Research Article

Tumor-Stromal Interactions Influence Radiation Sensitivity in Epithelial- versus Mesenchymal-Like Prostate Cancer Cells

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HS-27a human bone stromal cells, in 2D or 3D cultures, induced cellular plasticity in human prostate cancer ARCaP E and ARCaPM cells in an EMT model. Cocultured ARCaP E or ARCaPM cells with HS-27a, developed increased colony forming capacity and growth advantage, with ARCaP E exhibiting the most significant increases in presence of bone or prostate stroma cells. Prostate (Pt-N or Pt-C) or bone (HS-27a) stromal cells induced significant resistance to radiation treatment in ARCaP E cells compared to ARCaP M cells. However pretreatment with anti-E-cadherin antibody (SHEP8-7) or anti-alpha v integrin blocking antibody (CNT095) significantly decreased stromal cell-induced radiation resistance in both ARCaP E- and ARCaP M-cocultured cells. Taken together the data suggest that mesenchymal-like cancer cells reverting to epithelial-like cells in the bone microenvironment through interaction with bone marrow stromal cells and reexpress E-cadherin. These cell adhesion molecules such as E-cadherin and integrin alpha v in cancer cells induce cell survival signals and mediate resistance to cancer treatments such as radiation.

1. Introduction

Prostate cancer is the most frequent tumor in men, afflicting African American males to a greater degree than Caucasians. Morbidity and mortality are mainly attributable to metastasis; yet the mechanisms associated with progression are largely unknown. Localized carcinomas are readily removed surgically, but once a tumor has established metastases, current therapies are not curative and prolong survival by only a few years. Metastasis occurs through a multistep process, where metastatic cells must intravasate local tissues and enter into and survive in the blood stream. These cells then extravasate into the secondary tissue and initiate and maintain micrometastases at distant sites, with the end result being the development of a metastatic tumor [1, 2]. During each step of this process, cancer cells exhibit transdifferentiation properties that allow both the spatial and temporal expression of epithelial and mesenchymal properties in response to microenvironment signals and its own basic survival needs (e.g., motility and invasion versus proliferation). Thus, a model of cellular transitions, as opposed to a continual progression to permanent differentiation state, is emerging as a significant mechanism during metastasis. A greater understanding of these mechanisms will result in clinical improvements and a better control of the metastasis process.

Epithelial-mesenchymal transition (EMT) was first described during development [3, 4]; however an EMT-like phenotypic change has been observed in a number of solid tumors [5–7]. This transition is typically characterized by a loss in E-cadherin and cytokeratin expression. EMT in cancer, as in development, is associated with an increase in cell proliferation [8, 9] and the acquisition of a mesenchymal phenotype that includes vimentin, N-cadherin,
and osteopontin expression. In both normal development
EMT and cancer-associated EMT, the loss of E-cadherin
is critical to the differentiation and maintenance of the
epithelial phenotype and provides a structural link between
adjacent cellular cytoskeletons, which is important for tissue
architecture. Cells that have undergone EMT (E-cadherin
negative mesenchymal cells) subsequently become more
migratory and invasive and proceed to traverse underlying
basement membranes, with an acquired ability to intravasate
the surrounding local tissue and gain access to vascular
conduits. As such, the loss of E-cadherin is rate limiting for
EMT [10, 11]. Recent reports from this laboratory and others
have described a mesenchymal to epithelial reverting tran-
sition (MErT) to occur, where mesenchymal-like prostate
cancer cell lines reexpress E-cadherin to become epithelial-
like, and reestablish cellular adhesion during colonization
within the liver tumor microenvironment [12, 13]. These
findings are shared in clinical metastases of various cancer
origins including breast, colon, and bladder, where robust
membrane expression of E-cadherin was observed, and the
paired more differentiated primary tumors were E-cadherin
negative [6, 14]. Thus, a reversion of the mesenchymal
phenotype appears to be important in latter stages of
metastasis.

