetastatic niches. It has been hypothesized that PCa cells with stemness and mesenchymal signatures act cooperatively in metastatic progression and the inhibition of stemness genes, and that overexpression of androgen receptor (AR) and GnRH-R decreases the rate the metastasis and sensitizes tumors to hormone therapy. The aim of the present review is to analyze the evidence regarding this cooperative process and the possible influence of stem-like cell phenotypes, AR and GnRH-R in metastatic progression and hormone resistance. These aspects may represent an important contribution in the understanding of the mechanisms underlying metastasis and hormone resistance in PCa, and potential routes to blocking these processes, enabling the development of novel therapies that would be particularly relevant for patients with metastatic and castration-resistant PCa.

Contents

1. Introduction

Prostate cancer (PCa) is one of the major causes of male cancer-associated death worldwide (1). Over the last few decades, screening programs have increased early diagnosis and identified treatments with the potential to cure the disease (2-5). However, the high rates of recurrence and metastasis remain major challenges in treating PCa (6-12). During a long period of the disease, PCa can become sensitive to androgen treatment (13,14). Testosterone controls cell proliferation, tumor growth and, potentially, dissemination (15-17), which is an advantage in treatments that involve androgen deprivation (AD), when curative surgery cannot be performed (18,19). Pharmacological castration using gonadotropin-releasing hormone (GnRH) analogs to

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block the hypothalamus-hypophysis-testicular axis provides the first-line treatment for disseminated PCa (20-22). However, during AD therapy, PCa cells frequently become androgen-resistant, resulting in a castration-resistant form of the disease with a poor prognosis (23-25). The genetic and molecular mechanisms underlying androgen resistance remain poorly understood (26-28). Research suggests that, in certain cases, the androgen receptor (AR) is involved in this resistance (29-31). On the other hand, recurrence and metastasis progression are complex processes that involve several mechanisms and genomic modifications of malignant cells (32,33). It is well-known that epithelial-mesenchymal transition (EMT) is the main pathway via which malignant epithelial cells from carcinomas alter their gene expression profile to display a mesenchymal phenotype, acquiring, among other features, one of the hallmarks of cancer cells: Invasive behavior (34-38). However, increasing evidence indicates that tumors contain a phenotypically heterogeneous cell population, and that the cooperative action of these different types of malignant cells is potentially required to accomplish a successful metastatic process (39-42). In the past few years, a small subpopulation of malignant cells with stem-like properties has been identified in numerous types of cancer, including PCa (43,44). These cells have been termed tumor-initiating cells (TICs) or cancer stem cells (CSCs), and are hypothesized to be responsible for recurrence and metastasis (45-48).

2. Androgen deprivation and local effects of GnRH analogs in PCa

As aforementioned, GnRH analog therapy is the gold standard to treat disseminated PCa (20,49). This treatment induces AD by blocking the hypothalamus-hypophysis-testicular axis, resulting in pharmacological castration. This type of therapy is very efficient at delaying tumor growth until PCa becomes castration-resistant (20). Gene amplification, mutations and other alterations in the AR gene have been identified (29,50-52). In addition, overexpression or constitutive activation of other proliferation signaling pathways that overcome androgen control have been reported (53,54). In addition, alterations in androgen metabolism within the prostate gland have been associated with androgen sensitivity (55,56). It is postulated that castration-resistant PCa arises from a combination of these different mechanisms. Our previous research, as well as other studies, have reported the presence of GnRH receptor (GnRH-R) in PCa cells (57-59). Furthermore, it has been observed that GnRH analogs induce proliferation arrest and apoptosis in PCa cells in a primary culture system (57,58). GnRH-R expression increases from benign prostatic hyperplasia to medium histological grade (Gleason score 6-7), and subsequently decreases in samples from patients with higher Gleason scores (60). Local cellular effects of GnRH analogs may be of clinical relevance, as these effects remain despite cell androgen insensitivity (58,60). Concentrations >20 ng/ml are required to obtain significant *in vitro* apoptotic effects (61); however, the plasma concentrations in patients receiving AD treatment are below this level (62). This problem may be solved via intraprostatic administration of GnRH analogs. Unfortunately, patients who are castration-resistant often have a higher Gleason score and, as aforementioned, GnRH-R expression decreases with higher Gleason scores. There is evidence that GnRH-R in PCa, specifically in the gonadotropic cells, is retained primarily in the endoplasmic reticulum, where it can be moved to the plasma membrane using peptide-mimetic compounds called pharmacoperones (pharmacological chaperones) (60). Using this strategy, it is possible to increase GnRH-R expression in cultured PCa cells and sensitize them to the apoptotic effects of GnRH analogs (Fig. 1).

