Rat Prostate Tumor Cells Progress in the Bone Microenvironment to a Highly Aggressive Phenotype[1]

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Abstract

Prostate cancer generally metastasizes to bone, and most patients have tumor cells in their bone marrow already at diagnosis. Tumor cells at the metastatic site may therefore progress in parallel with those in the primary tumor. Androgen deprivation therapy is often the first-line treatment for clinically detectable prostate cancer bone metastases. Although the treatment is effective, most metastases progress to a castration-resistant and lethal state. To examine metastatic progression in the bone microenvironment, we implanted androgen-sensitive, androgen receptor–positive, and relatively slow-growing Dunning G (G) rat prostate tumor cells into the tibial bone marrow of fully immune-competent Copenhagen rats. We show that tumor establishment in the bone marrow was reduced compared with the prostate, and whereas androgen deprivation did not affect tumor establishment or growth in the bone, this was markedly reduced in the prostate. Moreover, we found that, with time, G tumor cells in the bone microenvironment progress to a more aggressive phenotype with increased growth rate, reduced androgen sensitivity, and increased metastatic capacity. Tumor cells in the bone marrow encounter lower androgen levels and a higher degree of hypoxia than at the primary site, which may cause high selective pressures and eventually contribute to the development of a new and highly aggressive tumor cell phenotype. It is therefore important to specifically study progression in bone metastases. This tumor model could be used to increase our understanding of how tumor cells adapt in the bone microenvironment and may subsequently improve therapy strategies for prostate metastases in bone.

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Introduction

Metastasis to bone is one of the most important clinical features of prostate cancer (PC). Approximately 70% of patients have prostate tumor cells in their bone marrow already at diagnosis [1]. Although the existence of such disseminated tumor cells (DTCs) is a predictor of recurrence [1,2], not all of the DTCs grow into clinically detectable metastases [1]. Why only subsets of asymptomatic and dormant micrometastases progress into clinical overt metastases is largely unknown.

Androgen deprivation therapy is a common first-line therapy for metastatic PC. Although the initial response to androgen deprivation is of significant palliative value, the metastases progress into an incurable and fatal state termed *castration-resistant PC*. A better understanding of the biology behind metastatic progression, from dormant asymptomatic micrometastases to clinically detectable metastases and from overt metastases to castration-resistant disease, is therefore a key to development of more effective treatments for metastatic PC.

The ability to grow as a clinically detectable metastasis could be acquired in the primary tumor [3–5]. It is also possible that neoplastic cells that are able to survive in the bone microenvironment progress at

the metastatic site in parallel with those in the primary tumor, regarding both intrinsic properties of the neoplastic cells and development of a metastasis stroma [3–5]. The prognosis may therefore depend on the ability of the initially dormant metastatic cells to grow and interact with the different microenvironment at the metastatic site.

PC cells have been shown to home to the hematopoietic stem cell niche in bone and replace the hematopoietic stem cells, and this niche appears to support tumor dormancy [6]. The formation of both osteolytic and osteoblastic lesions at the metastatic site in bone suggests that interactions with the microenvironment are crucial for the establishment of bone metastases [7]. Moreover, both adaptive and innate immunity may control the establishment of metastases. Low levels of the histocompatibility leukocyte antigen class I molecule on breast cancer cells have been suggested to be a way for DTCs to escape from T-cell–induced cytotoxicity and subsequently facilitate metastatic outgrowth [8]. The activity of T-cells may also be suppressed in the bone microenvironment in PC [9]. Better *in vivo* models that enable studies of metastatic progression in the factual microenvironment of fully immune-competent animals are therefore needed. Furthermore, bone marrow DTCs from breast, prostate, and esophageal cancer have been shown to display significantly fewer genetic aberrations than primary tumor cells [10–13], suggesting that they are disseminated early during primary tumor progression. Cell lines from more advanced metastatic tumors may therefore not be useful in studies of metastatic progression, as the mechanisms that are crucial for early colonization and adaptive selection may have been altered. Furthermore, neoplastic cells continue to evolve genetically at the bone metastatic site, and metastasis-to-prostate and metastasis-to-metastasis spread has been shown to be common in PC patients [14,15].

Here we implanted androgen-sensitive, androgen receptor (AR)–positive, and relatively slow-growing and poorly metastatic Dunning G (G) rat prostate tumor cells [16] into the tibial bone marrow of fully immune-competent Copenhagen rats. The aim of this study was to develop an *in vivo* model that reflects several aspects of human PC bone metastases and to determine whether the bone microenvironment can induce stable changes in prostate tumor cells, primarily regarding growth rate, the ability to colonize secondary organs, and response to androgen deprivation.