Numerous studies have shown that the underlying influ-
ence of these cellular transitions is a consequence of tumor-
stromal interactions [15, 16]. Coculture studies have found
that the survival and proliferation of cancer cells are inti-
mately linked to the soluble factors in the microenvironment,
such as EGF, TGF-β, IGF-1 that contribute to survival and the
subsequent formation of macrometastasis [17–20]. However,
these factors are not likely to have a direct effect during
initial metastatic colonization, and thus heterotypic and
homotypic cellular adhesion has been proposed to provide
the necessary survival signals for successful colonization [21,
22]. Current state-of-the-art technology does not provide
the necessary resolution to determine at the single cell
level in patients or experimental in vivo systems, individual
cells that have successfully colonized the secondary site.
However, numerous reports have firmly established that
cancer-stromal interactions in vitro or in three-dimensional
(3D) assays accurately mimic the drug sensitivity/resistance
behavior of those cells found within solid tumors in vivo in a
preclinical or clinical setting [23]. Thus, we employed a novel
coculture assay to determine the cellular plasticity of cancer
cells promoted by the bone stroma and the effect of tumor-
stromal interactions on irradiation therapy in prostate
cancer.

The ARCaP model is the only robust prostate cancer
bone metastatic model which demonstrates epithelial to
mesenchymal transition (EMT). The ARCaP progression
model consists of ARCaP_E (epithelial) and ARCaP_M (mes-
enchymal), where the ARCaP_E cells have a bone metastatic
potential of 12.5% and the ARCaP_M cells have a bone
metastatic potential of 100%. The ARCaP_E and ARCaP_M
cells express the classical markers of EMT [24, 25]. Herein
we present findings that ARCaP_M cells undergo MErT when
cocultured within the bone microenvironment in 3D and
2D cultures. Additionally, ARCaP_E cells that retained an
epithelial phenotype exhibited a measurable growth advan-
tage and retained ability to form colonies, however only
under coculture conditions with bone stroma. Furthermore,
blocking the ability of ARCaP_E or ARCaP_M cells from E-
cadherin-mediated cell-cell adhesion or integrin alpha v
beta-associated adhesion significantly affected ARCaP cell
survival within bone stroma and sensitized these cells to
radiation treatment.

2. Methods

2.1. Cell Culture. The human prostate cancer cell lines,
ARCaP_E, ARCaP_M, the HS-27a bone stromal cells (ATCC,
Manasss, VA) and the Pt-N or Pt-C human prostate stromal
cell. Isolation and characterization of the human prostate
cancer RFP-ARCaP cell lines has been reported [26]. Red
Fluorescent Protein- (RFP-) transfected cells were main-
tained in G418 (350 mg/mL) prior to experimentation. All
cell lines were grown in a 5% CO_2 incubator at 37 °C in
media consisting of T-medium (Invitrogen, Carlsbad, CA)
supplemented with 5% (v/v) fetal bovine serum and 1%
Penicillin-Streptomycin.

2.2. Cocultures. Initial cocultures were performed as previ-
osly described [12, 13] with modifications. Cocultures con-
sisted of 50 000 cells/cm^2 of HS-27a bone marrow stromal
cells and 2000 cells/cm^2 prostate cancer cells. Cocultures
were maintained in serum-free T-media and plated on tissue
culture dishes.

2.3. Clonogenic Assay. Cells were plated at low densities
in six-well plates for 24 hours and then were irradiated
with the appropriate radiation dose. Twenty-four hours
later, the media were changed and cells were incubated
until they formed colonies having at least 50 or more cells.
Seventeen days later colonies were rinsed with PBS, stained
with methanol/crystal violet dye, and counted. The colony
formation ability was calculated as a ratio of the number of
colonies formed, divided by the total number of cells plated,
times the plating efficiency [(# of colonies formed ÷ total
# cells plated) × plating efficiency]. For experiments with
cocultures, cells were initially incubated on a mat of stromal
cells for 24 hours and radiated; 4 hours later clonogenic
assay was performed. For antibody-based experiments using
anti-E-cadherin (15 μg/mL, DECMA or SHEP8-7, Sigma)
and anti-integrin alpha-v (20 μg/mL, CNT095) antibody,
cancer cells were treated with respective antibodies for
24 hours prior to plating them on a mat of stromal
cells.