3. EMT and CSCs in PCa

EMT is a process in which an epithelial genetic program switches to a mesenchymal program; as a result, an epithelial cell loses its polarity, proliferation, and differentiation control and positioning, changing to a mesenchymal phenotype (63-65). This is a physiologically normal process occurring primarily during embryonic development (66). During carcinogenesis, similar genetic changes occur in carcinomas that transform a malignant epithelial cell into a highly proliferative and invasive mesenchymal-like cell (65,67). Epithelial malignant cells progressively lose adhesion molecules, such as E-cadherin, syndecans and tight junction molecules, whereas gene-regulating factors, including Snail family transcriptional repressor SNAI1, SNAI2, zinc finger E-box-binding homeobox 1/2 and TWIST increase their expression, together with mesenchymal markers such as vimentin, N-cadherin and metalloproteinases, resulting in an invasive cell phenotype (35,38,68-70).

In PCa, syndecans are associated with Gleason score and EMT markers (71-73). It is hypothesized that this mesenchymal and invasive phenotype is responsible for the metastatic process (68,74). However, there is no direct evidence that these mesenchymal cells (MCs) also have colonizing abilities. Conversely, increasing evidence suggests that a small population of malignant cells present in most types of tumor, CSCs, may be responsible for the final step in recurrence and metastasis (75-77). Our previous study identified and characterized a CSC population in PCa samples and determined their molecular stem signature ($CD133^+/CD44^+/ABCG2^+/CD24^-$) (78). In addition, proliferative, migratory, invasive and clonogenic abilities have been evaluated in this cell population (79). It is possible to separate this CSC population from mesenchymal-like cells by changing culture conditions, followed by magnetic-associated cells sorting (MACS) (78). In adherent conditions, most cells remain in a mesenchymal differentiate state, which has been determined from using specific markers and functional assays. However, in non-adherent conditions, most mesenchymal adherent cells die by anoikis (anchorage-dependent apoptosis), whereas a few cells survive, and rapidly form spheres that grow and remain for several weeks (78). Following MACS, separated sphere-forming cells represent an enriched CSC population (78). These CSCs exhibit a low proliferation rate, increased resistance to apoptosis and drug treatments, reduced invasive properties and a high clonogenic capacity compared with that in mesenchymal-adherent cells (79). In addition, these PCa CSCs have no expression of GnRH-R or AR, nor of numerous differentiation markers (79). Preliminary experiments within our laboratory using CSCs with stable expression of AR and GnRH-R via lentiviral transduction suggest that these cells can become sensitive to androgens and GnRH analogs (unpublished data) (Fig. 2).