Materials and Methods

Cell Culture and Animals

Androgen-sensitive, AR-positive, low-metastatic rat prostate G R3327 tumor cells were grown in RPMI 1640 + GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS) and 250 nM dexamethasone [16]. Adult syngenic and fully immune-competent male Copenhagen rats (Charles River, bred in our laboratory) were used in all animal experiments. All the animal work was carried out in accordance with protocols approved by the Umeå Ethical Committee for Animal Studies (permit number A110-12).

Intraprostatic and Intratibial Implantation of G Prostate Tumor Cells

For intraprostatic implantation simulating primary tumor growth, the animals were anesthetized, and an incision was made in the lower abdomen to expose the ventral prostate lobes. G tumor cells were carefully injected into one of the ventral prostate lobes using a Hamilton syringe. For intratibial injections simulating metastatic growth, the animals were anesthetized, and the right leg of the rat was flexed. Using a drilling motion, a 23G needle was inserted via the knee joint into the bone marrow cavity of the tibia, and G tumor cells were then injected directly into the bone marrow cavity.

The same number of G tumor cells (2×10^5 cells in 10 µl of RPMI) was implanted into the prostate or bone marrow as described above, and the animals were sacrificed 8 weeks later ($n = 10$ rats with prostate tumors, $n = 10$ rats with bone tumors) and 12 weeks later ($n = 6$ rats with prostate tumors, $n = 6$ rats with bone tumors). The prostatic tumors were removed and fixed in formalin for 24 hours, dehydrated, and paraffin embedded. The right leg was cut above the knee and formalin fixed for 48 hours, decalcified in formic acid–sodium citrate solution (30% and 15%, respectively) for 48 hours, dehydrated, and embedded in paraffin.

Castration Treatment

Rats were castrated by scrotal incision or they were sham operated (control) 7 days before implantation of tumor cells. G tumor cells (2×10^6) were implanted into the ventral prostate of castrated rats ($n = 9$) or control rats ($n = 8$). In separate animals, G tumor cells were implanted into the bone marrow of both tibias (2×10^6/tibia) in castrated rats ($n = 5$) and control rats ($n = 6$) as described above. All animals were sacrificed 6 weeks after implantation of tumor cells, and 1 hour before sacrifice, the animals were injected with bromodeoxyuridine (BrdU, 50 mg/kg; Sigma-Aldrich), and the tumors were removed and formalin fixed as described above.

In addition, G tumor cells were implanted into the ventral prostate (2×10^3 cells) and the tibial bone marrow (2×10^5 cells) of the same animals. After 8 weeks, the animals were randomized to receive sham ($n = 7$) or castration treatment ($n = 8$). The animals were injected with BrdU as described above and sacrificed 14 days after the treatment was started.

To label hypoxic cells, additional animals that had been castrated ($n = 6$) or sham operated ($n = 5$) for 7 days were injected with Hypoxyprobe (Millipore) 1 hour before sacrifice, and the prostates, livers, kidneys, and tibial bones were formalin fixed and embedded in paraffin as described earlier [17]. Tissue oxygen was also measured directly in the bone marrow and prostate in anesthetized rats using electrodes (LIcox, Mediplast, Malmö, Sweden, probe CC1.2) inserted in the same way as for intratibial or intraprostatic tumor cell injection.

To measure dihydrotestosterone (DHT) and testosterone (T), animals that had been castrated ($n = 7$) or sham operated ($n = 7$) for 7 days were sacrificed, and the ventral prostate lobes and the tibias and femurs were immediately frozen in liquid nitrogen. In addition, EDTA plasma was collected. DHT and T were measured in bone marrow, prostate tissue, and plasma (at the Swedish Metabolomics Centre, Umeå, Sweden) using liquid chromatography–tandem mass spectrometry as previously described [18].

Morphological Analysis

Tumor area was assessed in hematoxylin and eosin–stained sections using Pannoramic viewer software version 1.15 (3DHistech, www.3dhistech.com). For G tumors in the prostate, the largest tumor area of each tumor represented tumor size. In bone, the G tumors usually grew as multiple tumors within the bone marrow cavity and sometimes also in the joint cavity. Representative bone sections were analyzed, and the section with the largest total tumor area (the sum of all G tumor areas present) in each animal was chosen to represent tumor size in bone. Sections were immunostained using