2.4. Radiation. External beam radiation was delivered on
a 600 Varian linear accelerator (Varian Medical Systems,
Inc.Palo Alto, CA) with a 6 MV photon beam. A 40 × 40 cm
field size was utilized and Petri dishes were placed on 1.5 cm
of superflab bolus. Monitor units (MUs) were calculated
to deliver the dose to a depth of dmax at a dose rate of
600 MU/min.
2.5. Statistical Analysis. Representative findings are shown for all experiments, which were performed in triplicate, repeated a minimum of three times. Student’s t-test was used to determine the statistical significance between groups.

3. Results

3.1. ARCaP EMT Model Undergoes a Mesenchymal-to-Epithelial Reverting Transition (MERT). Recently, the ARCaP model has been described to closely mimic the pathophysiology of advanced clinical human prostate cancer bone metastasis [25]. The ARCaP E cells were derived from single-cell dilutions of the ARCaP cells. These cells exhibit a cuboidal-shaped epithelial morphology with high expression of epithelial markers, such as cytokeratin 18 and E-cadherin. The lineage-derived ARCaP M cells have a spindle-shaped mesenchymal morphology and phenotype. ARCaP M cells have decreased expression of E-cadherin and cytokeratins 18 and 19 but increased expression of N-cadherin and vimentin. These cells have decreased cell adhesion and increased metastatic propensity to bone and adrenal glands [27]. The morphologic and phenotypic changes observed in the ARCaP M cells closely resemble those of cells undergoing EMT.

Previously, we have demonstrated a Mesenchymal to Epithelial reverse Transition (MErT) of metastatic prostate cancer cell lines within an experimental coculture model and confirmed in patients with liver metastasis [13, 28]. Our findings have recently been confirmed in prostate cancer bone metastasis where E-cadherin and β-catenin were robustly expressed in late stage carcinomas [29]. Therefore we sought to identify the significance of the bone microenvironment within the experimental ARCaP model. To assess cellular plasticity of the ARCaP EMT model, we cocultured ARCaP cells with HS-27α cells in 3D RWV (rotary wall vessel) system for 3 days. ARCaP E cells formed larger prostate organoids than ARCaP M cells (data not shown). Upon immunohistochemical examination of organoids, we observed that both ARCaP E and ARCaP M express E-cadherin and lack N-cadherin expression (Figure 1(a)). To further examine the influence of tumor-stroma interactions over a multiday period we utilized a similar 2D coculture methods. Utilizing immunocytochemical analysis, we observed a lack E-cadherin and robust N-cadherin staining after 1 day in both ARCaP E and ARCaP M cocultures. However by day 4, both ARCaP E and ARCaP M cells formed tumor nest that express E-cadherin and lack N-cadherin staining (Figure 1(b)). It is worthy to note that ARCaP M tumor nest appeared to develop at much smaller extent, compared to ARCaP E cocultures.

Since ARCaP E cells formed larger tumor nest and spheroids when cocultured with HS-27α cells compared to ARCaP M cells, we sought to further assess if HS-27α cells preferentially stimulated the growth of ARCaP E cells versus ARCaP M cells. Utilizing GFP-transfected HS-27α bone marrow stromal cells and RFP-transfected ARCaP E or ARCaP M cells (Figure 2(a)), we examined the proliferative ability of ARCaP cells in homotypic and coculture conditions.

3.2. Stromal Cells Influence Radiation Treatment in Prostate Cancer Cells. Mesenchymal cancer cells have been thought to be more tumorigenic, aggressive, and resistant to treatments when compared to epithelial cancer cells [30]. A similar trend was observed in both ARCaP E and ARCaP M cells after (4 Gy) irradiation treatment. ARCaP M homotypic cancer cells are more resistant to radiation treatment compared to ARCaP E homotypic cancer cells (Figure 3(a)). However, ARCaP M cocultures did not affect the radiation sensitivity of ARCaP M cancer cells. The highly sensitive ARCaP E cells exhibit a significant increased resistance to radiation therapy, up to 3-fold, as result of their interaction with bone stromal cells (Figure 3(a), P < .01).