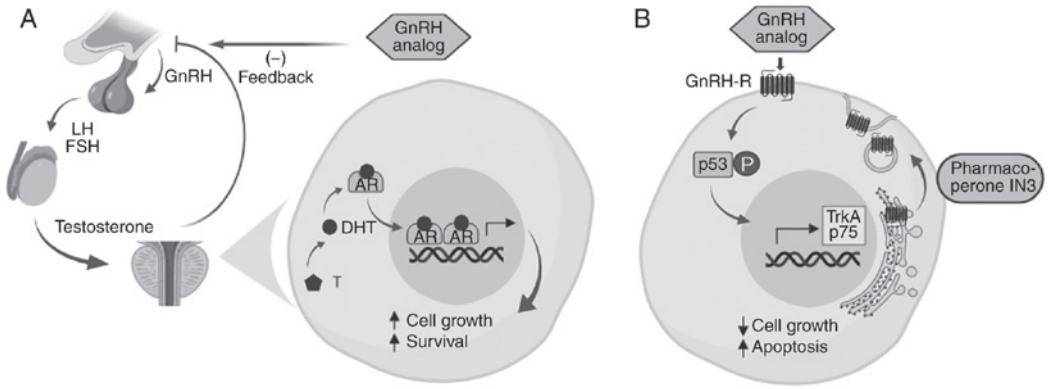

Figure 1. Comparison of systemic and local effects of GnRH analogs. (A) Systemic delivery of GnRH analogs blocks the hypothalamus-hypophysis-testicular axis, producing pharmacological castration that inhibits the AR-induced cell growth and survival of PCa cells. (B) Locally, in PCa cells, GnRH analogs activate GnRH-R, inducing phosphorylation of p53, and resulting in increased expression of TrkA and p75, which inhibits cell growth and stimulates the apoptosis of PCa cells. These effects can be potentiated with pharmacoperone IN3 by increasing GnRH-R availability in the cell membrane. AR, androgen receptor; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; GnRH-R, GnRH receptor; LH, luteinizing hormone; PCa, prostate cancer; TrkA, tropomyosin receptor kinase A.

4. Tumor cell phenotypic heterogeneity and metastatic processes

It is becoming apparent that tumors present a significant degree of cell heterogeneity (80,81). Tumor heterogeneity may be understood at the phenotypic and genetic level (82). Tumor cell phenotypic heterogeneity will specifically be discussed. Cellular and molecular mechanisms responsible for this heterogeneous cell population remain poorly understood. There remains controversy regarding the origin of CSCs, and several hypotheses have been suggested (76,83-85). However, regardless of the origin of CSCs, the relevant point, particularly for clinical application, is that such a population is present in the majority of cancers studied. The multifocal origin of cancer cells within the organ and the distinct differentiation fate during EMT process may explain, in part, this phenomenon (75,86). As with the process of microevolution, cancer cells adapt to different microenvironments, first within the tumor niche and subsequently in potential metastatic niches (87,88). Within the tumor, it is possible to find a hypoxic microenvironment, for instance in the center of a solid tumor, whereas in the periphery, where neoangiogenesis is occurring, a more oxygenated milieu is more prevalent (89,90); cancer cells adapt differently to these distinct microenvironments. Therefore, it is possible that during EMT progression, certain cells express a stem gene program, forming a stable CSC population within a tumor (91-93).

Metastasis is an inefficient process; it is estimated that <2% of total cancer cells entering the blood stream from a solid tumor will be able to colonize a premetastatic niche (94). Furthermore, <0.02% will be able to survive in that niche and support sustained growth to give rise to clinically evident metastatic foci (94). Evidence suggests that this is not a stochastic process, indicating that not all malignant cells are able to sustain metastasis (94). Furthermore, very few cells have the ability to colonize, survive and grow in a tissue or organ different to the one from which it originated (95). The majority of researchers investigating CSCs have concluded that these metastatic cells express stemness genes and exhibit little invasive capacity (96). Previous results from our laboratory in CSCs from PCa are consistent with this hypothesis (78). Instead, PCa CSCs, as with other CSCs, have a low proliferation rate, high resistance to drugs and apoptosis (particularly anoikis), sphere-growing ability and a high clonogenic capacity (97-99). Determining how these CSCs, with little invasive activity, can leave the tumor and colonize premetastatic niches will be subsequently addressed.