To further assess the role of the prostate stromal cells on tumor-stromal interactions influencing ARCaP cellular behavior, we cocultured paired prostate stromal fibroblasts isolated either from normal (Pt-N) or from cancer-associated regions (Pt-C) [31]. Again, ARCaP E cells cocultured with (Pt-N) or (Pt-C) exhibited a 7-fold and 8-fold increase in colony formation, respectively (Figure 3(b), P < .01). We also saw a similar trend in a growth analysis assay (data not shown). However when measuring clonogenic ability after radiation treatment, ARCaP E cells cocultured with either Pt-N or Pt-C had increased radiation resistance, with a 2-fold difference observed between homotypic cultured cells. Although a significant increase in clonogenic formation was observed in Pt-C versus Pt-N cocultures (P < .05), this did not significantly effect the radiation sensitivity of ARCaP M cells (Figures 3(c)). Taken together, both bone and prostate stromal cell have a grown inductive effect on ARCaP E cancer cells and mediate radiation resistance (up to 2-3 fold) in epithelial cancer phenotype, but not in ARCaP M mesenchymal cancer cells.
Figure 1: 3D cocultures of ARCaP_E or ARCaP_M with HS-27a cells show E-cadherin expression. (a) $1 \times 10^7$ ARCaP_E or ARCaP_M were cocultured with HS-27a cells in RWV for 3 days. Immunohistochemistry of organoids was stained with anti-E-cadherin or N-cadherin antibody. (b) 2D Cocultures of HS-27a were preformed utilizing a total of 50,000 cm$^2$/HS-27a fibroblasts, after which 20,000 cm$^2$ ARCaP_E or ARCaP_M were seeded on top of the fibroblast monolayer. The cocultures were maintained in serum-free medium for 1 or 4 days. Immunocytochemistry of cocultures over these time periods was performed utilizing anti-E-cadherin and N-cadherin antibodies. Shown are the EMT/MET of ARCaP_E cells (top panels) and MErT of ARCaP_M cells (bottom panels).

3.3. Blocking Adhesive Contact Effects Radiation Sensitivity of Cocultured ARCaP Cells. The importance of cell adhesion (i.e., cell-cell and cell-ECM adhesion) on the survival of disseminated cancer cells has been well documented as a requirement for colonization and survival within the metastatic microenvironment [32–34]. Therefore we utilized a well-known E-cadherin blocking antibody (SHEP8-7) and a pan-integrin antibody (CNT095) that targets human alpha-v-integrin and also was shown to block prostate tumor growth within bone [35]. Since ARCaP_E cells express high levels of the epithelial marker E-cadherin, and ARCaP_M cells can be microenvironmentally induced to express E-cadherin, we tested whether either of these blocking antibodies would affect the colony forming ability of either ARCaP_E or
ARCaP_M bone stroma-cocultured cells. Pretreatment with E-cadherin antibody did not affect the colony forming capacity of either ARCaP_E or ARCaP_M homotypic cultured cells; however it significantly reduced the ability of ARCaP_M- (P < .001) and ARCaP_E- (P < .01) cocultured cells to form colonies (Figure 4). Additionally, E-cadherin blocking antibody pretreatments further increased sensitivity to radiation treatment of ARCaP_M cells in homotypic and cocultured conditions, similarly (P < .01). E-cadherin blocking antibody-pretreated ARCaP_E cells showed the most significant increased sensitivity to radiation treatment in homotypic compared cocultured conditions (P < .001), however a significant reduction in colony formation, to a lesser extent, was observed in ARCaP_E cocultured cells.

Figure 2: ARCaP_E cells show a growth and colony forming capacity advantage in presence of HS-27a cells. (a) and (b) ARCaP_M cells were cocultured in the presence of GFP-HS-27a cells over a 6-day period. Growth of RFP ARCaP_E or ARCaP_M human prostate cancer cells was assessed by RFUs (relative fluorescent units) in the presence cocultures over a 6-day period. Results are means ± SE of three independent experiments. *P < .05 (students t-test) compared to cell number at day 1 ± SEM. (c) Clonogenic colony forming capacity of ARCaP_E and ARCaP_M prostate cancer cell after coculture ± SEM. ARCaP_M data were normalized to ARCaP_M control, and ARCaP_E data were normalized to ARCaP_E control (Note HS-27a induced slightly (1.35x) the growth of ARCaP_M cells but markedly (8x) the growth of ARCaP_E cells.). (d) ARCaP_E or ARCaP_M cells were cocultured with HS-27a cells. Shown are phase contrast images of colonies formed in the clonogenic assay.
Figure 3: Cocultured ARCaP_E cells gain cell colony forming capacity and radiation resistance when grown with bone and prostate stromal cells. (a) ARCaP_E or ARCaP_M cocultured cells were irradiated 24 hours after coculture with HS-27a cells and cancer cell colony forming capacity was assayed using clonogenic assay. Results are means ± SE of three independent experiments. ARCaP_M experimental data are normalized to ARCaP_M control and ARCaP_E experimental data are normalized to ARCaP_E control (a). ARCa_P_E cells cocultured with prostate stromal fibroblasts Pt-C (Cancer associated fibroblasts) or Pt-N (Normal/benign fibroblasts) were irradiated and compared to nonirradiated cocultures. Cell colony forming capacity was assayed by clonogenic assay. Data are normalized to ARCaP_E control levels. (b) ARCaP_M cells cocultured with Pt-C or Pt-N were irradiated and compared to nonirradiated cocultures (c). Cell colony forming capacity was assayed by clonogenic assay. Data are normalized to ARCaP_M control levels.

(Figure 4, \(P < .01\)). Therefore, targeting E-cadherin limited both epithelial and mesenchymal cells ability to form colonies after coculture with bone stromal cells.

To determine the influence of integrin alpha v cell adhesion with bone microenvironment, we performed similar clonogenic formation assay. Pretreatment with CNT095 antibody significantly decreased the clonogenic ability of both ARCaP_M and ARCaP_E cells in homotypic cultures (Figure 5, \(P < .001\)). Additionally, CNT095 significantly decreased bone stroma-induced radiation resistance in cancer cells in both ARCaP_M (\(P < .001\)) and ARCaP_E (\(P < .001\)) cancer cells, with the most significant reduction in cocultured conditions (\(P < .001\)) (Figure 5). Taken together, these results suggest that bone stroma-induced radiation resistance is mediated through both E-cadherin and integrin alpha v beta signaling in epithelial and mesenchymal cells. Thus, E-cadherin and integrin alpha v beta appear to present novel targets for metastatic and radiation resistant cells.

4. Discussion

It is well documented in prostate and others cancers that EMT is associated with initial transformation from encapsulated to invasive carcinomas. The mesenchymal phenotype, which is required for dissemination, has been suggested to revert to an epithelial phenotype in distant metastasis [13, 14, 29, 36]. This has been evidenced in the primary tumors which lack E-cadherin expression and, showing nuclear \(\beta\)-catenin expression, show strong membrane staining for both E-cadherin and \(\beta\)-catenin in metastatic liver [13] or bone microenvironment [28, 29]. We have previously shown, in commonly utilized prostate cancer cells lines DU-145 and
PC-3, that reexpression of E-cadherin and reversion of the mesenchymal phenotype is a rate limiting for metastatic seeding of primary rat hepatocytes [13]. Since bone metastasis is most prevalent in prostate cancers, we sought to extent these finding utilizing the ARCaP model, which is the first prostate cancer EMT model demonstrating histomorphological features and classical markers in a lineage-derived series of cells, to determine the functional relationship of this cellular transition. Whether this is accomplished through exposure to soluble growth factors or the bone microenvironment, the end result decreased differentiation with increased metastatic potential [25, 27, 37].