Metastasis is a complex process. Premetastatic niches are developed in advance by several signals originating from the initiating tumor determining the tissue tropism of the future metastatic foci (100,101). It is proposed that, once in the blood stream, CSCs are guided by homing signals from these premetastatic niches (102,103). Once colonizing a metastatic site has begun, CSCs can be induced by niche milieu factors to survive and proliferate, or to become quiescent (104,105). In the event of quiescence, future microenvironmental changes can subsequently induce cell proliferation and tumor growth, resulting in relapse, even if curative surgery was performed to remove the primary tumor (104). In human PCa, bone is one of the main sites of distant metastasis (106). Stromal-cell-derived-factor 1, acting through C-X-C chemokine receptor 4 on malignant cells, is hypothesized to promote cell survival in the niche (106). Secretion of several interleukins, tumor necrosis factor-α and other factors by cancer cells stimulates secretion of the receptor activator of NF-κB ligand (RANKL), which in turn stimulates osteoclast differentiation (107). Increased osteoclast activity releases bone matrix and growth factors that promote CSC survival and growth for metastatic progression (106-108). Exosomes secreted by CSCs and bulk cancer cell cultures derived from PCa contain various microRNAs (miRNAs/miRs). Comparing those miRNAs using next-generation sequencing followed by bioinformatics analysis, specific miRNAs, such as miR-100-5p, miR-21-5p and miR-139-5p were found to be overexpressed and, analyzed in an *in vitro* system, they increased the expression of metalloproteinases-2, -9 and -13, and RANKL, as well as fibroblast migration, supporting the idea that the different PCa cells contribute cooperatively to prepare the premetastatic niche (100).

Considering that CSCs appear to be the only cells within a tumor with the ability to form metastasis, it is reasonable to propose that any increase in circulating CSCs will raise the risk

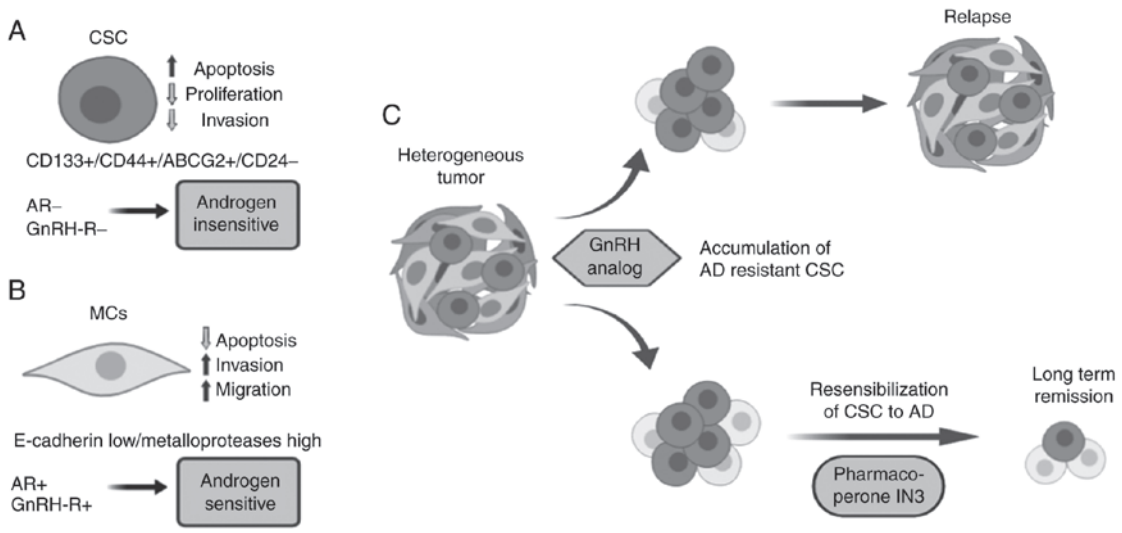

Figure 2. Resensibilization of CSCs to AD therapy could achieve long-term remission of metastatic CRPC. (A) CSCs are characterized by high resistance to apoptosis, low proliferation rates and low invasive capacities; as they do not express AR and GnRH-R, they are not responsive to AD therapies. (B) MCs have less resistance to apoptosis, and high invasive and migratory capacities; as they express AR and GnRH-R, they are responsive to AD therapies. (C) In a PCa tumor, characterized by heterogenous subpopulations, MCs represent the bulk of the tumor. CSCs and MCs cooperate to form metastases. MCs migrate and invade within the tumor stroma, allowing CSCs to escape to the circulation and grow in the metastatic niche, where they are able to grow and generate the full heterogeneity of the original tumor. After AD therapy with GnRH analogs, androgen-responsive MCs are in remission, but androgen-insensitive CSCs accumulate and the tumor returns. Resensibilization of CSCs to GnRH analogs using pharmacoperones or lentiviral transduction could lead to long-term remission of metastatic CRPC. AD, androgen deprivation; AR, androgen receptor; CRPC, castration-resistant PCa; CSC, cancer stem cell; MC, mesenchymal cell; GnRH, gonadotropin-releasing hormone; GnRH-R, GnRH receptor; PCa, prostate cancer.

of metastasis or recurrence (88,109-111). Our previous study investigated the expression of stem signatures in PCa samples of different histological grades, using a tissue microarray and quantitative immunohistochemistry (78). It was observed that the number of cells expressing stem markers increases with Gleason grade, reaching maximal levels at medium Gleason, and decreasing thereafter in high-Gleason grade, lymph node and bone metastatic samples (78). Considering that malignant cells begin to enter the blood stream shortly after the tumor becomes locally invasive (low-to-medium histological grade), it is possible that a patient with a localized tumor with a medium Gleason score will contain the maximal number of CSCs potentially leaving the tumor and spreading throughout blood stream. At this stage, the indicated therapy is surgical removal of prostate gland (112). However, if CSCs already released from the tumor have seeded the metastatic niches, recurrence risk would be high. This is an important point to consider, particularly in patients with localized tumors of low Gleason grade where the therapeutic recommendation is active surveillance (5,113). Therefore, identifying and quantifying CSCs in PCa biopsies may be a valuable prognostic factor for relapse.

5. Different malignant cell types in a solid tumor may collaborate to produce distant metastasis

Reanalyzing the problem of how CSCs with little invasive activity can leave the tumor and colonize premetastatic niches, it is reasonable to suggest that some type of collaboration with highly invasive mesenchymal-like cells occurs (45,96). Previously, Celià-Terrassa *et al* (114) provided evidence regarding this potential cooperative action. Using commercial cell lines derived from PCa (PC3) and bladder cancer (TSU-Prl), these were enriched with metastatic TICs, a cell population with a strong epithelial profile. In turn, they deprived TICs, a cell population with a mesenchymal profile. Overexpression of mesenchymal genes in the former cell population (epithelial phenotype) decreased its TIC ability, whereas knockdown of these genes in the latter cell population (mesenchymal phenotype) enhanced its TIC capacity (114). Using immunocompromised nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, it was observed that, injected in combination, mesenchymal-like cells increased the metastatic potential of epithelial TIC-enriched cell populations, suggesting a cooperative action between both cell types (114). Subsequently, the same research group described that secreted protein acidic and rich in cysteine (SPARC) mediates the metastatic cooperation between CSC and non-CSC cell subpopulations (39).