Our initial results show that ARCaPM cells maintained in 3D Rotary Wall Vessel (RWV) or 2D cocultures underwent
recent report has shown through RFP cell tracking that ARCaP M (1.35×) in ARCaP E cancer cells. Using E-cadherin neutralizing ARCaPE cells show a significant enhancement in colony and ARCaP M homotypic and cocultured cells. Our results indicate that blocking bone stroma-induced cellular plasticity gives rise to distinct populations of cancer cells within bone microenvironment, the mesenchymal phenotype and its kinetic characteristics (motility/invasive), and the epithelial characteristics necessary for secondary tumor development. The fact that the ARCaP M cells have an increase propensity for metastasis compared to ARCaP E cells suggests that dissemination from the primary tumor mass requires the mesenchymal phenotype. However a mesenchymal to epithelial transition is associated with initial metastatic seeding and subsequent formation of a cohesive tumor mass within the bone microenvironment. This hypothesis is supported in a bladder cancer model, where lineage-derived series of EMT-transformed mesenchymal-like cells exhibit increased lung metastasis in vivo; however secondary tumor formation is predominantly enhanced by the presence of epithelial cells compared to mesenchymal cells.

Since epithelial reversion enhances the growth of tumor cells in bone microenvironment, and this is observed in multiple experimental models and clinical metastases, there is a question of whether this transition is required for metastatic seeding and therefore an avenue for therapeutic intervention(s). To gain insight into the importance of this reversion, we utilized ionizing radiation on ARCaP E and ARCaP M homotypic and cocultured cells. Our results show that ARCaP E homotypic cultures when compared to ARCaP M homotypic cultures are more sensitive to radiation treatment (Figure 3(a)). However in the presence of bone or prostate stromal cells, ARCaP E cells gained increased radiation resistance, with increased proliferative and colony forming capacity (Figures 2(b) and 2(c)). This phenomenon was not observed in the ARCaP M cocultures. To determine the underlining causes of this observation, we hypothesized that cell-cell interactions through E-cadherin or cell-ECM interactions through integrins may mediate the stromal induced proliferative effect and radiation resistance in ARCaP E cancer cells. Using E-cadherin neutralizing antibody (SHEP8-7) and pan-anti-integrin alpha v antibody (CNT095), we were able to significantly block the stromal induced colony forming ability on ARCaP E cancer cells (Figures 4 and 5). Additionally, both antibodies significantly blocked the radiation resistance of ARCaP E in cocultured conditions (Figures 4 and 5). The E-cadherin neutralizing antibody also had an effect on homotypic ARCaP M-radiated cells and ARCaP M cells within cocultures (Figure 4). Thus it appears that blocking bone stroma-induced reexpression of E-cadherin in ARCaP M in the presence of bone stromal cells reduced the colony forming capacity of these cells (Figure 4). The decreased radiation sensitivity of E-cadherin expressing cells compared to cells lacking E-cadherin expression has recently been demonstrated in a cocultured model of MCF-7 (E-cad positive) and MDA-MB-231 (E-cad negative) cells with normal and radiation-induced senescent fibroblast [39], where radiation in MCF-7 cells showed enhanced resistance to radiation treatment compared to MDA-MB-231 cells. These findings are consistent with our model of a reepithelialization requirement within tumor microenvironment.

CNT095 antibody was toxic to both ARCaP M and ARCaP E homotypic and cocultured cells. Additionally CNT095 increased radiation sensitivity, even to a greater extent than E-cadherin neutralizing antibody treatment (Figure 5). These findings are consistent with our results of CNT095 treatment that causes a significantly reduced number of tumors generated by C4-2B cells, along with a concomitant increase of cortical bone in mice (unpublished data). Although C4-2B cells have not been observed to undergo EMT, this would suggest that targeting the cell-ECM interaction(s) that promote cellular plasticity in the tumor microenvironment, at least during initial metastatic seeding.

In conclusion, our data demonstrate that the E-cadherin and integrin alpha v adhesion interaction is a possible adjuvant therapy avenue for patients treated with radiation. Although an in-depth in vivo exploration of targeting epithelial-like versus mesenchymal-like cells is necessary to translate these findings to the clinical situation, our results indeed raise critical questions as to how we view prostate cancer metastasis and subsequently target metastatic tumor cells for therapy. Additionally, we have generated an in vitro model, that closely mimics the clinical situation, to delineate in a stepwise manner the dynamic tumor-host interaction(s) that promote cellular plasticity in the later stages of metastasis. The identification of further key molecules driving MErT in this system holds promise for the development of preventative and therapeutic strategies to minimize metastatic disease.
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