Recently, it was reported that SPARC induced EMT, increasing the invasive capacities of PCa cells (115). Collectively, these findings support the hypothesis that within a tumor, MCs become the predominant population via EMT, increasing the invasive capacity of the tumor. However, it has been proposed that a small cell population that expresses a stem-like program (CSCs) remains in the tumor and can escape passively with the bulk of MCs. Once in the metastatic niche, it is hypothesized that CSCs proliferate and produce progenitor cells that may further differentiate to an epithelial-like phenotype. This may explain certain findings revealing that in metastatic PCa samples, an increase in epithelial markers and a decrease in mesenchymal markers is observed, which has been called mesenchymal-epithelial transition (116,117). It is postulated that the metastatic foci will generate the full heterogeneity of the original tumor, in which epithelial-like cells will undergo EMT again, whilst a small number of CSCs are retained in the tumor. On the other hand, tumor cell plasticity influences the phenotypic heterogeneity of tumor cells,

with the varied cell abilities enabling cooperation to promote cancer progression and metastasis. Differential cell distribution within the tumor, and spatial and temporal patterns during EMT-stemness processes may influence cell frequencies and the results of the proposed cell cooperation (118). This may contribute to why different patients with PCa at the same stage may have different outcomes.

Personalized medicine should take into consideration this evidence to develop novel and innovative therapeutic strategies. In this context, resensibilization of PCa cells (including CSCs) to GnRH analogs using pharmacoperones or lentiviral transduction may provide an effective treatment against metastatic castration-resistant PCa. It is necessary to validate this hypothesis using CSCs and MCs derived from the tumors of various patients. Metastasis is, by definition, a process that occurs in a living organism. Therefore, there are no *in vitro* models for investigating this complex pathological process. In previous years, several *in vivo* models have been developed (119-124). The majority of these use immunocompromised mice, and several mouse strains have been obtained, a number of them via transgenic manipulation (124-126). One of the most used models, at present, is the NOD/SCID mouse (127).

6. Orthotopic model for the study of human PCa metastasis

The NOD/SCID mouse has been widely used to investigate the metastasis of several types of human cancer (128). A critical issue is the type of injection used to introduce human cancer cells. Numerous researchers use subcutaneous, intravenous or intracardiac administration, with varying results (114,129). Additionally, orthotopic models have been developed (injection in the same mouse organ or tissue from which human cells were derived). This model mimics the metastatic process more precisely (129). Reports of orthotopic models for human PCa have been published (130-132). A modification of the orthotopic model for PCa using a cell injection in one of the anterior lobes of the NOD/SCID mouse prostate has been developed by our laboratory (133,134). This orthotopic injection results in consistent and reproducible metastatic progression. First, a fraction of tumor cells injected in the mouse prostate survives and generates a tumor derived from surviving injected cells (transduced with luciferase and red fluorescent protein genes). The fluorescence allows the tracking of metastatic progression *in vivo* using *in vivo* imaging equipment. In a chronological sequence, metastatic foci begin to appear in the liver, lungs and the kidneys. Injection of cells into the anterior lobe, instead of the ventral prostate, has the advantage that it is possible to surgically remove the prostate tumor to evaluate the progression of metastasis, with or without the primary prostate tumor. In this orthotopic model, the utility of prostatectomy during metastasis progression has been demonstrated (134), as has the effect of knocking down the stemness gene Sox2 on metastasis (unpublished data). In current studies, progression towards a castration-resistant PCa mouse model using surgical castration as an AD strategy is being established.

7. Conclusions

In conclusion, it is proposed that there is cooperation between CSCs and MCs during metastatic progression. Further development of preclinical orthotopic models of PCa may provide additional evidence supporting this hypothesis. In addition, the role of stem genes, as well as AR, GnRH-R and differentiation genes, in metastasis progression and hormone resistance may have critical relevance. Further investigation of these aspects will contribute to the understanding of the cellular and molecular mechanisms of metastasis, recurrence and hormone resistance in PCa, which remain major challenges for the treatment of this disease. It is predicted that evidence obtained using preclinical models, will be beneficial for clinical purposes in the near future, identifying novel prognostic factors and therapeutic targets.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HRC and FLM contributed to reviewing and discussing the literature, and selecting relevant studies. EAC analyzed the subject and wrote the review.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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